Epidemiological and Pathological Correlates of Early Neonatal Mortality

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ABSTRACT

Introduction

Despite improved intrapartum monitoring of the fetus, the rate of cerebral palsy has not decreased. Many studies now suggest that the majority of children with such disability may sustain damage in utero. Current understanding and identification of antenatal brain injury is poor and individual vulnerability may be important. Specific genotypes such as Apolipoprotein E are disadvantageous to outcome in the context of adult brain injury and may also be important in early development.

Objectives

An aim of this project was to identify clinical and genetic risk factors for early neonatal mortality within the Scottish population with special consideration for those infants born in an asphyxiated condition. A further aim was to determine the prevalence of antenatal brain injury within this population and to correlate neuropathology with clinical factors.

Methods

Clinical data and neuropathological specimens were collected as part of the Scottish National Perinatal Neuropathology Study from all 22 delivery units throughout Scotland. Birth asphyxia was defined and infants were classified accordingly. Neuropathological examination was performed by a single observer in Edinburgh who was blind to clinical detail. Apolipoprotein E genotype was analysed and compared with known published data for adults

and healthy newborns. Comparisons of categorical data were made with the Chi-square test and numerical data were compared using the unpaired student's t-test or the Mann Whitney U test.

Results

Clinical data was collected from 191 early neonatal deaths. Complications of pregnancy were common in all neonatal deaths. The only predictive factors for asphyxia were indicators of intrapartum fetal distress. Neuropathological examination was possible in 59 infants surviving 3 days or less. Evidence of prelabour brain injury was observed in nearly half of this cohort, and this was significantly more common in asphyxiated infants and those who developed an encephalopathy. The only clinical associations of such damage were the presence of cardiotocograph abnormalities, meconium staining and severe depression at birth. Apolipoprotein E analysis was performed in 252 perinatal deaths. There was an over representation of the ε4 allele among healthy newborns compared to perinatal deaths and adults.

Conclusions

Brain injury occurring in utero is a common finding among neonatal deaths, particularly in those born with asphyxia. Current intrapartum indicators of fetal distress may signify a fetus who has already suffered compromise prior to the onset of labour. Differences in the Apolipoprotein ε4 genotype in perinatal populations suggest that the fetus may vary in its ability to withstand fatal injury.

Declaration

I declare that this thesis has been composed by myself. The results described herein are a product of work carried out by a research group of which I have been a member. I have made a substantial contribution to the research except where indicated to the contrary. This work has not been submitted for any other degree or professional qualification.

Acknowledgements

The Scottish National Perinatal Neuropathology Study was funded by the Scottish Home and Health Department and by Wellbeing and was designed and implemented by a research group consisting of Professor Neil McIntosh and myself (Section of Child Life & Health, University of Edinburgh) and Professor Jeanne Bell and Dr Jean Keeling (Division of Pathology (Neuropathology), University of Edinburgh). The publications resulting from the Scottish National Perinatal Neuropathology Study and referred to in the text of this thesis were jointly written by this research group.

I thank Professor Jeanne Bell, Dr Jean Keeling and Mrs Betty Wyatt who performed all the detailed neuropathology and placental pathology referred to in this text and who were responsible for many informed and inspiring discussions during the course of this thesis. Betty is remembered with fondness as she passed away sadly shortly after the study ended.

The study was dependent on the collaboration of Scottish Paediatric Pathologists and Neuropathologists. I am grateful to the midwives and medical staff in the 22 neonatal and obstetric units throughout Scotland whose enthusiasm in enrolling cases and collecting data made this study possible. I am also indebted to the families who, despite the tragedy of their baby's death and knowing that the results would not personally help them at their time of grief, allowed these detailed investigations to proceed.

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I dedicate this thesis to my family, whose patience and support during these last few years have allowed me the freedom to complete this project.

CONTENTS

Glossary

AFP	alpha-fetoprotein
APH	antepartum haemorrhage
ApoE/apoE	Apolipoprotein E (protein)
APOE	Apolipoprotein E (gene)
BA	birth asphyxia
CNS	central nervous system
CP	cerebral palsy
CTG	cardiotocography
ELISA	enzyme linked immunosorbent assay
ENND	early neonatal death
FAS	fetal anomaly scan
FT	full term
GFAP	glial fibrillary acidic protein
HCG	human chorionic gonadotrophin
HIE	hypoxic-ischemic encephalopathy
IUGR	intrauterine growth restriction
LB	live birth
MRI	magnetic resonance imaging
NND	neonatal death
NNE	neonatal encephalopathy
NNU	neonatal unit
OFC	occipitofrontal circumference
PBD	prelabour brain damage
PCR	polymerase chain reaction
PIH	pregnancy-induced hypertension
PM	post mortem
PROM	prolonged rupture of membranes
PT	preterm
SB	stillbirth
TNF	tumour necrosis factor

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Introduction

The three major causes of neonatal death are lethal malformations, prematurity and birth asphyxia[1]. Whilst major malformations and premature birth are generally regarded as unavoidable mischance, birth asphyxia implies a lack of care in labour. Although birth asphyxia is classically linked to intrapartum hypoxia-ischemia in full term infants, often proceeding to a neonatal encephalopathy, a proportion of preterm babies are also born in a neurologically depressed condition almost certainly related to poor oxygenation in labour. Asphyxia is acknowledged to be an imprecise term but is still used regularly by the profession and parents. It may be implied by one or more of the following features: a low Apgar score[2-5] a baby who is difficult to resuscitate, metabolic acidosis ascertained either in cord or in early neonatal blood samples[5;6], or the development of neonatal encephalopathy[7;8]. A history of these particular features may be sought retrospectively if an infant goes on to develop neurodevelopmental delay. None of these indicators, when applied prospectively to infants born in poor condition, has good sensitivity, specificity or predictive value for neurodevelopmental delay or disability, though in full term infants the development of neonatal encephalopathy is more specific[9]. It is clear that perinatal asphyxia is not likely to be an important factor in the development of every case of neonatal encephalopathy or in the majority of cases of cerebral palsy[7;10-14]. This view has recently been endorsed by a statement from the International Cerebral Palsy Task Force[15].

Obstetric care has seen dramatic changes over the last few decades. Most changes have contributed to the steadily falling stillbirth and neonatal death rates[1;16-18;18;19]. However despite better clinical care and widespread use of fetal monitoring and fetal blood sampling, full term infants continue to be born in a neurologically depressed condition. Such infants cause considerable distress to parents and staff. They contribute both to early neonatal mortality and to the pool of children who display later neurodevelopmental disability with cerebral palsy. Although in some cases obstetric risk factors can be identified, affected children also result from pregnancies and labours, which, even when scrutinized critically, appear to have been normal.

Litigation for perceived perinatal mismanagement is increasingly common, particularly in relation to infants born in a neurologically depressed condition– usually manifest by a poor Apgar score, and often reflexly labelled 'birth asphyxia'. Some recent anecdotal reports and small series of infants born in poor condition have shown neuropathological abnormality at autopsy that must have preceded the onset of labour. These generally represent the collected experience of specialist referral centres[20-22;22-24] or focus on a particular age group of infants, for example those born preterm[25-29]. Only rarely do the neuropathological studies include correlation with clinical factors[30-32]. Some insight into clinico-neuropathological correlation in the first weeks of life is now being achieved by neuroimaging[33].

Research into the genetics of brain damage in later life suggest that adults may have an individual vulnerability for neurological impairment following trauma and hypoxic-ischemia[34-37]. The Apolipoprotein E (APOE) genotype in particular is thought to affect different aspects of cell repair and death[38;39]. Little is known about the role of APOE in paediatric or neonatal brain injury but it is likely that mechanisms are similar. Recent work in normal infants suggests APOE genotype may predict neurodevelopmental scores at 24 months of age[40].

The aim of this thesis is to describe the neuropathology of the infant who dies in the first week of life and to attempt to delineate aetiological or predisposing factors. The role of these factors in fetal and neonatal brain injury may hold the answer to unexplained longterm neuromorbidity in children.

Aims and Objectives

The overall aims of this thesis are to:

1. describe the clinical characteristics of a population of infants dying within the first week of life.

2. describe the neuropathology of this population.

3. determine the allele frequencies of APOE in a population of perinatal deaths and compare these with

    a) a population of healthy newborns

    b) a population of healthy adults

More specific aims are to:

1. compare maternal sociodemographic, pregnancy and labour characteristics of infants born in an asphyxiated state with those born in good condition.

2. ascertain the prevalence of antenatal brain damage in a population of early neonatal deaths.

3. compare the prevalence of antenatal brain damage between asphyxiated and non-asphyxiated infants.

4. delineate any specific clinical risk factors for brain damage, particularly amongst asphyxiated infants.

5. determine any correlation between APOE genotype and abnormal neuropathology.

Chapter 1
The Scottish Perinatal Neuropathology Study

Design

The Scottish Perinatal Neuropathology Study was a prospective observational and experimental population-based study involving all twenty-two delivery units within Scotland between January 1996 and January 1999. The study was funded by the Scottish Office Home and Health Department (R26389 and K/MRS/50/C2307) and Wellbeing (M1/95).

Aims

The study had 4 overall research aims:

1. to assess the clinical characteristics of the mother, pregnancy, labour and infant where pregnancy ends in a perinatal death.

2. to determine whether the antepartum and intrapartum characteristics of asphyxiated early neonatal deaths were different to those of non-asphyxiated neonatal deaths

3. to investigate the neuropathological status in those infants in whom a post mortem was authorised, and to determine whether lesions could be of prelabour origin.

4. to correlate any neuropathology found with the clinical characteristics of the mother, pregnancy, labour and infant

Methods

The role of the author

The author was the clinical coordinator for the study. This role involved application to each hospital's local ethics committee, the setting up of a system for case identification, recruitment and acquisition of patient data and the creation and maintenance of the study database. The author needed to identify a link midwife and lead obstetric and paediatric consultants in each of the twenty-two delivery units involved and liase with these persons to ensure optimal case recruitment and completion of study data forms. The role involved regular presentations in each of the units to optimise staff awareness and the circulation of a bimonthly newsletter to promote the study. A list of delivery units and neonatal units who took part in the study can be found in Appendix 1.

Patients

Inclusions

The base study considered all perinatal deaths that were ≥ 24 weeks gestation at birth and ≤ 7 days of age at time of death delivered in Scotland over a two year period. This thesis concerns the epidemiology and neuropathology of the liveborn subset of the study cohort only. APOE analysis of perinatal deaths included the stillborn cohort also.

Exclusions

Infants with central nervous system (CNS) or cardiac malformations, major chromosomal abnormalities or CNS infection were excluded as it was felt that the neuropathological changes associated with such conditions might interfere with the interpretation of any changes superimposed by a perinatal insult.

Clinical Dataset

For each case a detailed questionnaire was completed by specially trained midwives or other local staff who recorded a battery of clinical information and the results of investigations relating to each pregnancy, labour, delivery and neonatal course. The dataset can be found in Appendix 2. The author met with the midwife and clinician involved in the case of a neonatal death to elucidate all the relevant details. Clinical data could be collected on all eligible cases irrespective of enrolment in the pathology study and data was entered into a central database (SPSS) by the author.

Ethical and consent procedures

Before starting the study, each delivery unit obtained approval from their local research ethics committee. As different units received ethical permission at slightly varying times, the spread of data collection was three years, although it was two years for each individual centre. Cases were enrolled at the time of post mortem request by the clinician responsible for the care of the infant during life. A detailed clinical dataset was collected on all infants regardless of post mortem authorisation. The purpose of the study

was explained to parents. Signed consent was obtained for autopsy, and on a separate consent form if authorised, for extended neuropathological research studies on the brain.

Classification

a) Maturity

Full term infants (FT) were those of 37 weeks gestation and above. All others were preterm (PT).

b) Definition of asphyxia

Infants were defined as having suffered 'birth asphyxia' (BA) if they had any one of the following features:

- an Apgar score at 5 minutes of ≤ 5.0
- a cord or initial blood pH of <7.1
- the presence of grade 2/3 neonatal encephalopathy. The grading of encephalopathy used was that of Sarnat and Sarnat[41].

Because of the diverse clinical circumstances not all criteria were available for assessment in each case. Infants who displayed at least one of these criteria were classified as showing clinical evidence of birth asphyxia (BA). If none of these criteria were present the infant was included in the non-asphyxiated group (noBA). This definition is deliberately liberal to include as many cases as possible where an element of intrapartum asphyxia may have been present. The reasons for this and the criteria chosen are discussed in the discussion of chapter 3. It is accepted that this definition may also

include infants who were acidotic or depressed at birth due to other factors such as prematurity or acidosis. The contribution of these factors to the study definition of birth asphyxia and the presence of prelabour brain damage are discussed in chapters 3 and 5. It is recognised that by current definitions, the presence of hypoxic-ischemic encephalopathy in the presence of acidosis is a more conservative definition and the neuropathological features of this subgroup were analysed separately.

Pathological Examination

a) Autopsies

Autopsies were conducted in six Scottish centres and the brain was retained in fixative for later examination. In the South East of Scotland the fixed brains were examined in the Department of Neuropathology at the Western General Hospital, Edinburgh. Elsewhere the fixed brains were sampled locally according to a previously agreed protocol (Appendix 3). Up to 20 representative paraffin embedded blocks were prepared in each case from all areas of the cerebrum (including temporal hippocampus), and from the basal ganglia and thalami, midbrain, pons, medulla, vermis and cerebellar hemispheres. These blocks were collected centrally for review and further investigation in Edinburgh. Paraffin sections were stained routinely with haematoxylin and eosin and luxol fast blue/cresyl violet (myelin). Selected sections were investigated immunocytochemically for astrocytic status, using an antibody to glial fibrillary acidic protein (GFAP) and for microglia/macrophages (antibodies to CD68 and MHCII) or stained with

Perls Prussian blue stain (haemosiderin). The neuropathological appearances in grey and white matter were assessed independently in all cases by two observers (Professor Jeanne Bell and Mrs Betty Wyatt) who were initially blind to the clinical history. Selected cases were also reviewed by Dr Jean Keeling. Recorded neuropathological features included neuronal eosinophilia and karyorrhexis, astrocytic hyperplasia, activated microglia and accumulation of macrophages, haemorrhage (recent and older), vascular responses, and foci of mineralisation and of infarction. The neuropathological features were then correlated with the gestational and postnatal age of the infant and with the criteria of birth asphyxia, in combination and individually.

A judgement of whether the damage dated from before the onset of labour (prelabour brain damage, PBD), was based in part on the presence of patently mature lesions such as established infarcts, previous haemorrhage or extensive mineralisation. However these features were present in the minority of brain damaged infants. More diffuse features such as definite macrophage infiltration/accumulation and/or prominent reactive astrocytic hyperplasia in white matter are thought to develop over a period greater than 3 days (Tables 5.2 and 5.3). It was estimated that the presence of prelabour brain damage (PBD) or absence (noPBD) could only be determined reliably in infants dying $\leq$ 3 days from the onset of labour.

b) Placenta

The placenta, cord and membranes were examined macroscopically and cord

length, placental measurements and trimmed weight were recorded. Any abnormality was described. Histological samples were taken to include a cross section of the umbilical cord, one strip of membranes (adjacent to the hole through which the baby was delivered, if identifiable), and two blocks of placenta with both fetal and maternal surfaces. Blocks and slides from the placenta and adnexa were submitted for central review in Edinburgh by a paediatric pathologist (Dr Jean Keeling). Histological evidence of infection, specifically chorioamnionitis in the extraplacental membranes, chorionic plate and funisitis were recorded, as was villitis if generalised.

Statistics

Descriptive statistics were used to examine the prevalence of clinical variables. The Chi square test and Yates correction (or Fisher's exact test where sample size was less than 20) was used to compare categorical variables and the unpaired t-test or Mann Whitney U test to compare the difference in continuous variables. Significance was assumed at the $p<0.05$ level.

The population

Of the 692 deaths in the 2 years of the study, 221 were early neonatal deaths corresponding with the estimated early neonatal death rate of 2.5/1000 live births in Scotland[42].

Figure 1 lists how the cohort of 692 qualifying perinatal deaths was reduced by various exclusions through the 221 liveborn infants to the 70 infants from

whom the brain was available for examination in this study.

Of the 221 liveborn infants, 47 met the exclusion criteria leaving 174 infants of whom 37 were not notified to the clinical coordinator until the end of the study period. There were 137 infants who met the study criteria and had full clinical detail. Of these, 90 were classified as BA and 47 as noBA according to the definition. Table 1 shows how they met the criteria for birth asphyxia.

Of the 137 eligible infants with a full clinical dataset, 88 had consent for post mortem and 70 of these had additional consent for the neuropathological samples required for the study. These 70 infants were divided according to whether they displayed birth asphyxia or not. Analysis of those 59 infants who died at 3 days or less after the onset of labour allowed identification of pathological features likely to have predated labour and birth. Damage seen could not be reliably ascertained as occurring 'prelabour' when the infant had lived for more than three days. Placentas were available for histological examination from 41 of the 70 infants.

Table 1. Clinical features of birth asphyxia in 137 early neonatal deaths.

	Full term	Preterm
Total infants	**38**	**99**
Single feature only		
Apgar ≤5 at 5 mins	9	35
Cord pH < 7.1	0	1
1st pH < 7.1	1	8
Encephalopathy (NNE)	1	0
Two features of asphyxia		
Low Apgar and low pH	7	9
Low Apgar and NNE	2	1
Low pH and NNE	0	1
Three features of asphyxia		
Low pH, low Apgar and NNE	11	4
Total with some indication of asphyxia	**31 (82%)**	**59 (60%)**

[Note - All infants had a 5 minute Apgar score measured. Only 12 full term infants and 11 preterm infants had a cord pH measured. An additional 22 full term infants and 35 preterm infants had a pH done on arrival in the local neonatal unit. Sixteen full term infants at 12 hours of age were not paralysed, and 14 of these had features of an encephalopathy. 19 preterm infants remained alive and had not receive muscle relaxants at 12 hours, 6 had an encephalopathy]

Figure 1. The Scottish Perinatal Deaths Cohort

692 Perinatal deaths

471 Stillbirths

221 Early neonatal deaths

47 with exclusion criteria (16 chromosome abnormality, 25 cardiovascular malformation, 6 CNS malformation, 0 CNS infection)

174 Early neonatal deaths meeting inclusion criteria

37 excluded as missed, these included - 24 (gestation 24-28 weeks) died from extreme prematurity, severe RDS or severe IVH; 3 others (gestation 29,30,35 weeks) died from complications of prematurity and 5 FT (3 BA, 1 MAS, 1 sudden collapse on ward); 5 infants of unknown gestation, 2 dying of cardiorespiratory, 2 of renal and 1 of cardiovascular failure.

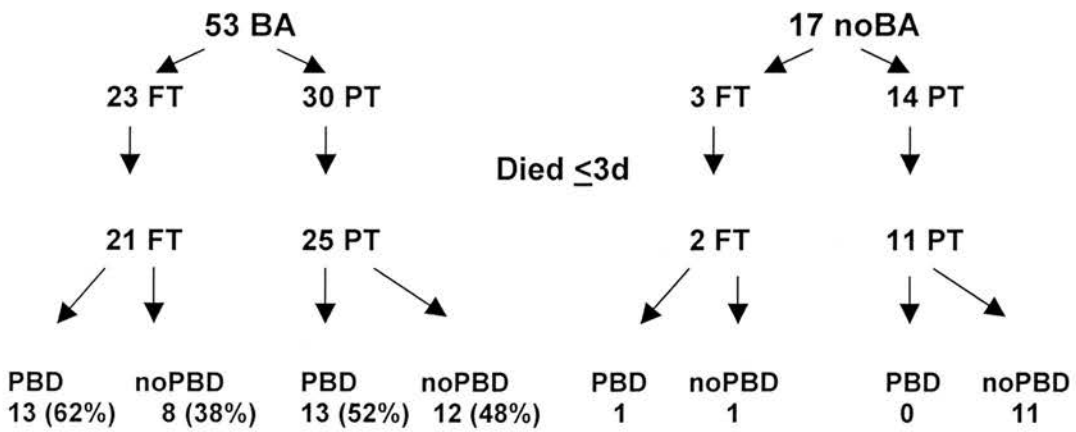
137 Early neonatal deaths with clinical detail

38 FT (31BA, 7noBA); 99 PT (59BA, 40noBA)

88 Consented for autopsy

18 with no additional samples –some without consent and some samples proving unsuitable for comprehensive neuropathological analysis. Only 50% of these 18 had 'BA' compared to 75% of the remaining 70: 16 of the 70 had grade 3 HIE compared with none of the 18 who didn't having extended neuropathology. Otherwise these 18 infants did not differ in any significant way from the 70 when considering social class, maternal age, complications of pregnancy, type of delivery, admission to NNU, Apgar scores, pH, age at death, resuscitation, gestation and birthweight

70 Consented for additional study samples



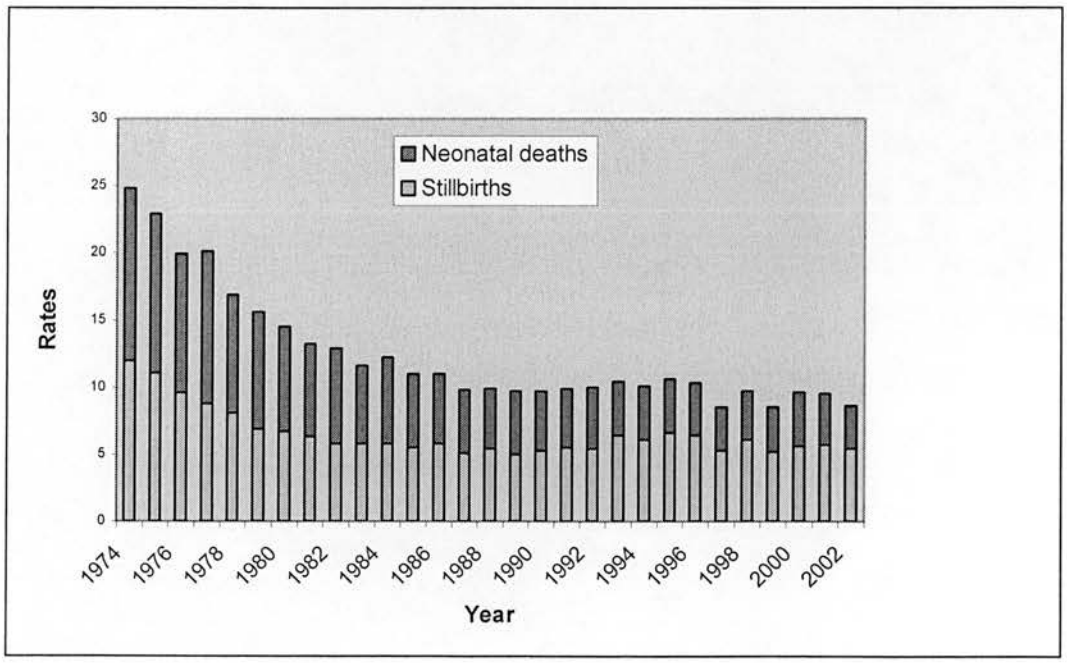
Key: FT= full term; PT= preterm; BA= birth asphyxia; noBA= no birth asphyxia; PBD= prelabour brain damage; noPBD= no prelabour brain damage; RDS= respiratory distress syndrome; IVH= intraventricular haemorrhage; MAS= meconium aspiration syndrome; HIE= hypoxic/ischaemic encephalopathy; CVS= cardiovascular system; CNS= central nervous system; NNU= neonatal unit

Chapter 2
Trends

1. Trends in perinatal mortality

The last sixty years have seen a dramatic reduction in perinatal mortality rates throughout the developed world. In the 1940s in Europe the stillbirth rate was around 20/1000 births and the early neonatal death rate around 22/1000 livebirths. Over the first thirty years the rate declined by 20-25% and subsequently in the latter half of this period by 55-60%[43]. Current Scottish mortality figures are 5.4/1000 total births for stillbirths and 2.2/1000LBs for early neonatal deaths. The figure below shows national trends in Scotland since 1974[44].

Figure 2.1 Scottish perinatal mortality rates (1974-2002)



The overall background frequency of congenital malformations in the newborn population, about 3%, has not changed much over 50 years but the contribution that malformations make to the perinatal mortality rate have increased by a factor of 5 due to reduction in other causes of death. This is true particularly for the late neonatal death rate where deaths due to malformations comprise around 40-50% of such mortality[44]. In contrast the proportion of malformations amongst stillbirths has increased only slightly despite the decrease in the overall stillbirth rate. The reason for this is in large part due to the reduction in neural tube defects (in particular anencephaly which is invariable fatal and is amongst the commonest defect in stillbirths), which has resulted in a decreasing stillbirth rate since the 1960s. This decline can only be partly accounted for by early termination of affected fetuses and the periconceptual use of folic acid. In fact the incidence of neural tube defects had begun to fall before such factors came into play and is still not fully understood. As many malformations occur sporadically and to low-risk women it is expected that the burden of malformations to the stillbirth rate will continue largely unchanged. However in contrast it can be expected that improved prognosis for congenitally malformed live births will continue to lead to decreases in the neonatal death rate at least until a core of unpreventable and uncorrectable congenital defects is approached.

The concept of 'preventable mortality' in perinatal mortality statistics is sometimes used to evaluate the effectiveness of care and distinguishes deaths from congenital abnormality ('unpreventable') from deaths due to other

causes ('preventable'). Deaths of infants weighing less than 500g are also generally thought to be unavoidable. Although there have been substantial reductions in deaths due to congenital abnormality and extreme low birth weight over the last ten years the 'preventable' perinatal mortality rate, inclusive of late neonatal deaths, is maintained around 5.7/1000 total births. The cause of 'preventable mortality' amongst stillbirths is still largely unexplained with around 65% of all cases in this category. Antepartum haemorrhage and hypertension of pregnancy contribute to the remainder of non-malformed stillbirths.

Among all early neonatal deaths, nearly 40% are due to problems of prematurity and over a quarter are due to congenital anomalies, with anoxia and/or birth trauma accounting for 17% and infection 10%. Amongst multiple births, prematurity as a cause soars to around 75% and congenital anomalies are responsible for about 10% of deaths. Although the relative risk of death from any cause is greater in multiple births, this increase in risk is greatest for deaths due to the problems of prematurity. In fact amongst neonatal deaths in multiples there is a ten fold increase in the rate of neonatal death due to lung immaturity and hyaline membrane disease[44]. Despite this, birthweight specific perinatal mortality has continued to fall over the last twenty years against a background of steady or even increasing preterm birth rate. This reduction is secondary to advances in neonatal care of the premature infant, in particular the use of antenatal steroids and surfactant in extremely low birth weight infants[45].

2. Trends in birth asphyxia and cerebral palsy

Birth prevalence of 'birth asphyxia' ranges from 1.8-7.7 per 1000 term live births[7;46-51] and contributes to around 10% of neonatal mortality. The rate is somewhat dependent on definition of the term 'birth asphyxia' and this may be defined purely on the basis of a low Apgar score[52;53] acidosis during or at delivery, prolonged depression of respiration[54], need for mechanical ventilation[55], neonatal encephalopathy[47;56] or a combination of these. The definition of the term 'birth asphyxia' has been repeatedly modified over the years in response to increasing obstetric litigation and assignment of blame, and the diminishing association between cerebral palsy and best indicators of intrapartum hypoxia. A consensus has evolved that for damaging intrapartum hypoxia to have occurred there must be documented evidence of ischemia in the form of a low pH and accompanying neonatal depression in the form of an encephalopathy[15]. In the longitudinal study of birth asphyxia in the Swedish population between 1985 and 1991 the incidence of 'birth asphyxia' as defined by an Apgar score of <7 at 5 mins (excluding congenital malformations, opioid related depression and intracranial haemorrhage) was 6.9/1000. When only those who developed encephalopathy were included the incidence of clinically significant birth asphyxia dropped to 1.8/1000 LBs[49]. Many studies of 'birth asphyxia' pre-1990 suggest that this rate has not changed appreciably over the last 30 years[47;49;56]. However, a recently published study of over 5 million births from California observed a 91% decrease in 'birth asphyxia' over the decade of the 1990s. This trend applied to all birthweight groups[51]. Similarly perinatal mortality in the USA due to

intrapartum asphyxia fell by 85% from 1970 to 1981[57]. However, because the clinical syndrome of birth asphyxia is not specific for hypoxic-ischemic brain injury or any other single cause, authors of more recent studies of birth asphyxia have based estimates solely on what they describe as post-asphyxial encephalopathy[58] where a classical neurological syndrome develops following evidence of intrapartum asphyxia as defined by reasonably stringent criteria.

The incidence of post-asphyxial or hypoxic-ischemic encephalopathy (HIE) ranges from 3-6/1000 livebirths[47;56;59;60]. Although the incidence of "birth asphyxia" is reported to have reduced only latterly, there appears to have been a more long-standing reduction in the incidence of HIE. A Canadian study [61] showed a halving in seizures secondary to severe encephalopathy over a twenty year period between 1960 and 1980. A British study[62] found a decline in the incidence of HIE from 7.7/1000 LBs to 1.9/1000 LBs in the period between two cohorts from the late 1970s and mid-1990s and Sheth and colleagues[60] in the USA showed a halving of the admission rate for HIE in the decade 1986-1995.

Cerebral palsy is a descriptive term for a heterogeneous collection of non-progressive disorders of movement and posture that become manifest early in life and are not the result of a recognised cerebral malformation, but the result of an insult or repeated insult to the developing brain. Interest in the incidence of cerebral palsy arises because it is often somewhat erroneously

used as an outcome measure for obstetric care.

The total prevalence of cerebral palsy in developed countries is around 2.5/1000LBs. This has not changed over the last forty years despite the more widespread use of obstetric interventions aimed at detecting and reducing intrapartum morbidity and neonatal management of the complications of extreme prematurity[63]. Moreover in underdeveloped and developing countries without such interventions, the cerebral palsy rate is remarkably similar to that of the rest of the world[64]. During the course of a Swedish study extending over nearly thirty years there were substantial reductions in perinatal mortality but disappointingly no parallel reduction in the rate of cerebral palsy[65]. In fact, a recent study from the USA showed a modest increase in cerebral palsy in infants with normal birthweight between 1975 and 1991[66] and data from the Western Australia cerebral palsy register suggests that the percentage of term children with severe forms of cerebral palsy is increasing (www.ichr.uwa.edu.au). Other studies from Western Australia[67], Ireland[68] and Northern England[69;70] have shown a small decreasing trend in heavier infants and steady or increasing rates amongst low birth weight infants that makes the overall rate harder to interpret. As birthweight specific mortality has continued to fall on a background of a steady preterm birth rate it is tempting to think that the increase in prevalence of cerebral palsy in preterm low birthweight babies has resulted from the development of neonatal intensive care and to think of them as 'casualties of technological development'[71]. There are three possible

explanations for this adverse side effect of special care: one that the immature brain of an intact preterm infant is susceptible to haemorrhage and ischemia and the subsequent neurological deficit. Another explanation is that an increasing number of preterm infants are surviving, this has generated more interest in routine follow up and has led to an improvement in the completeness of ascertainment especially in milder cases which previously may have been missed. Finally, cerebral palsy and preterm delivery may have similar origins in pregnancy and more of these children, who were compromised before birth, are now surviving[72]. The classical form of cerebral palsy associated with prematurity is spastic diplegia with preservation of intelligence. However the rise in cerebral palsy is in all spastic types and many are associated with other severe handicaps. This supports the hypothesis that modern technological advances have allowed the survival of preterm infant with established neurological damage.

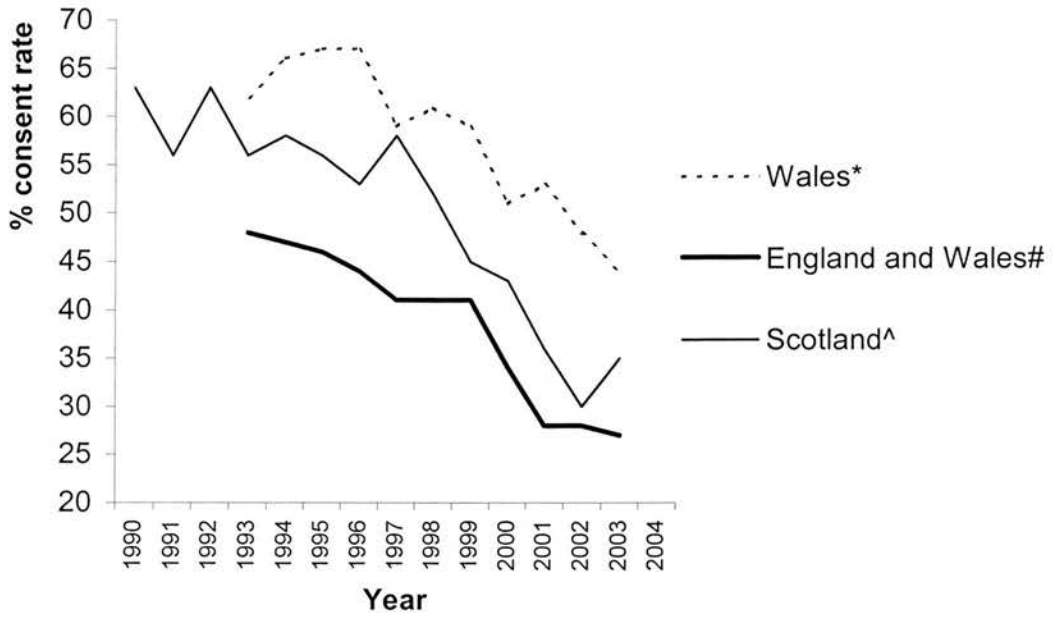
3. Trends in neonatal post mortem rates

Despite increased confidence in new diagnostic techniques, the usefulness of autopsy in establishing cause of death and determining unrelated diagnoses is well-documented[73-76]. Autopsy has other recognised benefits in assisting the grieving process[77] and providing information for genetic counselling or the management of future pregnancies[78]. Nevertheless the autopsy consent rate has been declining worldwide over the last three decades[79] and there has been much concern about public confidence in the process since well-publicised criticisms in the media surrounding organ retention. Within the United Kingdom there was a continuous decrease in neonatal post mortems throughout the 1990s and early 2000 (Figure 2.2).

In the late 1990s, the Bristol Royal Infirmary inquiry[80] into deaths of babies having heart surgery caused widespread public concern about the quality of information delivered to families about post mortem examinations, in particular the retention of whole organs for detailed laboratory examination. In January of 2001 the Redfern report[81] was published and followed an intensive inquiry into the removal, retention and disposal of human organs and tissues following post-mortem examinations in the Royal Liverpool Children's Hospital NHS Trust. The report criticised the 'systematic' removal of tissues at post mortem, often without consent and often without later histological examination.

The Human Tissue Act of 1961 which recommended that tissues or organs

Figure 2.2 United Kingdom neonatal post mortem rates 1990-2003



* Welsh data from the All Wales Perinatal Survey, personal communication

\# English and Welsh data from the 5th-8th Confidential Enquiry into Stillbirths and Deaths in Infancy reports [17-19;82]

^ Scottish data from Information and Statistics Division of NHS Scotland [83], personal communication

could be removed from bodies at autopsy as long as there was no objection raised to retention is now seen as paternalistic and new systems based on the recommendations of the report have subsequently been put in place to ensure no such repetition[84].

However the sensationalistic media handling of these two episodes almost certainly damaged the public's confidence in the post mortem process and consent rates fell substantially throughout the country over the next two years.

The Scottish Neuropathology Study took place during the years 1996-1999 when the Scottish neonatal post mortem rate fell below 50%. Our study was concerned with stillborn infants and early neonatal deaths and the combined post mortem rate for these groups was 64%. The study excluded late neonatal deaths, a group that is associated with a lower autopsy consent rate[85] and those with known congenital malformations or chromosomal abnormalities where, if the diagnosis is already established clinically, there may be less endeavour to obtain autopsy consent.

The consent process during the study period was less unambiguous and explicit than it is required to be today and of note is the discrepancy between the neonatal post mortem rate of 64% for brain retention and study consent rate of 51% where the only difference in protocol was centralisation of brain blocks for detailed neuropathology.

Since the time period of the study, post mortem rates have continued to fall around the United Kingdom, although in Edinburgh where the research team are based, there has been recent optimism over current rates approaching those seen at the beginning of the 1990s[86].

Chapter 3
Clinical characteristics of pregnancies resulting in asphyxia-related neonatal death

Introduction

Considerable improvements in antenatal care have resulted in a dramatic fall in maternal and perinatal mortality since the 1940s. However despite significant advances in fetal surveillance over the last twenty years there has been little further gain in the perinatal death rate within the UK[82]. The increasing ability to monitor the fetus in a high-risk pregnancy with ultrasound, Doppler studies and fetal blood sampling means that delivery can be expedited when a fetus appears compromised and as a result the caesarean section rate within the UK is now approaching 25%.

Despite such intensive supervision and intervention some babies continue to be born in a depressed condition. A small proportion of these children may show neurological symptoms in the neonatal period and some of these encephalopathic infants may go on to develop cerebral palsy. However most children with cerebral palsy have no identifiable problem at birth[87].

Over the last hundred years there has been increasing interest in the aetiology of cerebral palsy. Legal action against health authorities on behalf of neurologically handicapped children has escalated in the last few decades under the general supposition that negligence in the care of the mother and

fetus during labour has led to irreversible brain damage in the child[88]. However the measures put in place over that time to monitor the fetus in labour have not led to the expected decrease in the cerebral palsy rate (Chapter 2). Furthermore, continued research into the antecedents of long term neurological morbidity shows that a minority of such damage is attributable to intrapartum hypoxia. Instead epidemiological evidence suggests that intrauterine insult is more likely to be responsible[89-91], and this is supported by neuropathological confirmation of antenatal brain damage in the fetus and newborn[23;32]. Thus, although prevention of intrapartum asphyxia remains essential, limitation of much childhood neurodisability may depend on our ability to understand the fetal environment and its hazards.

There is little doubt that labour and delivery can be hazardous to the fetus and that intrapartum asphyxia does occur. It is also true that such infants may subsequently die or develop neurological impairment consequent to this injury. What is unclear is whether all such infants have been normal up until the onset of labour or whether they may already have been subjected to a earlier insult. What does seem apparent is that not all asphyxial injury is the same. Indeed, depending on the mechanism, duration and intensity of the insult, the distribution of brain injury varies dramatically and the condition of the newborn may differ accordingly[92-94]. It is likely that chronic or intermittent forms of asphyxia are far more common than the catastrophic asphyxiation of the experimental animal model, although less is known about subsequent adaptation at birth.

Although many children who later develop cerebral palsy have no adverse intrapartum features[89], it is possible that a fetus who has already sustained damage in utero may tolerate even the hypoxia of a normal labour poorly. Non-specific signs of fetal distress may herald such an infant who may be born in an 'asphyxiated' condition[95]. Obstetric intervention may not alter the prognosis in such cases, but the stress of a prolonged or even normal labour may put such a fetus at risk of further damage.

The first part of this chapter describes some of the difficulties in the definition of birth asphyxia and details the literature on the clinical associations of asphyxia and neonatal encephalopathy. The second part presents and discusses the clinical features of asphyxia-related neonatal deaths from the Scottish Perinatal Neuropathology Study.

Defining 'birth asphyxia'

Birth asphyxia refers to the end result of oxygen and nutrient deprivation to the fetal brain during labour. A normal fetus is well adapted to the inevitable process of intermittent 'asphyxia' that occurs during normal labour. These adaptations include switching to anaerobic metabolism during periods of relative hypoxia and redirecting blood flow to vital organs such as the brain and myocardium. If the adverse factors persist, the physiological adaptations become attenuated, with eventual decompensation resulting in increasing acidosis and a fall in cerebral blood flow. Hypoxic-ischemic damage to the

brain may occur as a result of these persistent abnormalities. No direct measures of asphyxia are available and various definitions, including fetal heart rate abnormalities, acidosis and low Apgar scores, have historically been used. However attempts to relate these measures to subsequent neurological impairment have consistently failed as they do not reliably predict which children will develop cerebral palsy.

Fetal cardiotocography was introduced in the 1960s in the hope that early identification of fetal compromise would lead to prompt delivery and avoidance of irreversible damage. The Dublin randomised trial of electronic fetal monitoring versus intermittent auscultation in 13,000 women led to a halving of the incidence of neonatal seizures in term infants, suggesting differential exposure to preventable intrapartum asphyxia. Disappointingly however follow up of this cohort to the age of four years did not reveal any differences in the rate of cerebral palsy[96]. Keegan and colleagues identified a group of full term and preterm newborns with convulsions and found a much higher incidence of CTG abnormalities compared to a control group. However when determining the obstetrical response to the abnormal heart rate patterns they found in all cases that it was entirely appropriate. Eventually many of the cases with neonatal seizures developed CP[97]. Some abnormality of the CTG has been described in as many as 40-80% of labours yet neonatal encephalopathy only occurs in 1-6/1000 [98]. Fetal heart rate abnormalities may not always equate with the presence of damaging asphyxia but instead may be the manifestation of pre-existing damage or

maldevelopment of the fetal brain. An abnormal CTG tracing can be the result of chromosomal abnormalities or congenital malformations[99]. Changes in fetal heart rate variability occur with increasing CNS maturation and conditions which result in delayed or disordered maturation may affect the CTG.

Correlation of the CTG with other markers of asphyxia is poor. Acidosis, said to be the hallmark of intrapartum hypoxic-ischemia[6] is only found in about one third of cases where the CTG is deemed to be abnormal but only one in six infants with severe acidosis may exhibit a normal fetal heart tracing during labour[100]. There have been attempts to refine interpretation of the CTG by using simultaneous electrocardiographic analysis of the ST segment and T wave which reflect the ability of the myocardium to respond to the stresses of labour. A Swedish randomised controlled trial of around 5000 maternities showed a reduction in neonatal encephalopathy and metabolic acidosis when fetal ECG analysis was performed in conjunction with cardiotocography[101;102].

Fetal blood sampling was developed to add precision to electronic fetal heart rate monitoring and to recognise those infants developing acidosis during labour. Although a low pH has been considered the best widely available measure for identification of asphyxia during labour, the level and duration of acidosis at which irreversible damage may occur has been notoriously difficult to define and may well vary between infants. The definition of a low

pH has been debated by many[5;103-105] and remains the subject of controversy. Although profound acidosis is found in many infants with neonatal encephalopathy, the vast majority of infants with acidosis are normal in the neonatal period and subsequently[106-109]. The association of pH with long-term outcome is even weaker. Ruth and Raivio conducted a prospective study of the predictive value of metabolic acidosis and Apgar score on perinatal brain damage[5]. Nine hundred and eight-two infants were studied and followed to one year of age. The sensitivity of a low pH for adverse outcome was 21% with a positive predictive value of only 8%. Only 4 of the cohort had any indicator of CP at 1 year of age and none of the four had a pH $\leq$ 7.0 at birth. Low found that only 13% of newborns with moderate acidosis had a major deficit at 1 year but that the incidence of any deficit increased to 80% the more severe and the longer the duration of the acidosis[110]. Neonatal acidosis is not necessarily specific to intrapartum asphyxia: it is found more commonly in fetuses who are growth restricted[111], those with sepsis[112] and amongst those with rare inborn errors of metabolism. Acidosis has also been reported more commonly in the preterm fetus[113]. It remains to be proven whether metabolic acidosis when present in a fetal, cord or early neonatal blood sample, is attributable to chronic or intermittent hypoxia of longstanding duration or whether de novo acute hypoxia has occurred during labour in a previously healthy fetus.

The Apgar score, first developed in 1952 by Dr Virginia Apgar, is a quick method of assessing the state of the newborn infant[114]. It is observer

dependent and usually performed retrospectively. As a tool for predicting outcome it performs poorly, especially amongst preterm infants[115]. The National Collaborative Perinatal Project (NCPP) found that only 8% of infants ≥2500g with a 5 minute Apgar score of three or less died in the first year and only 0.7% developed cerebral palsy. Among low birthweight infants the risk of death was far higher, 55%, but again only a small number, 7%, suffered from cerebral palsy on follow-up. In fact the risk of cerebral palsy amongst term infants only became significant when the Apgar score remained severely depressed by 20 minutes of age. It is important to note that seventy-five percent of all children with cerebral palsy within the NCPP had 5 minute Apgar scores of 7 or higher[52] and this is consistent across studies[107]. Although Levene found a depressed Apgar score at 10 minutes to be the most sensitive predictor of outcome[9], Naeye found that a low 10 minute Apgar score was more likely to be due to congenital abnormalities than birth asphyxia in children with cerebral palsy[91]. In fact the Apgar score may be adversely affected by a number of factors other than asphyxia. Drugs such as maternal opiates, infection, hypovolemia, congenital disorders and antenatal brain damage may all cause depressed respiration at birth and some of the components of the Apgar score are notoriously difficult to assess in lower gestations[54] who are far more likely to be attributed low scores compared to their mature counterparts.

The term 'birth asphyxia' is now considered to be imprecise and its implications are of dubious validity and seldom applied to problems of gas

exchange. In 1999 an international consensus statement was issued by the International Cerebral Palsy Task Force defining intrapartum asphyxia sufficient to cause death or neurological damage. Essential criteria include early onset of moderate or severe encephalopathy in infants ≥ 34 weeks gestation as well as evidence of acidosis in a fetal or early neonatal blood gas (pH<7.0 and base deficit ≥ 12mmol/l). Other non-specific criteria that together may be suggestive of damaging intrapartum asphyxia include Apgar scores of 6 or less for longer than 5 minutes, a sentinel hypoxic event occurring immediately before or during labour and evidence of neonatal multiorgan dysfunction[15].

The relative contribution of birth asphyxia to cerebral palsy

Controversy exists about the relative contributions of pre- and intrapartum factors on long-term adverse outcome. There is little doubt that intrapartum asphyxia occurs and that it can lead to death or cerebral palsy in survivors. The question is what proportion of neurological impairment in children can be attributed to birth asphyxia, and whether these infants have significant predisposing factors. That the majority of children with cerebral palsy have been damaged during labour and delivery has now been refuted on the basis of several studies. The National Collaborative Perinatal Study (NCPP) followed the course of 60,000 pregnancies in 12 medical-school affiliated hospitals from 1959 to 1966 prospectively. Many had no intrapartum risk factors for asphyxia and the majority of those who did had co-existing antenatal risk factors or stigmata which meant that either brain damage or an

increased vulnerability to such damage had occurred well before labour began. In fact Nelson and Ellenberg suggest that only 9% of all cerebral palsy cases within this cohort were damaged perinatally[89]. Naeye and colleagues analysed a sub-group of 43,000 full term infants to determine the role of intrapartum hypoxic-ischemia in the development of cerebral palsy in mature infants[91]. Although the newer methods of determining fetal well-being were not in use during that period, surrogate indicators of intrapartum asphyxia such as delayed onset of respirations, fetal bradycardia and neonatal seizures were collected. This study found that only 6% of cerebral palsy could be attributed to birth asphyxia. The authors suggested that even state-of-the art intrapartum management was unlikely to prevent cerebral palsy in these full term infants as congenital disorders were responsible for many more cases of cerebral palsy than birth asphyxia.

More recent data collected after the introduction of neonatal intensive care and more sophisticated intrapartum monitoring techniques, support this early data. Blair and Stanley in the Western Australian series, a time-ordered multivariate analysis of 183 cases and 549 matched controls, found that birth asphyxia was only likely in around 8% of cases of cerebral palsy[90]. Stanley and English report data on low birthweight infants within this cohort, 13% of whom were reported to have significant perinatal asphyxia. Only 10% of these infants, around 1% of the total cohort, later developed cerebral palsy[116]. Results from a British study reported a similar proportion: only 10% of full term infants who developed CP showed evidence of BA[117]. On the

other hand the Swedish longitudinal survey of cerebral palsy, reports intrapartum factors as being responsible for 52% of preterm and 28% of term infants with CP[118]. In this study however one of the criteria suggestive of intrapartum asphyxia was the requirement for mechanical ventilation after birth, a necessity which may be related to lung pathology and maternal sedation as well as birth asphyxia.

Supportive of a pre-partum origin of brain damage is the increased frequency of minor malformations, abnormal dermatoglyphics and developmental tooth enamel abnormalities in children with cerebral palsy[89;91;119-121]. Affected siblings are more common in children with cerebral palsy[122] and autosomal recessive and X-linked modes of inheritance have been reported[123]. Others have also found an increased incidence of birth defects amongst infants born with birth asphyxia and neonatal encephalopathy[124;125].

Imaging studies of the brain in children with cerebral palsy show that white matter damage predominates[126-128] even in term infants. White matter damage preferentially occurs in the second and early third trimesters[24] and its occurrence among term children with cerebral palsy emphasises that such damage frequently occurs prenatally.

Risk factors for birth asphyxia and hypoxic-ischemic encephalopathy (HIE)

Clinical studies of severe birth asphyxia show that most children who survive

with sequelae have clinical signs of encephalopathy during the neonatal period[129]. Similarly between a quarter and a half of children with encephalopathy following intrapartum asphyxia may go on to develop neurological deficits. It has been shown that damaging intrapartum asphyxia is regularly followed by neurological symptoms in the neonatal period[130]. However, indicators of intrapartum asphyxia are often absent in infants with so-called hypoxic-ischemic encephalopathy. Volpe noted that a third of infants described as having HIE had no recognised adverse features during labour[131]. Nelson and Ellenberg found that three quarters of term infants said to have HIE had meconium stained liquor or fetal bradycardia but this was also true of 61% of those infants without HIE. Among those infants without features of fetal distress during labour, 2.9% developed an encephalopathy compared to 3.3% of those with fetal distress[130].

Thus the contribution of intrapartum events to newborn encephalopathy remains unclear. Although neonatal encephalopathy may follow severe intrapartum asphyxia, it may be in some instances the manifestation of a problem originating before the onset of labour.

Finer and Robertson examined the perinatal risk factors of 95 full term infants with hypoxic-ischemic encephalopathy and found a high incidence of maternal complications and fetal growth restriction. Although abnormal cardiotocography was common amongst those mothers monitored, around 40% of encephalopathic infants had a five minute Apgar score of 8 or

more[46]. Gaffney and colleagues found that children with CP who had exhibited NNE were more likely to have had evidence of intrapartum complications than those who were asymptomatic in the newborn period but who later developed CP[117]. The authors inferred that NNE was a marker of intrapartum injury but did not rule out the possibility that encephalopathy may represent a more longstanding neurological disorder. Westgate and colleagues showed that nearly a quarter of full term infants with hypoxic-ischemic encephalopathy experienced a severe obstetric catastrophe during labour but that a further quarter only had evidence of antenatal hypoxia[132]. It was unclear in the remaining 50% when fetal compromise began.

One of the largest studies of risk factors in encephalopathy is the Western Australian case-control study which described the relative contributions of intrapartum and antenatal factors to full term newborn encephalopathy (NNE)[58;59]. One hundred and sixty four infants with moderate or severe encephalopathy were matched with 400 controls. The definition of encephalopathy was broad and not dependent on asphyxia criteria. Intrapartum factors alone were found in only 5% of cases, in 24% both antepartum and intrapartum factors were identified, and in 69% only antepartum factors. Two percent had no identifiable factors.

Among maternal features examined in this study, lower socio-economic status, increasing maternal age and a family history of neurological disease or seizures were positively associated with NNE. Risk factors during

pregnancy included infertility treatment, maternal thyroid disease, severe pre-eclampsia, bleeding, and a diagnosed viral infection. Maternal pyrexia in labour, an acute intrapartum event and a persistent occipitoposterior position were all associated with the development of NNE. Although there was no difference in overall caesarean section rate, elective caesarean section alone was found to be protective against NNE (OR 0.17). Babies who were below the tenth centile were far more likely to develop NNE than controls. Many of these results support data from a more historical cohort reported by this group[50] and other studies.

Small for gestational age infants are over-represented compared to controls in many studies of birth asphyxia and proportions range from 7.5% to 29%[47;49]. Many studies report an excess of growth restricted infants in cases of newborn encephalopathy ranging from 25-33%[46;47;58;133-135] suggesting an antepartum onset of fetal compromise. It has been shown that for a given gestational age, a higher proportion of those with cerebral palsy are growth restricted particularly those with diplegia and quadriplegia[10;136;137].

Bleeding in pregnancy and placental abruption have consistently been found to be associated with neonatal depression in the Western Australian study and others[138-140].

The association of pregnancy-induced hypertension (PIH) with encephalopathy is unclear: Haddad showed that PIH was commoner in

infants who had an Apgar of 0 at birth and were subsequently resuscitated[139] and others have found that PIH is strongly associated with neonatal encephalopathy[46;141]. On the other hand PIH has been associated with a reduced incidence of intrapartum asphyxia and intraventricular haemorrhage amongst extremely low birth weight infants[25]. Murphy et al showed a reduced risk of cerebral palsy in very preterm survivors when pre-eclampsia had complicated the pregnancy[142]. The reasons for these conflicting results are unclear but may be due to two factors. Because of its effect on fetal growth, PIH may appear protective against adverse outcome in birthweight analyses, if gestational age is not accounted for. Additionally many studies of the effects of PIH on neonatal morbidity and later handicap do not take into consideration the potential beneficial effects of any maternal antihypertensive treatment such as magnesium, mode of delivery or antenatal steroids.

The association of maternal thyroid disease with neonatal encephalopathy has also been reported[50;138;140] as has an association with cerebral palsy[89;143]. It has been postulated that thyroid dysfunction, its underlying cause or treatment may disrupt normal neuronal development.

A further Australian case-control study published in 2001 sought to determine obstetric antecedents in 83 full term infants ventilated because of perinatal asphyxia[138]. Again, there was a higher incidence of maternal thyroid disease as well as antenatal complications among case infants

compared to controls. In labour, prolonged rupture of membranes, maternal pyrexia and haemorrhage were all more common among cases who were also more likely to be growth restricted and male. Cases were more likely to show signs of fetal distress during labour and have severely depressed Apgar scores at birth.

In a Swedish cohort of over 42,000 infants, 75 developed hypoxic-ischemic encephalopathy. Both antenatal and intrapartum complications were more common in hypoxic-ischemic encephalopathy infants compared to the rest of the population. Specific predictive factors such as thyroid disease, pregnancy-induced hypertension, placental abruption, meconium and cardiotocograph abnormalities were consistent with previous literature. There was a non-significant trend for a protective effect of elective caesarean section[140].

The role of maternal infection and pyrexia has received increasing interest as it has emerged that infants of mothers with such features are more likely to show evidence of brain injury on imaging[144-146], neuropathological evidence of brain injury at post mortem, and are at greater risk of developing cerebral palsy[147]. Maternal pyrexia in labour has been found to be a strong predictor in many studies of adverse neonatal depression and encephalopathy[50;59;138;148-150] and it has been postulated that the production of maternal cytokines due to infection may be an important factor[148]. In these studies distinction is not made between infectious and non-infectious pyrexia

and other surrogates of infection, such as prolonged rupture of the membranes, have been variably associated. Lieberman investigated the role of pyrexia of a non-infectious origin on neonatal outcome. In a study of over 1200 non-infected women, 10% had intrapartum pyrexia and this was associated with depressed Apgar scores, hypotonia at delivery, need for bag and mask resuscitation and neonatal seizures. All these complications increased in frequency as the degree of maternal fever increased[151]. The mechanism of fetal damage in maternal pyrexia is not known and may involve cerebral infection, hyperthermia or the action of inflammatory mediators such as interleukin-6 and tumour necrosis factor-α which damage oligodendroglial cells in the developing brain.

In summary it appears that while the role of intrapartum factors may be important in the development of clinically significant asphyxia, many of these infants have pre-existing features which may either be directly responsible for damage occurring before the onset of labour or make an infant more vulnerable to the normal asphyxiating events during delivery.

Aims

1. to describe the sociodemographic, pregnancy, labour and delivery characteristics of a population of early neonatal deaths and describe the features of their life in the first week.
2. to determine any differences in the above characteristics between asphyxiated infants and those born in a good condition.

Methods

Refer to chapter 1 for methods.

Note

Throughout the results section, cross-reference is made to table 5.7 where an infant went on to have neuropathological examination. The maternal, pregnancy, labour and postnatal details of these cases are listed. Where case numbers are not given, such infants did not have neuropathological examination.

Results

Population (Figure 1)

174 infants greater than or equal to 24 weeks gestation and less than 7 full days of age met the inclusion criteria for the study.

137 infants had a full dataset available. 37 infants were not notified to the study coordinator until after the end of the study period. Retrospective collection of demographic data from most of these 37 infants showed that proportion of preterm infants was similar to the 137 infants with a full dataset.

There were 134 mothers in the study who gave birth to 137 infants. Twenty infants were multiple births, including two pairs where both twins died and two infants from a set of triplets who both died.

a) Sociodemographic characteristics (Table 3.1)

Thirty-seven percent of the cohort were primigravida and had an average age at booking of 27.6 years. A history of infertility or previous pregnancy loss was common affecting 29% of the population. Over one third of mothers were smokers at booking. Only one mother had known thyroid dysfunction (case 28) but required no medication. When the cohort was divided into those with asphyxia at delivery and those without, the mothers were comparable for all sociodemographic details.

Table 3.1 Comparison of maternal sociodemographic details

	All mothers (n=134)	BA (n=90 infants)	noBA (n=47 infants)	Sig
Maternal age at booking*	27.6(6.1)	28.2(5.9)	26.8(6.5)	0.192
Body Mass Index at booking (kg/m^2)*	25.3(4.8)	25.4(4.2)	24.7(3.6)	0.504
Social class 1,2#	55(42)	41(46)	17(37)	0.311
Unemployed#	16(12)	8(9)	8(18)	0.180
Married#	38(28)	21(23)	17(36)	0.111
Smoker at booking#	49(37)	35(39)	16(35)	0.606
Primigravida#	49(37)	30(33)	20(43)	0.287
No of pregnancies^	1(0,2)	1(0,2)	1(0,2)	0.308
[1]Relative infertility#	39(29)	31(34)	9(19)	0.468
Previous termination of pregnancy#	23(17)	14(16)	10(21)	0.403
[2]Previous uterine instrumentation#	32(24)	24(27)	9(19)	0.329

* mean, standard deviation and p value (independent samples t-test)
\# number, percentage and 2-tailed significance (Chi-square test)
^ median, interquartile range and asymptotic 2-tailed significance (Mann-Whitney U test)

[1]history of infertility, previous neonatal death, stillbirth or miscarriage
[2]including suction termination of pregnancy, cervical cone biopsy, previous caesarean section and fibroid surgery

b) Antenatal characteristics (Table 3.2)

Thirteen percent of all pregnancies were unbooked or booked after 16 weeks. Seventeen of 134 mothers (13%) had multiple pregnancies, 16 of which were twin and 1 triplet. Two mothers were known to have conceived following ovulation induction, and one by in vitro fertilisation. Although abnormal serum screening for AFP and HCG occurred in 14 pregnancies, only five of these went on to have amniocentesis. Five other amniocenteses were performed for amnioreduction (4) or at maternal request (1). Fifty-eight mothers had a fetal anomaly scan, either routinely or because of clinical concern. Twenty scans were abnormal and findings included oligohydramnios, polyhydramnios, intrauterine growth restriction, fetal

Table 3.2 Comparison of antenatal characteristics

	All mothers (n=134)	BA (n=90)	noBA (n=47)	Sig
Unbbooked or booked >15 weeks#	18(13)	12(13)	6(13)	0.926
Systolic BP at booking^	115(15)	117(16)	112(13)	0.075
Diastolic BP at booking^	68(12)	68(13)	68(10)	0.970
Multiple pregnancy#	17(13)	12(13)	8(17)	0.562
Assisted conception#	3(2)	3(3)	1(2)	1.000
Abnormal serum screening#	14(16)	7(12)	7(24)	0.212
Amniocentesis performed#	10(8)	6(7)	5(11)	0.509
Abnormal fetal anomaly scan#	20(34)	16(38)	5(25)	0.308
Antibiotics in pregnancy#	21(16)	12(13)	10(21)	0.229
Steroids in pregnancy#	33(25)	18(20)	17(36)	**0.039**
Hyperemesis#	17(13)	7(8)	11(23)	**0.010**
Placenta praevia >grade 1 #	7(5)	1(1)	6(13)	**0.006**
Pyrexia >38°C/flu-like illness during pregnancy#	13(10)	5(6)	8(17)	0.061
Oligohydramnios#	25(19)	13(15)	14(30)	**0.032**
Poor fetal growth#	19(14)	9(10)	10(22)	0.066
Anemia <11g/dl#	43(32)	27(30)	17(37)	0.437
Polyhydramnios#	11(8)	10(11)	1(2)	0.097
Loss of fetal movements reported#	14(11)	8(9)	6(13)	0.554
Premature rupture of membranes#	29(22)	20(23)	11(24)	0.850
Intrauterine or urinary tract infection#	16(12)	10(11)	8(17)	0.331
Pregnancy-induced hypertension#	14(11)	7(8)	7(15)	0.235
Antepartum haemorrhage in 2/3 trimesters#	38(28)	22(24)	18(38)	0.090
Any of the above complications of pregnancy#	109(81)	71(79)	41(87)	0.230

* mean, standard deviation and p value (independent samples t-test)
# number, percentage and 2-tailed significance (Chi-square test)
^ median, interquartile range and asymptotic 2-tailed significance (Mann-Whitney U test)

ascites, duodenal atresia and hydrops. Complications of pregnancy were common affecting 81% of the entire cohort, in particular oligohydramnios (19%), anemia (32%), suspected intrauterine growth restriction (IUGR, 14%), premature rupture of membranes (PROM, 22%) and second or third

trimester antepartum haemorrhage (APH, 28%).

Comparison of the asphyxiated and non-asphyxiated cohorts showed that mothers of the non-asphyxiated (noBA) cohort were more likely to have received steroids during pregnancy (p=0.039), were more likely to have suffered from hyperemesis requiring inpatient management or outpatient medical treatment (p=0.01) and were more likely to have a low lying placenta (p=0.06). The mothers of the non-asphyxiated cohort were also more likely to have had oligohydramnios diagnosed on antenatal scan (p=0.032). Antenatal concerns about fetal growth restriction were common and were more common in the noBA cohort (p=0.066). Polyhydramnios was present in 8% of the asphyxiated cohort.

c) Intrapartum characteristics (Table 3.3)

Markers of fetal distress, such as meconium staining and CTG abnormalities were significantly more prevalent amongst the BA cohort (26% vs 11%, p=0.04; 59% vs 33%, p=0.004) in particular a terminal fetal bradycardia (p=0.002). Intrapartum infection, indicated by positive vaginal swabs and maternal pyrexia, increased white cell count or increased C reactive protein, occurred in 12 cases (E Coli and other coliforms, group B Streptococcus and Staphylococcus aureus) but was not more common in the BA group. Only 5/44 women with an epidural had a temperature of >38°C in labour. Malpresentation was less frequent in the BA cohort (29% vs 47%, p=0.037). Fifty percent of all early neonatal deaths had instrumental or operative

Table 3.3 Comparison of intrapartum characteristics

	All mothers (n=134)	BA (n=90)	NoBA (n=47)	Sig
Induction of labour#	13(10)	8(9)	5(11)	0.764
No labour#	29(22)	21(23)	8(17)	0.391
General anaesthetic in labour#	26(19)	19(21)	7(15)	0.378
Epidural in labour#	44(33)	32(36)	12(26)	0.233
Opiates in labour#	45(34)	28(31)	19(40)	0.276
Pyrexia in labour >38°C#	11(8)	7(8)	4(9)	1.000
Documented intrapartum infection#	12(9)	7(8)	5(11)	0.533
Bleeding in labour#	30(22)	21(23)	11(23)	0.993

	All cases (n=137)	BA (n=90)	NoBA (n=47)	Sig
Cord prolapse#	9(7)	7(8)	2(4)	0.718
Meconium staining#	28(20)	23(26)	5(11)	**0.040**
Bradycardia#	20/98(20)	20/72(28)	0	**0.002**
CTG abnormality reported#	51/98(52)	41/71(59)	10/27(33)	**0.004**
1st stage duration (h)^	2.1(0,6.6)	2.5(0, 6.8)	1.7(0,6.3)	0.726
2nd stage duration (m)^	5(0,0.5)	4(0,27)	7(0,30)	0.472
Time of ruptured membranes (h)^	2(0,14)	3(0,17)	1(0,7)	0.314
Malpresentation of fetus#	48(35)	26(29)	22(47)	**0.037**
Ruptured membranes >24h#	28(21)	19(22)	9(19)	0.691
Forceps/Ventouse delivery#	10(7)	7(8)	3(6)	1.000
Emergency caesarean section#	57(42)	41(46)	16(34)	0.194
Delivery out of hours (21:00-08:59 and weekends)#	83(61)	57(63)	26(55)	0.362
Sentinel hypoxic event#	34(25)	26(29)	8(17)	0.092

* mean, standard deviation and p value (independent samples t-test)
# number, percentage and 2-tailed significance (Chi-square test)
^ median, interquartile range and asymptotic 2-tailed significance (Mann-Whitney U test)

deliveries and 22% were delivered before the onset of labour for fetal distress or maternal emergency. Two babies were delivered by elective caesarean section pre-labour: one for a twin pregnancy in a primigravida (case 32) and the other for a previous caesarean section: the poor condition of both babies was unexpected at delivery.

There were 34 instances where a sentinel hypoxic event was evident such as

Table 3.4 Comparison of characteristics of infants at birth

	All infants (n=137)	BA (n=90)	noBA (n=47)	Sig
Male sex#	84 (61)	55 (61)	29 (62)	0.946
Gestation*	31 (6.4)	31.7 (6.7)	29.2(5.4)	**0.017**
< 37 weeks gestation#	99 (72)	58 (64)	41 (87)	**0.005**
Weight (g)*	1779 (1193)	1971(1268)	1411 (944)	**0.004**
Weight < 3rd centile for gestational age#	12(9)	9(10)	3(6)	0.545
Occipitofrontal circumference (cm)*	27.8 (6)	28.9(5.9)	26.1(4.3)	**0.015**
Apgar 0 at 1 minute#	18 (13)	18 (20)	0	**0.001**
1 minute Apgar^	2(1,5)	1(1,2)	5(4,9)	**<0.001**
5 minute Apgar^	4(2,8)	3(1,4)	8(7,9)	**<0.001**
10 minute Apgar^	5(1,8)	2(0,5)	9(8,9)	**<0.001**
Time to establish regular respirations (m)^	1 (0,5)	3(0,14)	0 (0,2)	**<0.001**
Cardiac massage required#	40 (29)	37 (41)	3(6)	**<0.001**
Intubation required#	113 (83)	81 (90)	32(68)	**0.001**
Age at death (h)^	15 (1,51)	10.3 (1,35)	41.6 (10,55)	**0.002**
Admitted to SCBU #	106 (77)	65(72)	41(87)	**0.046**

* mean, standard deviation and p value (independent samples t-test)
\# number, percentage and 2-tailed significance (Chi-square test)
^ median, interquartile range and asymptotic 2-tailed significance (Mann-Whitney U test)

massive abruption (10, cases 46, 50, 55, 69), cord accident (9, cases 8, 9, 31, 38, 41), ruptured uterus (3, cases 1, 5, 14) and others (12 in total, including delivery into a toilet, cases 59 and 67; fetomaternal bleed, cases 11 and 13; and severe difficulty in delivery, cases 57, 62, 65). This was no more common in asphyxiated babies.

d) Infant characteristics at birth (Table 3.4)

There were 38 infants who fulfilled criteria for birth asphyxia and 99 who were born in a good condition (see Chapter 1, Table 1). The two cohorts of infants were different with respect to gestation. The non-asphyxiated cohort were of younger gestation (29 vs 32, p=0.002) and consequently lighter in weight and with a smaller head circumference.

Despite antenatal concerns about fetal growth, only 9% of all infants proved to be small for gestational age at birth. The BA cohort was more likely to have lower Apgar scores, and require more resuscitation as a result. Eighteen (20%) of BA infants were asystolic at birth but showed signs of life after extensive resuscitation. The BA cohort died earlier compared with the non-asphyxiated infants (10.7h vs 39.1h, p=0.006). Of 137 infants, only 106 survived to be admitted to a neonatal unit. Six of the original 47 non-asphyxiated cohort were never admitted to a neonatal unit: three were found dead in their cots (cases 33, 35) and 3 suffered a sudden acute deterioration in the labour ward following a normal delivery (case 2) and resuscitation was subsequently unsuccessful, one a preterm triplet who was alive and in good condition for gestation following initial resuscitation. Twenty-five infants with birth asphyxia, though with signs of life at or shortly after birth could not be resuscitated sufficiently to get them to their local neonatal unit.

e) Infant characteristics in the first week (Table 3.5)

Details of infants admitted to a neonatal unit were often incomplete because of early death. There remained a preponderance of immature infants amongst the noBA cohort and this group had a lower first mean arterial blood pressure (34 vs 41, p=0.004).

Within the first hour of birth, those infants with BA had a markedly lower initial arterial blood pH (6.96 vs 7.27, p= <0.001). Infants with BA were more likely to have renal dysfunction (26% vs 15%, p=0.015), to require assisted ventilation for poor respiratory drive (33% vs 3%, p=<0.001) and to

Table 3.5 Comparison of neonatal characteristics

	All infants (n=106)	BA (n=65)	noBA (n=41)	Sig
< 37 weeks gestation#	80 (76)	42(65)	38 (93)	**0.001**
Initial pH within 1st hour*	7.12 (0.25)	6.96 (0.23)	7.25 (0.15)	**<0.001**
Time to reach pH ≥7.25 (h)^	4 (2,7)	5 (3,8)	3 (1,6)	**0.048**
First mean arterial pressure*	38(15)	40(15)	36(13)	0.207
Hematuria in first 24h#	32 (74)	19 (76)	13(72)	1.000
Creatinine >120#	23 (41)	17 (55)	6 (24)	**0.020**
Inotrope required#	35 (36)	20 (34)	15 (39)	0.644
Colloid required#	82 (84)	51 (86)	31(80)	0.362
Surfactant given#	56 (53)	26(40)	30(73)	**0.001**
Respiratory distress syndrome#	46 (46)	20(32)	26(67)	**0.001**
Ventilated for poor respiratory drive#	21 (21)	20 (33)	1(3)	**<0.001**
Abnormal coagulation#	46 (80)	28 (80)	18 (78)	1.000
Abnormal infection screen#	15 (18)	9 (18)	6 (17)	0.839
Abnormal liver function tests#	7 (29)	7 (47)	0	**0.022**
Hypoglycemia <2.6 mmol/l#	40 (43)	22 (40)	18 (47)	0.480
Hyperglycemia >8 mmol/l#	36 (39)	22 (40)	14 (37)	0.759
Necrotising enterocolitis#	5 (5)	4 (7)	1 (3)	0.645
Seizures#	12 (11)	11 (17)	1 (2)	**0.027**
Muscle relaxant#	22 (23)	8 (14)	14 (36)	**0.011**
[1]Abnormal neurology#	22/30 (73)	19/21(90%)	3/9 (33%)	**<0.001**
Abnormal cranial USS#	48/68(71)	31/40 (83)	17/28(61)	0.135
Age at death (h)^	36 (9,69)	25 (8,70)	43 (13,68)	0.250

* mean, standard deviation and p value (independent samples t-test)
\# number, percentage and 2-tailed significance (Chi-square test)
^ median, interquartile range and asymptotic 2-tailed significance (Mann-Whitney U test)
1. alive >12hours, not receiving sedation, paralysis, anticonvulsants

have biochemical evidence of liver dysfunction (47% vs 0, p=0.022). In keeping with their shorter gestation the noBA cohort had a greater incidence of respiratory distress syndrome and were more likely to receive exogenous surfactant and muscular paralysis. In those infants who survived longer than twelve hours and who had not received sedation, paralysis or prophylactic anticonvulsants, abnormal neurology was documented more commonly in the BA cohort (p<0.001). Overt clinical seizures occurred almost exclusively in

asphyxiated infants. Twelve infants had an EEG performed; all were abnormal ranging from isoelectric trace in the majority to bilateral seizure activity. An abnormal infection screen was found in 15 of the total group, of which 12 were thought to have died of overwhelming sepsis. Pathogens included Group B streptococcus (7), staphylococcus aureus (2), Escherichia Coli (2) and others (4). The groups were comparable for other systemic dysfunction, in particular coagulopathy, necrotising enterocolitis, cardiovascular instability and glucose homeostasis. Cranial USS was performed in 48 babies: intraventricular haemorrhage was found in 27 infants, appearances suggestive of parenchymal damage in 8, and a mixture of these appearances in 4. Nine infants were reported as having slit-like ventricles and one infant had ventricular calcification. Most infants in the BA cohort died within the 24hrs while the NoBA cohort survived longer.

Table 3.6. Clinical cause of death in 137 early neonatal deaths

	BA (n=90)	NoBA (n=47)
Intrapartum hypoxic-ischemic injury	35 (39%)	-
Respiratory disease in preterm infant	25 (28%)	23 (49%)
Intracranial bleed	7 (8%)	7 (21%)
Infection	7 (8%)	5 (11%)
Congenital anomaly	4 (4%)	2 (4%)
Other: meconium aspiration, hydrops, pulmonary hypoplasia	3 (3%)	1 (2%)
Unexplained	9 (10%)	9 (20%)

No infants in the NoBA group died from supposed intrapartum hypoxia. Rates of infection and congenital anomaly were similar, and more infants in the NoBA group died from complications of prematurity. At the time of data collection, 13% of deaths remained unexplained (cases 2,4, 7, 9, 18, 19, 29, 32, 35, 61, 62).

Discussion

The aim of this study was to describe the maternal, pregnancy and delivery characteristics of a population where pregnancy ended in an early neonatal death and to ascertain whether any of these parameters were different in asphyxiated infants. Asphyxia-related death is generally regarded as avoidable mortality particularly when occurring in full term normally formed infants, and although new fetal monitoring techniques and intrapartum care have reduced the number of such deaths, longterm neuromorbidity, which is often attributed to 'birth asphyxia', has remained unchanged for decades.

This geographically-defined population-based study included all normally formed infants equal to or greater than 24 weeks gestation who died within the first week of life in all of the 22 delivery units within Scotland. Forty-seven infants had malformations which excluded them from the study. This was equivalent to 21% of the population, and does not include those 6 babies suitable for inclusion who died of malformations outwith the exclusion criteria, such as bilateral renal agenesis and laryngeal atresia. Ethical approval at local level allowed collection of anonymised clinical data in 137 infants irrespective of enrolment into the neuropathology arm of the study.

The risk factors for perinatal mortality, asphyxia and perinatal brain damage are heterogeneous and likely to involve complex interactions between the mother, the fetus, and their respective environments. It was therefore essential to study the risk factors within a population rather than individuals

or small cohorts. Although Scotland has different demographics to those of the entire United Kingdom, especially with regard to social deprivation scores and female smoking patterns, the population is generally representative of the developed world. Hospital-based studies may suffer from selection-bias and this is especially true of those conducted in specialist centres or tertiary neonatal units where there may be an excess of extreme prematurity or surgical conditions. Regional studies may not be truly representative of the entire population and may emphasise the role of particular risk factors or conditions found more commonly within that area. Population-based studies of larger numbers may reduce such statistical variation.

Definition of birth asphyxia

The study definition of 'birth asphyxia' may nowadays be criticised as overly liberal and not sufficiently discriminatory to detect infants who have suffered damaging asphyxia. One of the principle aims of the base study was to determine whether infants with antenatal brain damage might be predisposed to neurological depression at birth which might be labelled as birth asphyxia. Although evidence shows that Apgar scores and pH may be relatively poor predictors of long term neuromorbidity, these features, when present at birth, are often understood by the public and medicolegal profession to indicate fetal compromise during labour. An Apgar score at 5 minutes of ≤ 5.0 is the traditional assessment of birth asphyxia and it is widely recognised that a low 5 minute Apgar score has an association, though weak, with both

neonatal death and morbidity in surviving infants[107;137]. Although prematurity is known to affect assignment of the Apgar score and the classical signs of hypoxic-ischemic encephalopathy, this effect is less marked as gestation increases. Many infants of 34-36 weeks gestation are able to achieve normal Apgar scores while those of lower gestations may exhibit reduced tone and decreased respiratory drive under normal conditions. Prematurity was significantly commoner within the noBA cohort although two thirds of those who were asphyxiated were also preterm. Among those preterm infants who were classified as having birth asphyxia, a 5 minute Apgar score of ≤ 5 alone was found in 35 of 59 cases. Although some might argue that depressed scores are more common in lower gestations, a score this low is unlikely to be due to prematurity alone especially as 22 of these infants died before reaching the neonatal unit. Other conditions such as infection and neuromuscular conditions may also result in a low Apgar score and although there was no obvious clinical evidence of the latter among this population, infection may certainly have been common and may have contributed to depression at birth. Infection can also result in acidosis at birth, as can rarer congenital metabolic disorders. None of the infants who died were felt to have a primary metabolic acidosis.

Obstetric epidemiology has shown that a scalp pH of less than 7.25 is abnormal and delivery is indicated if less than 7.2[152]. The relationship between scalp and cord pH is good with a sensitivity of 93%[153]. However the neonate is rarely difficult to resuscitate unless the cord pH is less than 7.0. In

a large study of full term infants a pH of 7.1 represented 2SDs below the mean[103]. This value was chosen as indicating some degree of birth asphyxia within this project. Recognising the limitations (in the absence of a cord pH) a first blood gas with a pH less than 7.1 was also taken to indicate asphyxia. This was, in all cases, taken before an hour of age. A value for base deficit or plasma bicarbonate was rarely recorded and it was thus not possible to be certain that the cause of the acidosis was entirely ischemic in origin. As some of the infants went on to develop respiratory disease it is possible that in some cases carbon dioxide retention compounded the acidosis. The presence of acidosis is also not specific for asphyxia and may occur in the presence of infection, which affected at least 15 infants in the cohort.

The presence of grade 2/3 neonatal encephalopathy is now widely accepted as having a closer association with significant birth asphyxia and long term neurodevelopmental disability[9;9;107]. Although many infants in the study were classified as having abnormal neurology, in only a few was a diagnosis of hypoxic-ischemic encephalopathy possible. The main reason was that many infants died within a few hours of birth and a proportion of the remainder were treated with paralytic agents, sedation or prophylactic anticonvulsants. Some infants had neurological symptoms which did not fit the diagnosis of hypoxic-ischemic encephalopathy: for example preterm infants who had a normal neurological examination in the first days of life, followed by seizures related to an intraventricular haemorrhage thereafter. Other preterm infants were described as being very lethargic or abnormally jittery but

without progression of symptoms or associated neurological signs. One term infant had hypoxic-ischemic encephalopathy and multi-organ dysfunction but with an Apgar score at 5 mins of 6 and a cord pH of 7.11 with normal cardiotocograph monitoring. This baby had a CT scan in the first few days of life which showed widespread severe cerebral oedema with absent supratentorial perfusion and haemorrhage. This infant was thought to have suffered a recent antenatal hypoxic-ischemic insult.

Clinical data and ascertainment

Review of the relevant literature about perinatal mortality, birth asphyxia and asphyxia-related neuromorbidity highlighted many risk factors which the study sought to examine. Although many data points needed to be collected for each case, ascertainment of accurate and complete data was ensured by individual review of maternal and neonatal notes for each death. Where appropriate, data points carried a definition derived from well-recognised texts to ensure standardisation of responses. A meeting between myself, the link midwife, obstetrician and paediatrician in each case within two weeks of the death of the infant allowed accuracy of data collection to be verified. Each unit was awarded a small fee for the time invested by midwives in completing each form. Although the Information and Statistics Division of the National Health Service in Scotland collects national annual perinatal mortality statistics, direct validation of study numbers with these statistics could not be performed, as the date that each delivery unit started recruiting cases was different for each centre. However, study numbers did approximate

national statistics. The study only included deaths within neonatal or delivery units. Those infants who died in the first week of life at home, in a surgical unit or in a paediatric hospital were not included. Such infants may have died from infective causes or for surgical reasons but almost certainly not from intrapartum asphyxia. The number of these infants is likely to be very small.

Inclusion and exclusion criteria

All early neonatal deaths equal to or over 24 weeks gestation and equal to or less than 7 days of age included in the study. Enrolment in the study involved detailed neuropathological examination for evidence of antenatal hypoxic damage. Cellular reaction to hypoxia is increasingly difficult to substantiate in progressively immature brains and a minimum of 24 weeks was chosen. There were a few liveborn infants of twenty-three weeks gestation who died during the study period but these were not included. With increasing duration of survival, superimposed recent damage may complicate interpretation of more longstanding damage and only infants less than 7 days were included for this reason.

Infants with CNS malformations and chromosomal abnormalities were excluded from enrolment as these are often accompanied by disordered brain architecture. CNS infections may result in widespread infiltration of inflammatory cells and these changes may be difficult to distinguish from the cellular reaction seen following hypoxia. Infants with cardiothoracic

malformations were also excluded as circulatory disturbance in these conditions may itself result in hypoxic or ischemic damage in the brain.

Discussion of results

The epidemiological background of this cohort is similar to other recent studies from the developed world[50;58;59]. Mothers in the study had similar demographics to those of all Scottish maternities resulting in a stillbirth or live birth in 1996-1998, in particular maternal age, height, marital status and parity[83]. The proportion of pregnancies where booking women were smokers was considerably higher than that of all Scottish maternities (37% vs 29%)[83].

Around a third of women had experienced pregnancy loss or infertility in previous pregnancies and this is in agreement with previous studies which have shown increased perinatal mortality[154;155] and neonatal encephalopathy[58] in subsequent pregnancies. Lilienfield and Parkhurst found an association between previous infant loss and cerebral palsy and postulated the existence of a 'continuum of reproductive wastage' with lethal (pregnancy loss) and sublethal manifestations (cerebral palsy)[156]. A more recent study showed an increase in cerebral palsy in children born after in vitro fertilisation [157].

Most complications studied in this cohort were more common than those found in all Scottish maternities. For example, antepartum haemorrhage, intrauterine infection, intrauterine growth retardation, polyhydramnios,

hyperemesis and oligohydramnios each affected less than 4% of all maternities over the study period. Premature rupture of the membranes affected around 5%, and anaemia, 14% of Scottish maternities over the study time period[83]. Pregnancies with antenatal complications are known to be at high risk of neonatal morbidity and mortality[138;142;158-161] and high risk obstetric clinics are in place in many centres to identify a fetus at risk of compromise and pre-empt poor outcome. In deciding the best option for a fetus at risk, a balance has to be met between expeditious delivery from a hostile uterine environment and the hazards of premature existence. Both options may have disadvantages for the fetus and may result in mortality. Eight-one percent of study mothers had pregnancy complications identified and despite such surveillance, the pregnancy ended in neonatal death. Death may have resulted due to late presentation, inadequate surveillance or iatrogenic prematurity. Although not significantly different (statistically), most complications were commoner in non-asphyxiated infants suggesting that identification of these infants may have led to improved condition at birth. Despite having no recognised antenatal complications, nineteen percent of study infants died. This is consistent with other data which show that the absence of maternal complications does not protect the infant from a neonatal complication or death[161-163].

Analysis of the detailed data from the mother and her pregnancy did not identify any reliable predictors for birth asphyxia. Significant placenta praevia, oligohydramnios and hyperemesis were protective against asphyxia

in general, possibly because these mothers were more intensively monitored. Similarly those mothers who received steroids were less likely to produce an infant with signs of asphyxia, perhaps because they were under medical care before and after steroid administration. A history of viral illness in pregnancy has previously been found to be associated with NNE[58]. Our series did not show this association and pyrexial illness in pregnancy was more common in those pregnancies resulting in non-asphyxiated infants. Intrauterine growth restriction has previously been strongly associated with NNE[50;58;132;134;139] and affected 9% of our population most of whom were non-asphyxiated infants.

Around 20% of the cohort were delivered before the onset of labour for reasons of fetal or maternal compromise. Many of these infants had signs of asphyxia at birth despite avoidance of labour suggesting that they may have been subject to a hypoxic-ischemic process antenatally. The incidence of meconium staining in all Scottish deliveries of that time period was 7% compared to the 20% seen in the neonatal deaths of this study. Although meconium staining alone has a high false positive rate[164] it is associated with increased perinatal mortality and morbidity[165]. It has been hypothesized that intra-amniotic meconium may cause vasoconstriction of the umbilical vessels inducing fetal hypoxia/ischemia[166]. This is difficult to substantiate after delivery. Although meconium stained liquor affected mostly term deliveries, it also complicated around 10% of preterm deliveries. Although historically, preterm in utero passage of meconium was thought to be pathognomic of

Listeria infection, a recent study has shown meconium staining to affect around 5% of all deliveries less than 33 weeks gestation and is associated with prolonged rupture of the membranes but not listeriosis. These infants were found to be at an increased risk of intraventricular hemorrhage[167]. Within our cohort, only two of the 13 preterm infants with meconium staining had ruptured membranes for more than 24 hours. Listeria was not isolated in any infant. Ten of these infants were classified as having birth asphyxia. Only two infants survived long enough for a head USS to be performed and in both intraventricular haemorrhages were present.

The need for induction of labour was low and reflects the high number of preterm deliveries that were spontaneous in onset. It has been postulated that spontaneous preterm labour is an adaptive response initiated by the fetus to escape an unfavourable uterine environment. Emergency caesarean section for fetal compromise was common and more than double the national caesarean section rate. Abnormal presentation which is associated with birth asphyxia in term infants in the literature[140] was almost exclusively confined to infants <32 weeks gestation and was in fact less common in the asphyxiated cohort.

Intrapartum infection and pyrexia have strong associations with both 'birth asphyxia' and neonatal encephalopathy[59;148;149;151]. Incidence of documented intrapartum infection in Scottish maternities is reported as 0.16%. This is likely to be an underestimate as recording of data takes place soon after

delivery and before culture results may be available. Despite this, the incidence of intrapartum infection within this study is considerable. All cases had isolation of organism in the mother associated with pyrexia in labour, histological chorioamnionitis or maternal white cell changes with clinical suspicion of infection. However there was no evidence within the cohort that maternal infection or pyrexia were commoner within the asphyxiated group. Epidural analgesia may result in maternal pyrexia but this association was not seen in this study where epidurals were common. Prolonged rupture of membranes for more than 24 hours before delivery (PROM) has been associated with neonatal encephalopathy[50] but not in other series[149;151]. There was a high incidence of PROM within our cohort but not more so within the BA group. PROM was associated with prematurity, documented intrapartum infection and pyrexia in labour.

Perinatal mortality is associated with delivery at night or at weekends[168-170]. This study did not show an excess of deaths following delivery at these times. However, 80% of infants who were the most severely depressed at birth (stillborn but showed some signs of life following prolonged resuscitation) were delivered out of normal working hours. In only one case was a consultant obstetrician present at delivery. Although intrapartum asphyxia is often attributed to an acute catastrophe during labour, a sentinel event such as this was only present in a quarter of all deaths and not significantly more so in infants with signs of asphyxia. Seventeen percent of non-asphyxiated infants had an acute perinatal event which had the potential

to result in hypoxia-ischemia. These cases were all preterm infants who had normal Apgar scores and no evidence of acidosis.

Examination of intrapartum factors revealed no significant differences between BA and noBA cohorts apart from meconium staining of the liquor and CTG abnormalities. Both these features are well known associations of birth asphyxia. The fact that there is more intensive monitoring during labour than at any other time during pregnancy means that any injuries sustained antenatally may not become manifest until the commencement of labour or until the normal stresses of labour uncover signs of a compromised fetus. This leads to the assumption that the fetus was healthy until labour and it was only at this point that problems occurred. If a fetus has experienced neurological insult or maldevelopment during pregnancy, this damage may affect parts of the brain responsible for autonomic control of activities such as heart rate and respiration[171]. Thus the classical signs of intrapartum stress such as reduced heart rate variability, and meconium passage in utero and low Apgar scores may all represent the first recognised signs of a previously compromised fetus. It is not possible to recognise the point at which brain damage becomes irreversible in such chronic compromise. It is possible that this point may be reached during a labour if the fetus has been able to compensate until that time. It could then be argued that if delivery were to be expedited by caesarean section for example, further irreversible damage could be prevented.

There was a preponderance of male infants at birth in keeping with the increased mortality for boys at all ages[172]. The majority of deaths were in preterm infants who made up three quarters of the study cohort. Non-asphyxiated infants were more likely to be preterm and this might reflect the fact that immature and more simple brain may be more resistant to an asphyxial environment. Asphyxiated infants were in poorer condition, required more extensive resuscitation and were less likely to survive long enough to be admitted to a neonatal unit. Neonatal deaths are concentrated increasingly at the start of the neonatal period and one third of infant deaths occur within the first 24 hours of life[173]. Most infants in the study died within the first day of life and asphyxiated infants died earlier.

Many infants received sedation or paralysis so the true incidence of neurological symptoms is likely to be underestimated. In addition preterm infants may not exhibit the classical signs of encephalopathy as often as mature infants. Neurological examinations were performed by the clinician in charge of the infant's care and were not standardised. However, review of the infant's examination took place between the clinician and the author following the death of the infant. Neurological abnormalities were defined as profound hypotonia, prolonged jitteriness, brainstem release phenomena, lip smacking, prolonged clonus, hypertonia, seizures, coma. Feeding difficulty was common to most infants within the study and was not included as a neurological symptom. Infants admitted to a unit who survived less than 12 hours of age were generally obtunded throughout their short life and in most

cases this was due to severe cardiorespiratory insufficiency. Although only 12 of our cohort exhibited seizures, recent work suggests that many subtle clinical seizures may be missed[174] without a full electroencephalographic record. This may be especially true among preterm infants or where seizures are brief or focal. Similarly, abnormalities of neurological examination may not be appreciated in the sick preterm infant especially if their condition prohibits much handling. Most units do not have easy access to EEG monitoring and at the time of the study only one neonatal unit had cerebral function monitoring in use. All infants who had seizures had an EEG performed and in all cases this was profoundly abnormal.

This study reports the maternal and pregnancy characteristics of a population of early neonatal deaths and describes the condition of these infants at birth and their progress over the first week of life. Attempt was made to identify features in the mothers, pregnancies and labours which might predict the delivery of an asphyxiated infant. Despite 66% of infants born in an asphyxiated state, no reliable indicator could be found to identify such infants apart from abnormal CTG monitoring and meconium staining in labour. This might suggest that the processes leading to neonatal death are similar for the two groups or that other factors not examined might be more definitive. It is possible for example that genetic or environmental influences may be involved. Infertility was common among our cohort as was a previous pregnancy loss. Family history of seizures, learning disability or cerebral palsy was sought among mothers in the study but many midwives

and clinicians felt unable to approach mothers for this information at a time of great distress. Similarly a detailed dietary, smoking, alcohol and recreational drug history was not possible.

Despite the identification and surveillance of high risk pregnancies many infants continue to be born in a depressed condition and eventually die. Although many infants in this study did not respond to resuscitative measures over the first day of life, many others had sustained neurological damage manifest as hypoxic-ischemic encephalopathy and intraventricular haemorrhage which was so severe that it was thought to be incompatible with any meaningful existence. In these cases a decision was made involving the parents to withdraw life-prolonging treatment. Although the majority of neonatal deaths occur in premature infants or in infants with congenital anomalies which are incompatible with life, many others may be preventable. Both perinatal infection and asphyxial injury remain important potentially avoidable causes of neonatal death. Identification of such infants can be difficult and neonatal management of infection and asphyxia have changed little over the last thirty years. Accurate detection of maternal infection is difficult and routine screening results in many mothers and babies treated unnecessarily. Detection of fetal hypoxia during labour depends largely on non-specific signs none of which inform of damage to the fetus. Fetal injury during pregnancy is even more difficult to ascertain. Newer rescue strategies such as immunoglobulin for neonatal sepsis and hypothermia for hypoxic-ischemic encephalopathy are being researched currently.

This study has demonstrated that the current battery of pregnancy and labour-associated investigations remain blunt instruments in accurately predicting the arrival of an asphyxiated infant. Current evidence suggests that neonatal encephalopathy may be the manifestation of a number of different morbidities, some occurring antenatally, others during labour and still others as chronic or repeated insults spanning both periods. It is highly unlikely that one causal pathway will be determined and instead there is likely to be considerable heterogeneity among aetiologies. Future work must address the development of methods for detecting fetuses at risk so that optimal intrapartum and postnatal management can be planned.

Chapter 4
Apolipoprotein E genotyping and brain damage in early neonatal deaths*

Introduction and background

Apolipoprotein E polymorphism and genetics

Apolipoprotein E (apoE) is a 299 amino acid glycosylated protein which has a major role in cholesterol and lipoprotein metabolism[175]. The major isoforms of apoE (apoE2, apoE3 and apoE4) are products of three alleles (ε2, ε3 and ε4) located on the long arm of chromosome 19[176]. Expression of these alleles result in six APOE genotypes: ε2/ε2, ε3/ε2, ε3/ε3, ε4/ε2, ε4/ε3, ε4/ε4. Although the isoforms differ only by single amino acid substitutions, this distinction has profound functional implications at a cellular level.

In all populations the ε3 allele is the most frequent, ranging from 50-90%, with the frequency of ε4 and ε2 ranging from 5-35% and 1-15% respectively. Within the UK the relative frequencies of the APOE alleles are approximately as follows: ε3 77%, ε4 15% and ε2 8%[177]. ε3 seems to be the normal isoform in all known functions, and is also the most common isoform. ε2 and ε4 can be dysfunctional and have a definite impact on lipid and lipoprotein levels.

* This chapter contains some text from published work reproduced with consent from co-authors and publishers[347;348].

ApoE and lipid metabolism

ApoE is an intracellular cholesterol and fatty acid transport protein that plays an important role in lipid metabolism. It is synthesised and secreted by many tissues throughout the body but particularly by tissue macrophages in the liver, brain and skin. The principal role of apoE is to transport lipids from one tissue to another. ApoE is a component of VLDL particles as they are excreted from the liver and is acquired by chylomicrons on synthesis by the small intestine.

Much of the early clinical interest in apoE related to hyperlipidemia, atherosclerosis and the risk of coronary heart disease[177]. Isoform-specific differences include the association of ε2 with type III hyperlipidemia and with both increased and decreased risk for atherosclerosis and the association of ε4 with an increased risk of high cholesterol levels and atherosclerosis.

Apolipoprotein E and the nervous system

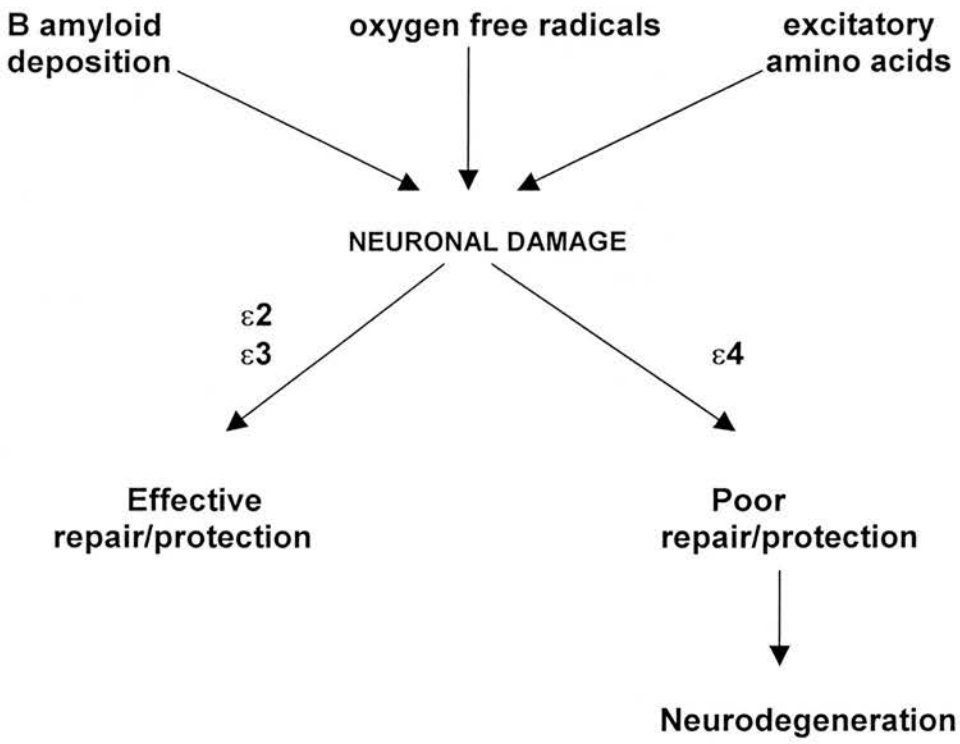
By the middle of the 1980s, evidence began to emerge that apoE played an important role in neurological diseases. It is unique among lipoproteins in that it is involved in the recovery response of injured nerve tissue.

The brain is the second major site of apoE messenger RNA expression in humans and contains one third of the levels found in the liver. ApoE is synthesised by the central nervous system glia and in particular by astrocytes[178]. ApoE is the principal lipid transport vehicle in cerebrospinal fluid and appears to play a key role in neuronal repair by distributing lipids

to regenerating axons and to Schwann cells during remyelination. This lipid transport system provides injured neurons with cholesterol and phospholipids allowing the potential for repair of cell membranes, growth of neurites, dendritic remodelling and synaptogenesis. From the study of animal models of acute nerve injury it is proposed that degenerating cellular components are taken up by astrocytes and packaged together with apoE into lipoprotein complexes[179]. These complexes are secreted into the extracellular space and bind to specific receptors on neurons and oligodendroglial cells and are subsequently internalised. The lipids obtained in this way may be used for cellular repair. Following injury there is a coordinated increase in expression of apoE by astrocytes and LDL receptors by neurons, which facilitates transport of lipids by this system. For example, apoE synthesis is induced dramatically (250-350-fold) following neuronal injury, in particular following injury to peripheral nerves[38], optic nerves[180]and spinal cord[181]. In cerebral ischemia, apoE has been found in neurons in both rat and gerbil models[182;183] where it is thought to have been internalised following secretion by astrocytes. However, apoE appears to also have more complex roles than that of lipid transport in traumatic or ischaemic nerve injury: it has been found localised in the cytoplasm of cortical hippocampal neurons in patients with Alzheimer's and Parkinson's disease, and in epilepsy[184;185]. There is growing evidence that apoE, lipid metabolism and immune responses are related not only systemically but also in the central nervous system[186]. ApoE appears to suppress the production of various pro-inflammatory cytokines, including TNFα, IL1β and IL-6, in neural cells[37;187].

Accumulating evidence supports a role for APOE isoform-related effects on the neuronal repair mechanism and the maintenance of synapses and dendrites. In vitro work shows that ε3 enhances neurite outgrowth whereas ε4 decreases neurite outgrowth[39]. This effect is mediated by changes in microtubule assembly affecting the neuronal cytoskeleton. Such observations raise the possibility that individuals may vary in their susceptibility to the effects of acute brain injury according to their APOE phenotype (Figure 4.1).

Figure 4.1. Isoform-specific effects on neuronal repair and protection



(Modified from Weisgraber and Mahley[188]and reproduced with permission of the Federal Academy of Experimental Biology)

In 1993, Corder and colleagues discovered that APOE is a major susceptibility gene associated with over half of cases of sporadic and familial Alzheimer's disease. ε4 was the isoform responsible for this effect, increasing the occurrence and lowering the age of onset of the disease[189]. More recent work has shown that individuals have a genetic predisposition to amyloid β-protein deposition following head injury conferred by ε4[190]. Deposition of β-amyloid protein in the brain plays a key role in the pathogenesis of Alzheimer's disease suggesting a possible association between APOE genotype and head injury in the development of Alzheimer's disease. Research on mice has shown APOE isoform-specific effects on learning and memory[191] and in humans evidence is beginning to emerge that ε4 expression may negatively affect cognitive function in middle aged adults before any symptoms of Alzheimer's disease are present[192;193]. A higher frequency of ε4 has also been reported in patients who do not recover from post-traumatic coma compared to those who recover consciousness[194]. In another study, patients who died after spontaneous intracerebral haemorrhage had an excess of ε4, and of the survivors those with ε4 had poorer neurological function[195]. Head injury in an ε4 carrier is more likely to lead to death[196] and ε4 carriers undergoing cardiac bypass recover neuropsychological functions less efficiently than those without an ε4 allele[197].

Although often supposed to be the 'good allele' a link has been established between ε2 and amyloid angiopathy[198], sporadic Parkinson's disease[199] and

argyrophilic grain dementia[200]. Little is known about the function of the APOE gene on the brain in childhood.

The most likely time to sustain a neurological insult is before or during birth. At least 60% of term and 20% of preterm[10] infants with subsequent cerebral palsy are probably disabled by antepartum factors. Although many etiological factors, including metabolic, mitochondrial and genetic factors, play a role, intrapartum hypoxic-ischemic encephalopathy remains an important cause of childhood cerebral palsy in the term infant. Many infants who suffer a brain insult recover with no long-term effects[201]. However, a proportion die in the neonatal period due to the severity of their injury, and others survive but with a range of permanent neurological abnormalities which manifest as cerebral palsy and/or cognitive deficits. There are few markers to predict the degree of neurological sequelae after such an insult.

Much of what is known of the role of apoE in the central nervous system relates to mature brain tissue. There have been no studies looking at the effect of APOE genotype on the developing infant or its nervous system. In this study we examined the distribution of APOE genotype in healthy infants and perinatal deaths and hypothesised that isoform–related differences in APOE might predispose to brain injury in the perinatal period. We also sought to determine whether the APOE genotype was associated with differences in fetal/infant birth weight and other clinical parameters.

Methods

Aims and objectives

The aims of the study were

1. To compare the relative frequencies of the Apolipoprotein E allele in 3 Scottish populations:

    Group 1: healthy newborn infants

    Group 2: perinatal deaths (early neonatal deaths and stillborn infants)

    Group 3: healthy middle aged adults[202]

2. To investigate the relationship of Apolipoprotein E genotype of perinatal deaths with neuropathology

3. To compare the Apolipoprotein E genotype of perinatal deaths with clinical features

Design

This study was a retrospective cross-sectional cohort analysis.

Subjects

Adults: 400 healthy late middle aged men and women who were randomly selected from General Practitioner lists in the North East of Scotland[202]. No other demographic information regarding this population has been published.

Healthy newborn infants: dried blood spots were collected anonymously and randomly from the placentas of 371 healthy infants born in the Simpson Memorial Maternity Pavilion, Royal Infirmary of Edinburgh. All infants

were >35 weeks gestation and had a birthweight greater than 2 kg. All infants were healthy at birth and all left hospital when due to do so. The blood was sampled anonymously and no other demographic information was collected.

Perinatal deaths: DNA was extracted from formalin-fixed paraffin embedded brain blocks of 252 perinatal deaths enrolled in the population-based Scottish Perinatal Neuropathology Study (Chapter 1). APOE genotyping was performed in 68 early neonatal deaths and 186 stillbirths. Two pairs of twins were enrolled in this study, one pair liveborn and one stillborn. For the purposes of this study, the second twin was excluded from analysis. In other multiple pregnancies occurring in the study, only one infant of the siblings had died. No other sibling pregnancies were entered in this two year study. Therefore this study investigated 261 perinatal deaths (67 early neonatal deaths and 185 stillbirths), each in a different family.

Ethics

This study was approved by the Lothian Research Ethics Committee (LREC 1998/6/53).

Funding

This study was funded by the Scottish Office K/MRS/50/C2739 £63,972. Role of apolipoprotein E genotype in perinatal hypoxic/ischaemic encephalopathy. Principal grant holder: Professor Jeanne E Bell. Other grant holders: Professor Neil McIntosh, Dr Julie-Clare Becher, Dr Jean W Keeling.

Sample analysis

The hot start polymerase chain reaction (PCR) method for APOE genotyping was based on that described by Hixson and Vernier[203] and was adapted for analysis of formalin fixed, paraffin embedded tissue. DNA was extracted in each case from two or three 5μm sections using the Qiagen's DNeasy tissue kit (Catalogue No: 69506). The sections were placed in Qiagen tissue lysis buffer and vortex mixed before incubating with proteinase K at 50^0C in a water bath for 48 hours. After further vortex mixing incubation was performed at 70^0C for 10 minutes before mixing with absolute alcohol. The mixture was subjected to centrifugation at 13,000rpm in Qiagen DNeasy spin columns. Finally the eluted DNA solution was added to a master mix solution containing customised primers (MWG Biotech) as follows:

Downstream primer: ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A

Upstream primer: TCC AAG GAG CTG CAG GCG GCG CA

The master mix also contained 5μl of x 10 PCR buffer, 8μl of 5mM nucleotides, 1.5μl 50mM Mg Chloride and 1.25μl Taq polymerase (Invitrogen) as well as 20pmol of each primer.

DNA cycling was run on a Techne Flexigene Thermocycler at 95^0C for 3 minutes followed by 45 cycles at 94^0C for 1 minute, 62^0C for 1 minute and 72^0C for 2 minutes with a final extension of 72^0C for 8 minutes. 10μl of each PCR reaction was run on a 3% agarose gel and visualised with ethidium bromide under ultraviolet light. This revealed the PCR product of 227 base

pairs. PCR product was mixed with 1µl Hha1 restriction digest enzyme (New England Biolabs) at 37^0C overnight. Samples were then run on a 4% Metaphor gel and again visualised with ethidium bromide under ultraviolet light for photography.

The genotype for each sample was determined from the migration pattern of bands in the gel. PCR was repeated for cases in which the band pattern was faint or equivocal until a definite result was obtained. In the small number of cases in which paraffin embedded brain tissue failed to yield a result despite repeat investigation, further attempts to extract DNA switched to non-brain tissue blocks and these occasionally provided a result when the brain had proved negative. Paraffin blocks of spleen were used for this purpose.

This work was performed by Ian Croy and Ian Anthony in the Molecular Biology Department of the Western General Hospital, Edinburgh.

Statistics

Descriptive statistics were used to examine the prevalence of the different alleles. Hardy-Weinberg expectation was calculated for each cohort to ascertain population equilibrium. The Chi square test for trend was used to compare allele frequencies between groups and to compare allele frequency with neuropathology and clinical parameters. Significance is assumed at the $p<0.05$ level.

Results

Unequivocal results with clear APOE banding patterns were obtained for 67 of 70 liveborn infants who died at 7 days of age or less and 186 of 191 stillborn fetuses of at least 24 weeks gestation. Of the two pairs of twins in this study, all four infants proved to be APOE 2/3. The second twin of each pair was excluded from further analysis. APOE genotypes of 371 healthy newborns were performed. Spleen blocks were used in 8 cases all other results were from brain tissue.

1. Comparison of allele frequencies between cohorts

The distribution of APOE alleles and genotypes for these groups is shown in Tables 4.1.A-C which also include previously published data for 400 Scottish adults[202]. Table 4.1.C shows the Hardy-Weinberg expectation for each population and shows that departure from this expectation is trivial. Table 4.2 compares the different groups and shows significance values as calculated by the chi-square test for trend.

APOE ε2

There is an over-representation of ε2 in perinatal deaths (13%) compared with healthy newborns (8%, p=0.012) and with adults (8%, p=0.007). Taking the stillbirths alone, the increased prevalence of the ε2 allele remains significant compared with healthy newborns (p=0.019) and with adults (p=0.011). The prevalence of all genotypes possessing one or more ε2 alleles (2/2, 2/3 and 2/4) was higher among perinatal deaths than among healthy

liveborns and adults (Table 4.1.B).

APOE ε3

The prevalence of the ε3 allele is higher in adults than in healthy newborn infants (p=0.042) and early neonatal deaths (p=0.023).

APOE ε4

The prevalence of ε4 is higher in healthy liveborns than in both normal adults (p=0.026) and stillbirths (p=0.012). Review of Table 4.1.A suggests that liveborn infants who die in the perinatal period have over-representation of both ε2 and ε4 alleles compared with normal adults but the number of cases (n=67) is too small to place reliance on statistical analysis.

Table 4.1. Comparison of Apolipoprotein E Alleles and Genotype

4.1.A. Distribution of APOE Alleles

Cohort	ε2	ε3	ε4	Total alleles
Adults	66 (8%)	616 (77%)	118 (15%)	**800**
Healthy Newborn	63 (8%)	538 (72%)	141 (19%)	**742**
Perinatal Deaths	65 (13%)	365 (72%)	74 (15%)	**504**
Early neonatal deaths	17(13%)	91(68%)	26(19%)	**134**
Stillbirths	48(13%)	274(74%)	48(13%)	**370**

4.1.B. Distribution of APOE genotypes

Cohort	2/2	3/3	4/4	2/3	2/4	3/4
Adults (n=400)	2 (0.5%)	233 (58.2%)	4 (1%)	51 (12.8%)	11 (2.7%)	99 (24.8%)
Healthy Newborns (n=371)	2 (0.5%)	199 (53.6%)	15 (4%)	44 (11.9%)	15 (4%)	96 (25.9%)
Perinatal deaths (n=252)	5 (2%)	138 (54.8%)	3 (1.2%)	38 (15.1%)	17 (6.7%)	51 (20.2%)
Early neonatal deaths (n=67)	2 (3%)	28 (41.8%)	0	11 (16.4%)	2 (3%)	24 (35.8%)
Stillbirths (n=185)	3 (1.6%)	110 (59.5%)	3 (1.6%)	27 (14.6%)	15 (8.1%)	27 (14.6%)

4.1.C. Distribution of alleles with Hardy-Weinberg expectation

	ε3/ε3 (homozygotes)	ε3/- (heterozygotes)	Others (ε3 null)	Total	p value
Adults					
Observed	232	152	16	400	0.63
Expected	237	142	21	400	
Healthy Newborns					
Observed	192	139	31	362	0.58
Expected	188	146	28	362	
Perinatal deaths					
Observed	133	85	24	242	0.55
Expected	129	95	18	242	

Table 4.2. Comparison of APOE allele frequencies and significance values

Groups compared	ε2	ε3	ε4
Adults vs all infants	0.127	**0.022**	0.134
Adults vs healthy newborns	0.865	**0.042**	**0.026**
Adults vs perinatal deaths	**0.007**	0.062	0.973
Adults vs early neonatal deaths	0.095	**0.023**	0.167
Adults vs stillbirths	**0.011**	0.272	0.418
Healthy newborns vs perinatal deaths	**0.012**	0.973	**0.048**
Healthy newborns vs early neonatal deaths	0.121	0.276	0.914
Healthy newborns vs stillbirths	**0.019**	0.584	**0.012**
Early neonatal deaths vs stillbirths	0.932	0.173	0.072

2. APOE genotype and neuropathology

Detailed neuropathology was available for 66/67 neonatal deaths and all 185 stillborn infants where the genotype was known. Because of the low numbers of infants with the genotypes ε2/ε2, ε4/ε4, and ε2/ε4 (see table 4.1.B), subjects with the ε2 allele (ε2/ε2 and ε2/ε3) were combined into one group, ε3/ε3 homozygotes made up another group, while the final group was comprised of subjects with the ε4 allele (ε4/ε4, ε3/ε4, and ε2/ε4), as is the convention[177]. Tables 4.3 and 4.4 show the distribution of APOE genotype according to the presence or absence of brain damage in these cohorts. No correlation was detected between APOE status and hypoxic brain damage in live born or stillborn babies. However among the stillborn cohort with

Table 4.3. Distribution of APOE alleles in hypoxic brain damage in liveborn infants

	ε3/ε3 homozygotes (n=28)	ε2/ε2, ε3/ε2 (n=13)	ε4/ε4, ε4/ε3, ε4/ε2 (n=25)
Brain damage (n=36)	14(39%)	7(19%)	15(42%)
No brain damage (n=30)	14(48%)	6(20%)	10(34%)
Healthy newborns	199(54%)	46(12%)	126(34%)

Table 4.4. Distribution of APOE alleles in hypoxic brain damage in stillborn infants

	ε3/ε3 homozygotes (n=110)	ε2/ε2, ε3/ε2 (n=30)	ε4/ε4, ε4/ε3, ε4/ε2 (n=45)
Brain damage (n=123)	76 (62%)	17 (14%)	30 (24%)
No brain damage (n=62)	34 (55%)	13 (21%)	15 (24%)
Healthy newborns	199(54%)	46(12%)	126(34%)

evidence of brain haemorrhage there was a significant excess in ε3 homozygotes (72% compared with 53% of stillbirths with no haemorrhage, p=0.025 and with 54% of healthy newborns, p=0.001, table 4.6). This was not observed in the neonatal death cohort (table 4.5).

Table 4.5. Distribution of APOE alleles in brain haemorrhage in neonatal deaths

	ε3/ε3 homozygotes (n=28)	ε2/ε2, ε3/ε2 (n=13)	ε4/ε4, ε4/ε3, ε4/ε2 (n=25)
Haemorrhage (n=33)	14(42%)	7(21%)	12(36%)
No Haemorrhage (n=33)	14(42%)	6(18%)	13(39%)

Healthy newborns	199(54%)	46(12%)	126(34%)

Table 4.6. Distribution of APOE alleles in brain haemorrhage in stillborn infants

	ε3/ε3 homozygotes (n=110)	ε2/ε2, ε3/ε2 (n=30)	ε4/ε4, ε4/ε3, ε4/ε2 (n=45)
Haemorrhage (n=67)	48(72%)*	8(12%)	11(16%)
No Haemorrhage (n=118)	62(53%)	22(19%)	34(29%)

Healthy newborns	199(54%)	46(12%)	126(34%)

*p=0.025 compared to stillbirths with no haemorrhage and p=0.01 compared to healthy newborns

3. APOE genotype and clinical features amongst perinatal deaths

A full clinical dataset was available for 66/67 of the neonatal deaths and all 185 stillborn infants where the genotype was known. Table 4.7 shows the variation in allele distribution across gestational groups. Although there appears to be an increase in genotypes containing ε2 in extremely preterm gestations this difference is not statistically significant. Comparison of other clinical detail (eg birthweight centile, baby:placenta ratios etc) between different alleles revealed no significant differences in either cohort.

Table 4.7 Allele distributions by gestational group in perinatal deaths

Perinatal deaths (n=251)	**ε3/ε3 homozygotes**	**ε2/ε2, ε3/ε2**	**ε4/ε4, ε4/ε3, ε4/ε2**
24-26 weeks (n=46)	23(50%)	12(26%)	11(24%)
27-29 weeks (n=31)	18(58%)	6(19%)	7(23%)
30-32 weeks (n=28)	15(54%)	-	13(46%)
33-35 weeks (n=38)	24(63%)	9(24%)	5(13%)
≥36 weeks (n=108)	58(54%)	16(15%)	34(32%)
Healthy newborns ≥ 36weeks (n=362)	199(54%)	46(12%)	126(34%)

Discussion

Allele frequencies in the Scottish population

This study sought to determine the APOE allele and genotype distribution in Scottish perinatal populations and compare these to published Scottish adult data. In the case of perinatal death, a relationship was sought between APOE genotype and both neuropathology and clinical features. The results of the study showed significant differences in allele distribution between adults, healthy newborns and perinatal deaths and an association between genotype and brain haemorrhage in stillborn infants.

The relative frequency of different alleles of APOE has been calculated for many population groups. The ε3 allele is the commonest in all published studies. In most populations the next commonest is ε4. Amongst Caucasian populations, whose allele frequency has been studied in many countries, the relative frequency of alleles is remarkably similar, apart from Finnish subjects. All studies in Caucasians have shown an ε3 frequency of <0.79, lower than the frequency amongst American Indians, Chinese and Japanese (>0.82). There are however, differences in ε4 and ε2 distribution between geographically separated Caucasian groups, most notably a significant reduction of ε2 frequency with an excess of ε4 in the Finnish population[177].

Few investigators have examined the effect of age on APOE allele frequency. Ordovas et al found no difference in the distribution of APOE alleles

between young and older adults[204] although Schachter et al found that centenarians had a lower incidence of ε4 and higher incidence of ε2 compared to young and middle aged adults[205]. No differences have been found between parents and their offspring[206]. Herrmann at al examined 199 newborns and found an excess of APOE ε3 and ε2 with reduced prevalence of ε4, a result that contrasts with the present findings[207].

The adult population was selected randomly in the mid 1980s and was composed of late middle-aged men and women. Although this is a historical cohort, the distribution of allele frequencies is similar to that of other Caucasian populations in the literature. In the review of all published data by Davignon and colleagues, it was found that in 5,805 Caucasians the APOE allele frequency was 0.08 for ε2, 0.77 for ε3 and 0.15 for ε4, a distribution which is precisely the same as that reported for the Scottish adult population used in this study[177]. The perinatal subjects were born a decade later than the adults reported, effectively a difference of 60-70 years.

The Hardy-Weinberg Law states that gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. Evolution involves changes in the gene pool but a population in Hardy-Weinberg equilibrium shows no change from generation to generation. The study data show that each population was in Hardy-Weinberg equilibrium and therefore the results cannot be explained by evolutionary change.

The study data showed an excess of the ε4 allele and a corresponding decrease in the ε3 allele amongst healthy babies at birth when compared to healthy middle-aged Scottish adults. The differences observed cannot have occurred by evolutionary forces as both populations are in Hardy-Weinberg equilibrium. While it is possible that these differences have occurred by chance it is unlikely as the numbers in the study are not small. Other reasons for the differences observed may include sampling bias: the Scottish adults were from the North of Scotland and the healthy newborns from Edinburgh. As the relative frequencies of the APOE alleles remain fairly constant throughout most Caucasian populations it is unlikely that two populations within a limited geographical area such as Scotland would exhibit such differences. However the adult cohort did have to consent to participation in the study which involved venepuncture whereas the newborns were truly sampled randomly by a method which did not involve consent or a noxious procedure. It is possible therefore that the results may represent differences in education or social class between populations, with the adult cohort better educated and more able to make an informed choice about participation. Although such differences between APOE genotypes have not been extensively explored in the literature there are studies which show an association between ε4 and both higher educational achievement and cognitive function in early to mid adulthood[208;209]. This is in contrast to the decline in cognitive function associated with ε4 in elderly subjects[210]. This study however shows a decreased frequency of ε4 among the middle-aged consenting cohort compared with infancy and therefore would not be

consistent with this theory. However, the results of this study are supported by research that centenarians have been found to have a lower frequency of the ε4 allele and a higher frequency of the ε2 allele[205].

The decline in ε4 between birth and middle age in the Scottish population may reflect an increased mortality or morbidity of ε4 carriers between these ages. The ε4 allele is known to be strongly associated with hypercholesterolemia and atherosclerosis[177]. Both these conditions may lead to ischemic heart disease which is a leading cause of premature mortality in the Scottish population[211].

Comparison of both perinatal cohorts reveal that there is an excess of ε2 and a corresponding decrease in ε4 in perinatal deaths compared to those infants who are born healthy and survive. Infants who are born alive but who die in the first week of life appear to have the lowest prevalence of ε3, with over-representation both of ε2 (resembling stillbirths) and of ε4 (resembling healthy newborns).

Support in the literature for a developmental association with different APOE alleles comes from two studies in children and spontaneous abortions. Zetterberg and colleagues found a decreased prevalence of ε4 in early pregnancy loss in Crete, 87% of which were in the first trimester[40]. They proposed that ε4 might have a protective effect in pregnancy. This suggestion would be consistent with the study findings in healthy newborns. In addition

the excess of ε2 in extremely preterm perinatal deaths compared to ε4 suggests that ε4 may promote normal pregnancy outcome. Wright and colleagues showed that infants with ε4 have a better mental developmental index at 2 years of age suggesting that the effects of ε4 at an early age may be beneficial rather than detrimental[212]. A similar effect of ε4 on cognitive function has also been shown in young children with chronic diarrhoea in South America[213] and in young adults where ε4 has been associated with higher educational attainment and improved mental arithmetic scores[208;209].

In contrast in this study, possession of one or more ε2 alleles appears to be detrimental to pregnancy outcome. ε2 has historically been regarded as the "good" gene with respect to Alzheimer's disease and ischaemic heart disease. ε2 was reported in an early study to be associated with pre-eclampsia[214] but this was not upheld in a subsequent study[215]. Although maternal genotyping was not possible in the context of this study, no association between offspring genotype and maternal pregnancy-induced hypertension was found. The allele ε2 is less often transmitted to babies who are small for gestational age[216] and such infants are more likely to develop cardiovascular disease later in life[217]. This study however did not find any association between birthweight centile and genotype among perinatal deaths.

The deleterious effects of ε4 present late in life when the effects of natural selection are weak. The negative effects of ε4 at older ages may be counterbalanced by beneficial influences during embryogenesis, infancy and

the reproductive years in order to explain the relatively stable frequency in adult populations. According to the antagonistic pleiotropic theory of ageing, natural selection favours genes which confer short term benefits at the expense of deterioration in later life[218]. Such opposing effects of the ε4 allele may result in beneficial effects in early development but detrimental effects in adulthood.

The nature of this early protective mechanism is unknown. APOE appears to have a wider relevance than simply its influence on lipid and cholesterol metabolism. Mahley and Rall have speculated about a role for ε4 in reducing susceptibility to infection[175]. APOE knockout mice have an impaired immune response and display a susceptibility to bacterial infections, in particular Listera[219] and Klebsiella[220]. ε4 may also have an anti-infective role against viruses, protozoa and fungi. It is well recognised that infection of the chorioamnion is a common cause of spontaneous onset of labour in the second and third trimester of pregnancy although fetal infection is a lesser but nevertheless important cause of intrauterine death.

ApoE is critical to synaptogenesis in the developing brain and genetic variants of APOE may therefore be associated with differential effects on neurodevelopment. Although it has been shown that ε4 may have detrimental effects on the maintenance of healthy neurites and neuronal cells[221;222] compared to ε3, other studies have shown potentially beneficial effects on neuronal survival. In a recent study by Ohkubo and colleagues, ε4 and not ε3

was responsible for the induction of many genes including the cell-protective gene, Bcl-2[223]. In addition, the appreciation that APOE is involved in some intracellular signalling pathways and may have a role in apoptosis presents a wide range of opportunity for influencing embryonic development[175].

During the past two decades the role of essential fatty acids and cholesterol in the developing brain has received increasing interest[175]. Although work has concentrated mainly on the role of polyunsaturated fatty acids in growth and myelination, there is mounting evidence to suggest that cholesterol is essential to neurodevelopment. Apolipoprotein E is a key regulator of lipoproteins and cholesterol. Isoform-related effects of APOE on cholesterol may influence myelination and synaptogenesis in the developing brain.

This study has found an excess of ε3 homozygosity among stillborn infants who showed brain haemorrhage at autopsy. The relationship of APOE genotype and haemorrhage and ischemic brain injury in adults appears to be complex: ε2 predisposes to haemorrhage in cerebral amyloid angiopathy but this condition is commoner in carriers of the ε4 allele[224]. Other forms of intracranial haemorrhage do not share the same association. Transgenic adult mice expressing human ε3 have smaller cerebral infarcts after ischemia than mice expressing ε4[225]. In humans, ε4 is a risk factor for ischemic stroke[226] and results in a larger ischemic lesion on MRI[227]. Much of the isoform-related risk in both ischemic and haemorrhagic stroke is thought to be related to chronic disease processes, such as atherosclerosis and microangiopathic

changes within the cerebral circulation. The association between APOE genotype in the fetus and haemorrhage is more difficult to explain. A Finnish study found that adolescent boys with the ε4 genotype demonstrated less evidence of cardiovascular stress, such as heart rate variability, when subjected to a series of mental stressors[228]. Genotype-specific effects on cardiovascular stability may predispose to haemorrhage within the developing brain. However, it is important to note that grouping of APOE genotypes may be an artificial approach and there may be confounding or synergistic effects depending on the combination of alleles. Moreover the assignment of ε2/ε4 to either ε2 or ε4 groups may be misleading.

Although the results of this study show differences between the studied populations these differences in general are not large. It is highly possible that these findings have occurred by chance. It is also impossible to exclude confounding effects of neighbouring genes without performing whole genome and linkage disequilibrium studies. In addition the causes of perinatal mortality are notoriously heterogeneous and it is fanciful to presume that a single genotype might hold the answer to all perinatal death. A recent review in the Lancet has highlighted the thousands of genetic polymorphisms which are tested for disease associations each week around the world and that it would be expected for many associations that are significant at 5% or less to have arisen by chance[229]. These authors suggest a much lower threshold of significance for such studies. It is emphasised that genes act through complex networks involving gene-gene and gene-

environment interactions and not in an additive manner and argue that association studies will always be 'hopelessly simplistic and reductionist'.

Accepting that this may be true, the results of this study are however consistent with others that show protective effects of ε4 in early development and these findings may warrant further investigation. It is increasingly recognised that genetic factors that alter the host response to its environment may be important during critical developmental windows that occur during early life[230]. Both cholesterol and fatty acids are critical to nervous system growth and maintenance, and genetic factors that regulate their metabolism may influence this development. However the mechanism by which the APOE gene influences survival in early development remains unclear and future studies should address this. One approach may be to study the APOE status at different gestations in the presence or absence of specific pathological disorders, both generalised and organ-specific. Such studies may increase our understanding of the diverse roles of apoE and of the specific mechanisms involved.

Chapter 5
The neuropathology of a population of early neonatal deaths**

Introduction

The origins of childhood neurological deficits are still poorly understood. Complications of pregnancy and adverse peripartum events are common and of uncertain but poor predictive value. Perinatal asphyxia in the majority of cases is not followed either by neonatal encephalopathy or by cerebral palsy[7;10-14] and there is international consensus that encephalopathy and cerebral palsy are infrequently caused by perinatal asphyxia (the International Cerebral Palsy Task Force)[15;231]. Despite the clinical and medicolegal significance attached to perinatal asphyxia, the neuropathological basis of this condition remains obscure.

Major published cohort studies of neonatal encephalopathies and related clinical conditions have shown that the associated factors are surprisingly diverse[58;59] but these studies seldom include neuropathological findings of the deceased infants. On the other hand, reported perinatal neuropathological studies usually represent the collected experience of specialist referral centres[20-24;232;233], or focus on particular age groups such as preterm infants[26-29]. Only a small number of studies have investigated the clinical correlates of identified neuropathological features[30-32;234;235]. Although in recent years

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neuroimaging has proved to be a powerful tool for studying the extent and location of lesions in the perinatal brain[33], neuropathological examination may be better at defining the nature of lesions. There is a complete dearth of studies designed to match detailed neuropathology with equally detailed demographic data in unselected groups of neonates.

Perinatal neuropathology

Brain development is a continuum which starts at about three weeks after conception with the formation and closure of the neural tube continuing into the third decade. Damage may occur at any point during this period. The fetal brain appears to be particularly vulnerable to injury and the patterns of injury seen are quite distinct from those seen in adults and older infants. A range of damage has been extensively documented over the last thirty years and haemorrhage and damage to both white and grey matter are well–described. Influential perinatal studies are described in table 5.1 and the incidence of various neuropathological features is given where published.

Table 5.1. Major perinatal neuropathological studies

Authors	Year	Subjects	Number	Findings
Banker & Larroche[236]	1962	NNDs <13 mo	117	GMH-5 (4%), WMD (PVL)- 22 (18%), neuronal loss- 3%
Terplan[237]	1967	NNDs <14d & SBs	936	GMD- 28% PT and 9% FT SBs WMD- 22% PT and 12% FT SBs ICH- 17% SBs hypoxic neuronal change- 14% PT and 37% FT NNDs WMD- 35-49% PT and 27-28% FT NNDs ICH- 40-50% PT focal WM necrosis- 39% of PT with ICH
DeRueck et al [238]	1972	LBs 36h-4yrs	13 with PVL	IVH-2, SAH-5
Friede[239]	1972	NNDs <1mo	230	PSN- 22
Armstrong & Norman[240]	1974	LBs 1-84d	24 with PVL	Haemorrhage into PVL-6 (25%)
Grunnet et al[241]	1974/9	PT NNDs <1500g, <32wks	93	IVH- 50 (54%), high freq of assoc lesions in cerebellum, hippocampus, subiculum
Leech et al[242]	1974/9	NNDs with RDS		IVH- 117 (68%), 91% subependymal PVL- 24 (14%) intraparenchymal haemorrhage- 15 (9%) focal WM necrosis- 9 (5%) as gestation and postnatal age increased, IVH less and PVL more frequent

Authors	Year	Subjects	Number	Findings
Leviton & Gilles[145]	1984	SB	165	hypertrophic astrocytes- 35% acutely damaged glia- 16% amphophilic globules- 26% focal necrosis- 4%
Sims et al[234]	1985	SBs 25-42 wks	433	CNS injury- 74 (17%) WM gliosis- 20 (5%) ICH, 25 (6%)
Nakamura et al[243]	1986	NNDs severe asphyxia ≤7d, PT and FT	26- 13 PT, 13 FT	White matter astrocytosis- 13 (50%), PVL- 3 (12%), macrophages- 3 (12%) PT had ICH and WMD commonly, but deep GMD only FT had widespread cortical GMD and WMD, few ICH PSN- 12 and all had other associated lesions
Takashima et al[244]	1986	NNDs ≤1500g	86	PVL- 39 (45%) ICH- 66 (77%), 35(53%) of these had PVL
Skullerud & Westre[28]	1986	NNDs <38 wks, <30d	96	IVH- 48 (50%), 75% of these had associated lesions PVL- 23 (24%) PSN- 57 (59%), 70% of these had associated lesions
Armstrong et al[245]	1987	NNDs ≤7d with IVH	24	associated cerebral abnormalities- 22 (92%) PVL- 18 (75%) choroid plexus haemorrhage- 11 (46%), PSN- 11 (46%)
Bejar et al [146]	1988	PT infants	11	AND- 3 (27%), PVL
Hope et al[246]	1988	PT <33wks	56	PVL- 21 (38%)
Ellis et al[32]	1988	NNDs <7d, >500g	89	AND- 22 (25%) – 10 (16%)PT, 12 (48%)FT
Dambska et al[247]	1989	NNDs <12d, >28wks PT and FT	14	focal WM necrosis- 5 diffuse WMD- 9

Authors	Year	Subjects	Number	Findings
Low et al[248]	1989	SBs and NNDs asphyxia-related death	120	ICH- 52 (43%) GM+/or WM necrosis- - 16 (14%) AND- 7(6%) no abnormality- 52(43%)
Paneth et al[249]	1990	NNDs ≤1600g, ≤32 wks, ≥6d	22	WMD- 15 (68%) – diffuse in 10, focal in 5 11 ischemic WMD, 4 haemorrhagic WMD IVH- 17 (77%) basal ganglia infarcts- 10 cortical infarcts- 7
Squier & Keeling[23]	1991	SBs, NNDs <1wk, TOPs	165	AND- 15 /90 NNDs (17%) ischemic brain damage- 17/39 SBs (44%) widespread ischemic damage to cerebral hemispheres- 10 SBs (26%) ICH- 5 (13%) 2 ICH in utero without associated ischemic lesions
Low et al[250]	1992	NNDs and SBs asphyxia-related death	208	AND- 17 (8%) neuronal/WM necrosis- 30 (14%)
Grafe & Kinney[235]	1994	SBs 18-41wks	98	WM damage- 28 (28%), neuronal necrosis- 6 (6%) ICH- 15(15%)
Gaffney et al [30]	1994	SB and NNDs 17-42wks, <3d	274-139SBs, 135 NNDs)	AND- 56 (20%): 34(24%) SBs and 22(16%) NNDs cystic lesions- 11% major congenital anomaly- 89 (32%)
Eken et al[251]	1994	NNDs with HIE	30	AND- 10%
Wigglesworth and Bridger[252]	1994	SBs >34 wks	103	AND- (33%) SBs anoxic/ischemic lesions in: 8/24 (33%) fresh SBs 25/37 (68%) SBs with early maceration 36/42 (85%) SBs with established maceration

Authors	Year	Subjects	Number	Findings
Burke & Tannenberg[253]	1995	SBs	175	ischemic WMD- 70(40%), 40(23%) periventricular, 4 ICH, grey matter ischaemic change- 8 (4%)
Murphy et al [254]	1996	NNDs <32wks	83	cerebral damage- 47 (57%), ischemic WMD- 39 (83%) AND- 12(31%) PND- 12(31%) UK- 15(38%) large areas of cystic damage- 16
Golden et al[26]	1997	NNDs <1500g, <52d	67	AND- 4/19 (21%) NNDs< 24h ICH- 60 (90%) germinal matrix haemorrhage- 45 (67%) parenchymal ICH- 25 (37%) coagulative necrosis WM- 20 (30%) haemorrhagic necrosis WM- 6(9%)
de Vries et al[255]	1998	NNDs ≤34wks	68	PVL- 17 (26%)
Gilles et al[27]	1998	NNDs <1500g	67	focal WM necrosis- 9 (14%) diffuse WM necrosis- 29 (44%) germinal matrix haemorrhage- 45 (67%) intraventricular haemorrhage- 33 (49%) subarachnoid haemorrhage- 33 (49%) parenchymal haemorrhage- 25 (37%) GMD- 2 (3%)
Cowan et al[33]	2003	FT NNDs with HIE	21	AND- 3 /21 (14%) focal PVWM gliosis

AND- antenatal brain damage, FT- full term, GMD- grey matter damage, ICH-intracranial haemorrhage, GMH- germinal matrix haemorrhage, HIE- hypoxic-ischemic encephalopathy, IVH- intraventricular haemorrhage, LB- live birth, NND- neonatal death, PVL- periventricular leucomalacia, PSN- pontosubicular necrosis, PV- periventricular, PND-postnatal brain damage, PT- preterm, RDS- respiratory distress syndrome, SB- stillbirth, TOP- termination of pregnancy, UK- unknown, WMD- white matter damage

What has emerged from both human and animal descriptive studies has been the differential vulnerability of the developing brain to hypoxia and in particular maturational, structural and functional differences. The brain of the immature newborn has a greater susceptibility to subependymal/ intraventricular haemorrhage and white matter damage compared to the full term infants in whom the grey matter of the cortex is preferentially affected. Differences in the development of the brain blood supply means that the germinal matrix with its rich network of fragile, poorly supported blood vessels is a prime area for haemorrhage in the preterm infant whereas the thin, compact, relatively poorly vascularised cortical ribbon is protected. Reduced effectiveness of the cerebral autoregulation mechanism in infants, particularly in the face of immaturity, systemic hypoxaemia and hypercarbia, means that the watershed areas of the brain's arterial supply are particularly susceptible to ischemic damage.

In addition to vascular factors, the metabolic activity and differentiation of cells within an area affect the propensity for damage. For example, the immature periventricular white matter exists on relatively active anaerobic glycolysis. With ischemia, the relatively high glucose demand readily outstrips the substrate supplies. The regional distribution of synapses using excitatory amino acids plays a critical role in the distribution of hypoxic injury throughout gestation. Glutamate is a major mediator of fast excitatory neurotransmission and acts at various receptors, including the NMDA receptor. The NMDA receptor is especially abundant in the developing brain,

particularly in the striatum, hippocampus and neocortex. When this receptor is stimulated in the rat brain with exogenous NMDA, the toxicity varies according to the developmental stage of the brain. Damage proved to be most severe when concentration of NMDA receptors is at its highest[256]. Not only increased receptor density but receptor sensitivity or differences in the compensatory or modulatory mechanisms may be important in glutamate-induced toxicity.

Undoubtedly the response of the developing brain to injury is complex and depends not only on individual vulnerability and ontogenetic differences but also on the nature of the insult. Variation in the nature and severity of insult can result in a spectrum of damage. In particular, classic experiments have shown acute total anoxia results in severe injury to the brain stem nuclei whereas partial anoxia causes parasagittal cortical damage[93;257;258]. These experiments showed that animals may survive short periods of total anoxia without histological change, but that damage increases with increasing duration of insult[259;260]. Others have shown that frequent brief episodes of ischemia may result in more neuronal loss than one single insult of the same total duration[94].

Consequently, results of experiments which use a standardised insult may not be easily extrapolated to the real life situation. In human gestation, an isolated injury to a fetus may occur acutely at delivery, or at an early stage of gestation. On the other hand injury may occur chronically or intermittently

throughout gestation. Such a chronically compromised fetus may also sustain an acute injury at delivery. The range of neuropathological changes may be complex as a result, with lesions of different ages superimposed in one region or co-existing in different areas in the brain. In addition, secondary degenerative processes occurring from the time of damage until death may complicate the neuropathological picture further.

Post-hypoxic cellular changes in the developing brain

A well-described sequence of events occurs within neurones and central nervous system glia of the developing brain following a hypoxic insult. This sequence has been documented in both human infants and animal models and the merits and limitations of both situations are discussed.

The difficulty of relating pathological change to a hypoxic-ischemic injury in the human infant is the uncertainty of knowing when exactly the insult occurred and although many of studies of human infants have attempted to document the sequence of cellular change following a clinical incident or sentinel event, it is recognised that it is impossible to say whether the brain was normal up until that point, when exactly during the peripartum period an insult occurred and the exact nature, of the insult. As a result the response to such heterogeneous injury observed may vary considerably.

Animal studies, on the other hand, have the advantage of a known timing and severity of insult and presuppose normal brain development until the point of

injury. Animal models should be expected to mimic the aetiological basis through which such injury occurs, reflect the histopathological spectrum of injury to the developing brain and ideally express the functional outcomes seen in the newborn infant and child.

Since the classic experimental work of Windle in the late 1950s[261;262] and Myers in the 1970s [263] a variety of animal models have been studied including sheep, pigs, rodents and non-human primates. A variety of insults have been studied, from pure hypoxia-ischemia (HI) to experiments where induction of seizures and hypotension have been introduced to more closely mimic the human condition. The duration of insult has varied from acute to chronic and intermittent insults.

Through such experiments, some difficulties in working with the animal model have been encountered. The various stages in brain development occur at different times across species. Myelination is complete by term in the fetal sheep whereas in the human it begins midgestation and progresses until early adolescence. In the rat, myelination only commences postnatally. Proliferation, migration and the development of subplate neurons occur earlier in humans than in most animal models. There are also ontogenetic differences in neurotransmitters with development of the NMDA receptor peaking at around 24 weeks in the human and 1-2 weeks postnatally in the rat [264]. In addition, rodent models of HI show wide interstrain variation in susceptibility to HI damage after an identical insult suggesting genetic

factors may be important [265]. Piglets and sheep generally mature quickly and therefore there may be difficulty in establishing relevance to the full term newborn. Non-human primates may be more similar to humans but there remain unresolved issues about scarcity, ethical issues and the relative maturity of their brain at birth[266]. Many have found difficulty in keeping animals alive long enough after a sustained hypoxic-ischemic insult to investigate the cellular response and clinical outcomes.

Despite these reservations about the use of animals to study HI injury, there has been tremendous progress over the last forty years enhancing our understanding of the mechanisms of perinatal brain injury particularly at biochemical and molecular levels and many experimental models have contributed to this progress.

Over two decades of investigation into various degrees of intrauterine asphyxia in Rhesus monkeys, Myers et al described 4 patterns of brain injury closely resembling the changes observed in perinatally damaged humans. [263]: total hypoxic-ischemia with acidosis produced damage predominantly in the brainstem; partial asphyxia with acidosis resulted in widespread cortical necrosis; partial asphyxia without acidosis produced selective white matter injury and partial prolonged asphyxia culminating in terminal total asphyxia resulted in damage in the basal ganglia and thalamus. The non-human primate model has also been studied in relation to white matter damage in the immature brain. Inder et al have developed a premature baboon model which

shows diffuse white matter damage and haemorrhage in the absence of an experimental interventional insult and correlates well with human findings[267].

The sheep has also been used to study both grey and white matter damage. Gunn et al subjected fetal sheep to repetitive and brief occlusion of the umbilical cord of and showed that intermittent asphyxia resulted in greater grey matter damage than a single asphyxial insult of the same duration. When the episodes were repeated at much shorter intervals the damage became more diffuse and extensive [268 269]. Others have shown maturational differences in response to asphyxia in the white matter in the sheep which parallels that seen in humans[270;271].

Following the introduction of the Rice-Vannucci 7 day old rat model in the 1980s, this animal has become the most commonly used model for perinatal asphyxia [272]. Histologically a gradation of injury is observed that correlates in a linear fashion with the duration/severity of the insult [273;274]. This model has been used extensively to study cerebral metabolism and the proinflammatory response occurring secondary to asphyxia. Work in the rodent white matter injury model has identified the oligodendrocyte progenitor cell as the major target and as such has correlated the 2^{nd}-5^{th} postnatal day in rats with 24-32 weeks gestation in the human as being the most vulnerable for such injury [275].

More recently interest has focussed on mechanisms of white matter injury and the sheep has been used to investigate the role of infection and inflammation in brain injury [276;277] by injecting bacterial endotoxin into the fetal sheep leading to preferential damage of white matter and in particular oligodendrocytes. On the other hand, umbilical cord occlusion alone resulted in neuronal necrosis in the hippocampus and striatum. More recently there has been evidence of a synergistic effect between infection and hypoxia. Mallard et al in the immature rat model showed that a combination of intraperitoneal endotoxin and a mild asphyxial insult resulted in extensive white matter damage, which could not be explained by simple additive effects of infection and hypoxia[278].

Other animal models have proved useful in studying the effect of fetal growth retardation and placental insufficiency on the developing brain. In both guinea pigs and lambs, chronic intrauterine hypoxia results in poor brain growth and myelination with reduced neuronal numbers in the cortex cerebellum[279;280]

The reactions detected in fetal animal brains in response to a variety of insults including acute and chronic hypoxia, ionizing radiation, glutamate analogues and ischaemic injury all lead to a similar sequence of responses suggesting that the fetal brain responds to injury in a stereotyped fashion. It is likely that a variety of insults trigger a final common pathway of damage in the perinatal brain. The initial trigger event is more difficult to identify in

the human fetus. Despite the limitations of the animal model, the sequence and timing of post-hypoxic cellular changes are remarkably similar to those of the human infant brain and have been documented comprehensively by Ellis et al [281]. The spectrum and evolution of post-hypoxic histopathological changes are documented in tables 5.2 and summarised in table 5.3. Both tables have been derived from the literature on human infants and key cellular changes are elaborated below.

Table 5.2. Timing of CNS injury after cerebral insult in human infants

Pathological Features	Timing of Onset after Injury	References
Neuronal Eosinophilia	6 – 24 hours	Norman (1978) described eosinophilia in differentiated neurons 24 -36 hours after hypoxic insult (such as delay in establishing respiration) in a classic study of perinatal brain damage. In quoting the results of animal studies, she points out that these may not be applicable directly to human infants. Low et al (1989) suggests that eosinophilia requires at least 18 hours after a documented insult (results in 16 of 120 perinatal deaths). A major current text of neuropathology (Graeber et al, in Greenfield's Neuropathology 2002) quotes a period of "more than 6" hours after insult with reference to observations in the rat.
Neuronal Karyorrhexis	12 – 48 hours	Friede (1972) described neuronal karyorrhexis in mature infants surviving at least 22 hours after an insult. Low et al (1989) suggested that nuclear pyknosis requires 18 hours to become visible. This study documents the duration of labour as well as postnatal survival but suggests that histological changes do not indicate precisely the timing of the insult. Wigglesworth & Bridger (1994) suggested this change required a time lapse of more than 24 hours from their own studies of perinatal deaths. Squier (2001) suggested a time interval of 12-48 hours from her own experience and a survey of the literature. Del Bigio and Becker (1994) note that neuronal karyorrhexis occurs prior to any microglial change occurring at 4 days or more
Infarcts – Necrosis	3 – 8 hours	Banker (1967) quotes 3 hours as the period needed for coagulation necrosis to become evident but a further 9 hours required for commencing cellular reactions. Norman (1978) describes smudgy eosinophilic coagulation necrosis with axonal balls and pyknotic glial nuclei arising 3-8 hours after a cerebral insult; most likely due to a failure of tissue perfusion. Squier (2001) describes coagulation necrosis and retraction balls occurring within 3 hours of insult, quoting other studies and her own observations.

Infarcts – Cavitation	14 – 42 days	Banker (1967) described cysts appearing in areas of damaged white matter two weeks after the insult. Ellis et al (1988) drew on their own experience to conclude that 14 days was required. Squier (2001) quotes the literature and her own experience in suggesting a period of 14 – 42 days. Kinney & Armstrong (2002) described a delay of "a few weeks".
Reactive Gliosis (white matter)	3 – 11 days	Roessman & Gambetti (1986) thought that 4 days were required (for the appearance of hypertrophied astrocytes identified categorically by GFAP immunocytochemistry; sometimes present in isolation but is taken as evidence of brain damage). Astrocytic hyperplasia could be detected as early as 20 weeks gestation but there was a more pronounced response with increasing gestational age. They reported focal and diffuse patterns. The diffuse pattern commonly affects the cerebral/cerebellar white matter where they often saw massive proliferation of astrocytes. Low et al (1989) indicated that 3 – 5 days were required. Banker and Larroche described the earliest astrocyte proliferation at 12 hours Gilles & Murphy (1969) thought that hypertrophic astrocytes required 3 days to appear and were able to attribute brain damage to the prenatal period on this basis, provided that the astrocytosis was accompanied by other evidence of white matter damage such as glial pyknosis. Ellis et al (1988) examined very carefully a series of infants in whom astrocytosis was graded as early, established or late. Subtle early astrocytosis could be detected by one day after insult whereas enlarged and hypertrophic astrocytes required 3-5 days. Norenberg (1994) concentrated on astrocytic reactions and thought these were maximal by 4 days after injury in humans compared with 24 hours in a rat model – he emphasised that the two situations were not comparable in that the human brain was likely to suffer more widespread damage and required to recover before mounting cellular reactions. The consensus is that reactive astrocytosis requires around 3 days and on this basis, damage has been ascribed in several series to the prenatal period.

Reactive Gliosis (grey matter)	3 – 5 days	Friede (1972) described a glial response occurring in pontosubicular necrosis 3-5 days after the insult. If infants with pontosubicular necrosis survived only 1-2 days, the grey matter glial response was very slight. Del Bigio & Becker (1994) described the glial response in damaged dentate gyrus "lagging behind" a microglial response which itself required 1-4 days. Marin-Padilla (1999) emphasised that grey matter damage was repaired by gliosis, unlike white matter which cavitates, but found this phenomenon only in comparatively longer survivors (weeks and months after the insult). Describes subpial and GM/WM gliosis and post-injury transformation of uninjured adjacent GM. Kinney & Armstrong (2002) suggested that 3-5 days were required for grey matter gliosis to follow on neuronal necrosis in both preterm and mature infants.
Microglial Upregulation	3 hours –3 days	Banker (1967) observed microglial infiltrate 8 hours after the onset of coagulative necrosis. Norman (1978) found rod cells in necrotic white matter foci only after 2-3 days following an anoxic episode. Ellis et al (1988), quoting animal studies compared with their own human studies, observed microglia 1-3 days after an insult. Low et al (1989) observed rod cells 36-72 hours after a hypoxic episode. Del Bigio & Becker (1994) observed an increased number of rod cells only when survival exceeded 4 days following an insult which had produced grey matter infarcts. This study quotes results in animal experiments where microglial upregulation was observed to commence approximately 1 day after the insult, becoming maximal at 4 days.
Macrophage Infiltration	3 – 7 days	Banker (1967) observed macrophages only 1 week after a documented insult. Friede (1972) observed macrophage responses (foamy cells) in relation to pontosubicular damage 4-5 days after the onset of presumed clinical insult. Ellis et al (1988) documented early, middle and late stages from their own observations of macrophage formation and related this to animal studies. Squier (2001) observed macrophages only 4-5 days after injury. Kinney & Armstrong (2002) suggested 3-5 days were required for a macrophage response in relation to pontosubicular necrosis, presumably based on their own experience.

Haemosiderin Deposits	2-3 days	Ellis et al (1988) found that haemosiderin staining macrophages accompanied the appearance of macrophages within and around haemorrhagic lesions and that this change required 3 days to appear. Squier & Keeling (1991) suggest that the presence of pigmented macrophages around haemorrhages requires at least 2 days. Vanesis (2001) states that haemosiderin containing macrophages are found within the brain 3-4 days after injury and this is later than in some other tissues. Vanesis emphases that no reliance should be placed on animal studies in this regard since there is very considerable interspecies variation.
Mineralisation	3 – 14 days	Norman (1978) believed that ferrugination of neurons could occur 3 days post insult. However other authors have all assumed a longer interval. Ellis et al (1988) observed that a period of 14 days was required for the appearance of perivascular mineralising foci in the kitten model. These foci, and amphophilic globules, may be found in association with white matter gliosis (Gilles & Murphy, 1969) and have been used to date the onset of damage in the prenatal period. Ellis et al (1988) thought that neuronal ferrugination required more than 14 days. Squier (2001) thought that mineralisation required 8-14 days. Amphophilic globules in and around the walls of small vessels may represent leaked plasma proteins.

Table 5.3. Timing of CNS Injury after cerebral insult

Pathological Feature	Timing of Onset after Injury	References
Neuronal eosinophilia	6-24 hours	282-284
Neuronal karyorrhexis	12-48 hours	283;285-287
Infarcts – necrosis	3-8 hours	281;282;287;288
Infarcts – cavitation	14– 42 days	32;289-291
White matter gliosis	3 – 11 days	26;27;232;248;292;291;293;294
Grey matter gliosis	3 – 5 days	22;239;291;295
Microglial upregulation	3 hours – 3 days	32;248;289;292;295
Macrophage infiltration	3 – 7 days	32;239;289-291
Fresh haemorrhage	minutes	32
Haemosiderin deposits	2-3 days	23;32;296
Mineralisation	3-14 days	32;232;292;297

Neuronal eosinophilia and nuclear karyorrhexis

Following neuronal ischemia, vulnerable neurons undergo a sequence of characteristic morphological changes. Neurons show microvacuolation in the first hours followed by marked cytoplasmic eosinophilia from around 6 hours. Nuclear karyorrhexis ensues from 12 hours onwards. Karyorrhexis is the process by which the nucleus of a dying cell breaks up into a number of rounded particles. Primary cell necrosis may result in nuclear karyorrhexis as well as nuclear karyolysis, or pyknosis. Karyorrhexis may also occur as the end stage of apoptosis, a form of active cell death mediated by a cascade of intracellular enzymes. The nucleus initially becomes round and shrinks with intense nuclear basophilia. Breakdown of nuclear chromatin ensues at around 12-24 hours and membrane-bound fragments of the cell are subsequently phagocytosed by adjacent cells. Apoptosis is the mechanism of programmed cell death by which cell numbers in the brain are regulated. Although most cell death following hypoxic-ischemic insult occurs by primary necrosis, it is increasingly recognised that cell death also occurs due to activation of apoptosis. This is especially true of immature, less differentiated cell types but may also occur within the context of milder injury.

Astrocytic change

Astrocytes are the most numerous cell element of the brain, outnumbering neurons 10 to 1 and occupying one third of the cerebral cortex[294]. They are not structurally independent but organised in a network interconnected by gap junctions. They are not morphologically static as their shape may be

influenced by interactions with neurons, neurotransmitters and by state of hydration. They have important roles in neuronal migration, neurite outgrowth, synaptogenesis and synaptic plasticity, maintenance of the blood brain barrier, provision of energy and nutrient support of neurons, regulation of water ion and neurotransmitter metabolism, modulation of immune and inflammatory responses and phagocytic functions. There are also specific enzyme systems which enable astrocytes to metabolise ammonia, glutamate and free radicals, protecting the brain from the toxicity of these agents.

Astrocyte swelling is very common and is probably the earliest response to CNS injury, occurring in response to a wide variety of insults[294]. This is followed by enlargement and proliferation of astrocytes a process which is thought to require between 3 and 5 days. The nucleus is often enlarged and irregular with multiple small nucleoli and an increase in chromatin. Although astrocytic hyperplasia has been detected in brains of fetuses as early as 20 weeks gestation, the magnitude of the response increases with increasing maturity and postnatal age[293]. (Prior to 20 weeks, the predominant reactive cell is the macrophage). Protrusions of long astrocytic cytoplasmic processes that will later form the 'glial scar' subsequently occur. Distinction must be made between reactive astrocytic proliferation and immature glia, immature migrating neurons and 'myelination gliosis' (oligodendroglia precursors)[23;292]. Myelination glia have a rim of pale basophilic cytoplasm and the cells may be arranged in rows. Reactive astrocytes on the other hand are less orderly in their arrangement, have abundant eosinophilic cytoplasm

and characteristically have long cytoplasmic processes[24;32;293]. In addition, reactive astrocytosis is usually accompanied by macrophage reaction, haemorrhage, coagulation necrosis and the presence of amphophilic globules. Amphophilic globules are small deposits seen most commonly in vessel walls but also in the parenchyma, and are most frequently seen in more mature infants who have survived longer[26;27]. They are thought to represent mineralising plasma proteins which have leaked through damaged blood vessel endothelium. However accurate identification of reactive astrocytes is best done using immunocytochemistry for glial fibrillary acidic protein[293] which is the main biochemical constituent of the glial filaments found in astrocytes.

Microglial proliferation

The microglia are derived from blood monocytes that migrate into the brain tissue during fetal development and subsequently remain after complete formation of the blood-brain barrier. In the fetal brain they are present from the twenty-first embryonic day and increase steadily in number through gestation and postnatally. In addition to astrocytes and oligodendrocytes, microglia represent the third major population of glial cells within the CNS. They form a network of resident macrophages with a capacity for immune surveillance and control. Microglia are distributed ubiquitously throughout the brain and spinal cord, and a key function is to monitor and sustain neuronal health. Microglia are extremely sensitive to virtually any insult within the CNS and proliferate accordingly within hours[32;236;248;295]. When

activated they are small elongated rod shaped cells with two or three branches emanating from each end. In most pathological settings the microglia are aided by infiltrating haematogenous macrophages which are evident within the CNS 4-5 days following injury[239;290]. Activated microglia are mainly scavenger cells, ingesting cell debris and contributing to cyst formation. Phagocytosis can be observed at 4-6 days and the accumulation of lipid material within these cells gives them a 'foamy' appearance. They take up and break down red cells, and the resulting haemosiderin-laden macrophages may persist for years after injury as a lasting record of previous haemorrhage.

Capillary proliferation

The endothelial cells of capillaries become swollen in the first few days following an injury, they show evidence of reduplication and their nuclei become more rounded. There is subsequent proliferation of capillaries at 5-8 days.

Mineralisation

Mineralisation is identified as basophilic granules within macrophages, neurones and processes and is first seen around 7 days of injury[32;290]. Although classically associated with viral infections, it is also seen in the thalamus and in areas of old infarction secondary to hypoxic-ischemic damage.

Incidence of prenatal brain damage

Studies determining the incidence of prenatal brain damage have applied the literature on post-hypoxic cellular changes to establish the duration of injury observed (table 5.1). Injury occurring in utero prior to the death of a fetus has been frequently seen in studies of stillborn infants, manifest as ischemic white matter damage, established infarcts, widespread cystic change and old haemorrhage[23;30;237;248;252;253]. In fact, any neuropathological changes observable in the brains of stillborn infants must have predated death by some hours. The exception to this is haemorrhage which may occur acutely as a terminal event. The incidence of such established damage ranges from 1-44% [23;234] of all stillbirths and up to 85% of macerated stillbirths[252].

Among studies of neuropathological abnormality in neonatal death there is much heterogeneity of subjects. Some studies have focussed only on preterm or low birth weight infants [26;27;254;298], others have restricted examination to all early NNDs[32;245]. Some researchers have selected deaths related to particular conditions such as asphyxia[33;248], respiratory distress syndrome[237;242] or intraventricular haemorrhage[245;299]. Timing of damage however may be difficult to ascertain where the infant has survived for many days or weeks and where duration of labour is unknown. Incidence of damage occurring prior to the delivery has been found in these studies in up to 75% of preterm neonatal deaths[245] and in up to 48% of full term neonatal deaths[32].

There are three neuropathological studies dedicated to asphyxia-related death in the literature. Nakamura et al studied 26 preterm and full term infants dying from severe asphyxia at less than a week of age. Five infants had evidence of periventricular leucomalacia, and 13 infants who had survived for 2 days or less had pathological astrocytosis in the white matter as observed with GFAP staining. Macrophages were present in three of these infants[243]. Low et al studied 120 asphyxia-related stillbirths and early neonatal deaths. 'Asphyxia' was not defined but the authors allude to acid-base studies. In all cases the duration of labour was known and clinical antepartum detail recorded. Sixteen cases had evidence of CNS injury other than haemorrhage. Seven (6%) infants had evidence of established brain injury characterised by histopathological changes which predated the onset of labour[248]. A Dutch study attempted to correlate neuropathological observations with USS[251] and subsequently with MRI findings[33]. Three out of 21 (14%) infants dying from severe HIE showed evidence of antenatal brain damage.

Antenatal brain damage as detected by fetal imaging has been widely reported. In particular, ultrasound has demonstrated the presence of fetal intraventricular haemorrhage and parenchymal echodensities[300]. A large French study of antenatal magnetic resonance imaging in 400 patients demonstrated the presence of ischemic and haemorrhagic lesions as well as the more subtle disorders of neuronal migration[301]. Postnatal MRI and ultrasound have also identified the presence of established antenatal brain

injury in newborn infants, confirmed subsequently at post mortem[33;146;255].

Some studies have attempted to correlate radiological diagnoses made during life with post mortem neuropathological features[33;251;302-304]. Newer technology and increased user experience has led to improved detection of perinatal brain damage during life. It is accepted that MRI has superior sensitivity for neuropathological damage than ultrasound examination[305;306] which may miss up to 72% of parenchymal abnormalities. With newer MR modalities such as diffusion-weighted imaging (DWI), acute disturbances within brain tissue can be observed. However post mortem remains the gold standard in the diagnosis of brain injury. In particular, histological changes such as inflammatory cell infiltration and increased cell death are easily missed by MRI[302]. Even with T2 sequencing and DWI around half of all histologically ascertained damage may be underestimated or overlooked by MRI[303;304].

Clinical correlation of neuropathological change

The fact that brain damage can occur in utero is undisputed. Both neuropathological and imaging studies have clearly demonstrated this. In addition, the majority of full term children with cerebral palsy have a normal perinatal history but investigation into their mother's pregnancy often reveals adverse features[89]. The aetiology of this damage, however, is less well understood. The fetus within the womb is relatively protected from the

investigative eye and though our ability to monitor the fetus with cardiotocograph monitoring, acid-base studies, ultrasound and Doppler studies has advanced significantly over the last quarter of a century, many adverse events occur unnoticed. Although obstetricians are adept at identifying high risk pregnancies, many stillbirths occur in pregnancies deemed to be low risk or uncomplicated[161;307].

Few researchers have sought to correlate neuropathological findings with antenatal clinical features. Larroche categorised lesions depending on whether maternal, fetal or placental/cord factors were responsible. In particular she discusses the contribution of twinning to disrupted morphogenesis early in gestation and to ischemic lesions in the second and third trimesters[308;309]. Necrosis of the cerebral white matter is well described in multiple gestations and is far commoner in monozygous twins[310]. In a review of the 53 surviving monozygotic twins whose pair had died in utero, 72% had an abnormality of the central nervous system[311]. This predilection for poor neurodevelopmental outcome in the surviving twin persists when one twin dies in infancy[312]. Placental anastamoses may lead to imbalance of flow between twins throughout gestation, resulting in single or recurrent episodes of hypoperfusion. Ischemic damage to the brain and other organs or even death may subsequently occur.

Sims and co-workers examined the histories of 25 stillborn infants found to have evidence of gliosis with or without haemorrhage and found that 36% of

these had evidence of chorioamnionitis compared to 20% of the 408 stillborn infants without any brain damage[234]. One infant with haemorrhage was found to have cerebral artery emboli and the authors suggest that these could have originated in association with placental vasculitis. Leviton found that 85% of infants who had evidence of antenatal white matter necrosis at post mortem, had had a terminal gram negative bacteraemia[313].

Murphy and colleagues reviewed the histories of 83 very preterm infants who died in the first six months of life[254]. They could find no clinical associations with antenatal ischemic white matter brain damage but neonatal sepsis, necrotising enterocolitis, pre-eclampsia and IUGR were all associated with the development of postnatal damage (ORs 4.6, 25, 11 and 5 respectively). This association with pre-eclampsia is in contrast to studies that show an inverse association with intraventricular haemorrhage[314] and with the later development of cerebral palsy[142;315]. Studies examining the association of pre-eclampsia with brain injury often fail to consider treatment effects of drugs such as magnesium sulphate. In addition, studies analysing risk by birthweight may overlook the effect of pre-eclampsia on fetal growth.

Gaffney and colleagues investigated the clinical association of prenatal white matter injury in 274 cases of perinatal death. Twenty percent of this cohort had evidence of prenatal brain damage. The mothers of these infants were more likely to be unmarried (OR 2.5) and to have had an antenatal diagnosis of intrauterine growth retardation (OR 2.7) or oligohydramnios (OR 2.9).

Indicators of fetal distress such as meconium staining and abnormal cardiotocograph were more common in infants with antenatal brain injury (ORs 6.8 and 12.4 respectively) as was a severely depressed 5 minute Apgar score (OR 3.2)[30].

Ellis and co-workers studied 89 early neonatal deaths and found prenatal brain damage to be more common in full term infants compared to preterm infants (48% vs 16%). Oligohydramnios appeared protective while polyhydramnios predisposed to prenatal brain damage among the full term cohort. The group found no other clinical associations with prenatal brain damage[32].

These studies show that despite the relatively high prevalence of antenatal brain damage in infants dying around the time of birth there are conflicting and inconsistent reports of factors responsible for this damage. Imaging studies of brain injury diagnosed during life suggest that infection and multiple gestations are important risk factors. Bejar et al classified 18% of preterm infants as having sustained antenatal white matter necrosis as diagnosed by postnatal ultrasound scan. Clinical associations were low birth weight, placental anastamoses in multiple gestations, purulent amniotic fluid and funisitis[146]. Others have found associations between ultrasound abnormalities and infection parameters such as chorioamnionitis, funisitis, increased inflammatory markers and neonatal sepsis[316-318].

As previous pathological studies have studied selected groups of infants, or have been hospital-based, therefore representing the experience of specialist centres, the aim of this study was to examine the prevalence of brain damage occurring within an entire population of neonatal deaths. Previous published literature on the clinical associations of perinatal brain damage is scarce and this study aimed to provide additional information about the clinical associations of brain damage within this population of perinatal deaths, with a particular focus on the differences between asphyxiated infants and those born in a good condition.

Aims

1. to investigate the neuropathological status in a population of early neonatal deaths in whom a post mortem was authorized, and to determine whether lesions could be of prelabour origin

2. to determine whether infants who have pre-existing brain damage are more likely to be born in an asphyxiated condition

3. to compare the antepartum and intrapartum course of early neonatal deaths born with birth asphyxia, with and without pre-existing damage

Methods

Refer to chapter 1 for methods.

Results

1. Population

Of 137 early neonatal deaths with a full clinical dataset, 88 (64%) had consent for autopsy and 70 (51%) had consent for the additional neuropathological investigations required by the study protocol. Of these 70, 53 were classified as asphyxiated (BA, including 23 mature infants ≥ 37 weeks and 30 preterm, 24-36 weeks) and 17 did not appear to be asphyxiated (noBA, three mature ≥ 37 weeks, 14 preterm infants 24-36 weeks) (Figure 1, Chapter 1). The mature infants lived for between 15 minutes and 7 days with only three surviving for more than 3 days. The preterm infants lived between 5 minutes and 6.8 days with only eight infants surviving for more than 3 days. The ratio of asphyxiated (77%) to non-asphyxiated (23%) was slightly skewed though not significantly in the group of autopsied infants towards asphyxiated cases when compared with the whole cohort of liveborn infants included in the detailed epidemiological survey (n=137, 66% asphyxiated, 34% non-asphyxiated).

2. Neuropathological findings

The prevalence of neuropathological findings of the cohort of 70 infants are shown in table 5.4, subgrouped according to whether they showed asphyxia or not (53 vs 17) and according to their gestation (mature vs preterm).

Table 5.4. Histological evidence of brain damage in 70 early neonatal deaths

	Asphyxiated infants (BA) n=53		Non-asphyxiated infants (noBA) n=17	
	Mature n=23	Preterm n=30	Mature n=3	Preterm n=14
Neuronal Eosinophilia	14 (61%)	9 (30%)	2	5 (36%)
Neuronal Karyorrhexis	11 (48%)	8 (27%)	0	0 (0%)
Grey Matter Infarcts	1 (4%)	3 (10%)	0	0 (0%)
White Matter Gliosis	11 (48%)	14 (47%)	2	0 (0%)
Grey Matter Gliosis	7 (30%)	5 (17%)	1	0 (0%)
Microglial Upregulation	9 (39%)	14 (47%)	1	1 (7%)
Macrophages	9 (39%)	14 (47%)	0	0 (0%)
Haemorrhage, Fresh	11 (48%)	19 (63%)	0	8 (57%)
Haemosiderin Deposits	0 (0%)	1 (3%)	0	0 (0%)
Mineral Deposits	2 (9%)	8 (27%)	2	1 (7%)

Assessment of neuronal damage was initially undertaken in sections stained with haematoxylin and eosin. Recent onset hypoxic change as indicated by neuronal eosinophilia [Fig 5.1], page 134, was seen in some or all differentiated nerve cell bodies in the cerebral cortex, basal ganglia, brain stem, particularly in the ventral pons and inferior olive, and in the cerebellar dentate nuclei and Purkinje cell layer. Neuronal karyorrhexes [Fig 5.2] were observed in a similar distribution but the dentate gyrus of the hippocampus and the cerebellar granular layer was also affected in occasional cases. If

neuronal karyorrhexes were only present infrequently, they were considered indistinguishable from the normal apoptosis known to occur during brain development and were disregarded. Even when present in large numbers, the changes affected only a subpopulation of neurons in the neocortex but were more numerous and concentrated in the subiculum of the temporal lobe (spreading to involve the entorhinal cortex and the neighbouring sectors of the cornu ammonis) and in the ventral pons. The most severely affected cases showed loss of most Purkinje cells [Fig 5.3] within the cerebellum. Neuronal eosinophilia was identified in 43% of the cohort and karyorrhexes in 27%. Cortical infarction with prominent neovascularisation and perivascular fibrosis [Fig 5.4] was observed in occasional infants. Figs 5.5-7 show focal and diffuse macrophage responses in the context of neuronal loss, not all of which had been suspected on routine staining but which were identified with immunocytochemistry [Fig 5.6]. A conspicuous glial response was also seen in conjunction with cortical damage [Fig 5.8].

Evidence of white matter damage in routinely stained sections was present in 24% of cases. This ranged from widespread homogenous eosinophilia of the neuropil to areas of clear focal infarction. In three cases of infarction, axonal retraction bulbs [Fig 5.9] were identified with more widespread surrounding white matter damage. Moderate numbers of karyorrhectic glial nuclei were identified within damaged white matter, and these were accompanied by infiltration of macrophages and astrocytic hyperplasia [Fig 5.10]. Even if white matter appeared normal [Fig 5.11], unexpected astrocytic hyperplasia

was sometimes revealed by GFAP immunostaining [Fig 5.12]. This was different from the normal white matter astrocytic immunoreactivity in this age group [Fig 13]. No clear foci of cystic periventricular leucomalacia were observed.

Small foci of perivascular mineralisation were present in the central white matter in 19% of the total cohort, more commonly in the preterm infants. One infant displayed extensive mineralisation in the basal ganglia and internal capsule (case 20).

In nine cases, glial nuclei in both white and grey matter were enlarged, pale and prominent [Fig 5.14] resembling the Alzheimer type II astrocytes observed in hepatic encephalopathy. Although the cytoplasm of such cells was not generally prominent, they were GFAP positive [Fig 5.15] and in most of these cases a reactive astrocytosis was confirmed in the white matter. Some mature neonates showed prominent changes of so-called myelination gliosis [Fig 5.16] but such cells did not prove to be GFAP reactive in contrast to juxtaposed normal astrocytes [Fig 5.17]. Interpretation of such cases sometimes proved difficult on routine staining especially in the presence of incipient white matter damage and GFAP staining proved essential for the detection of true astrocytic hyperplasia.

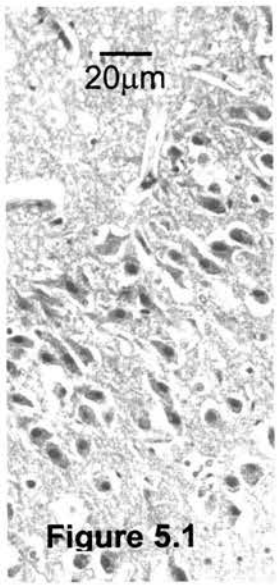


Figure 5.1

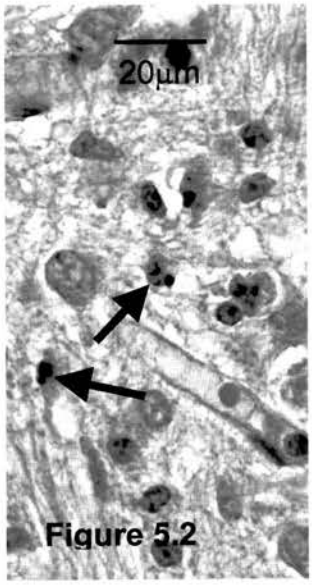


Figure 5.2

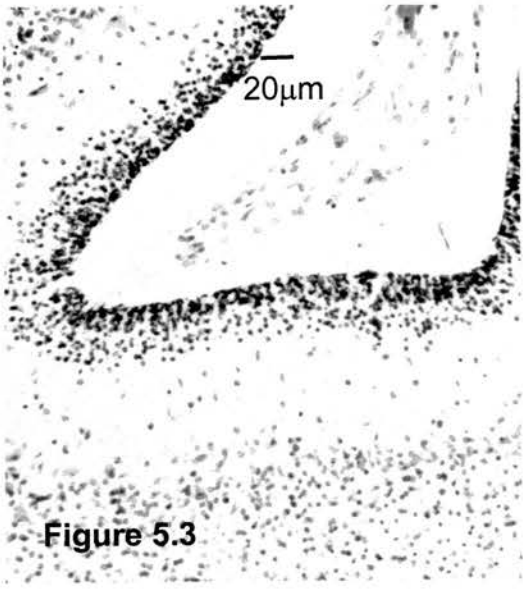


Figure 5.3

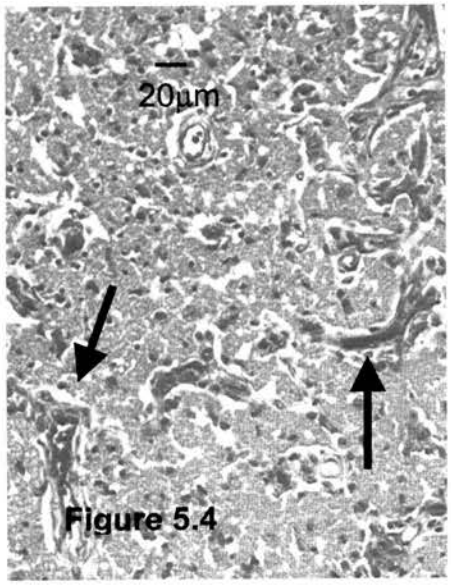


Figure 5.4

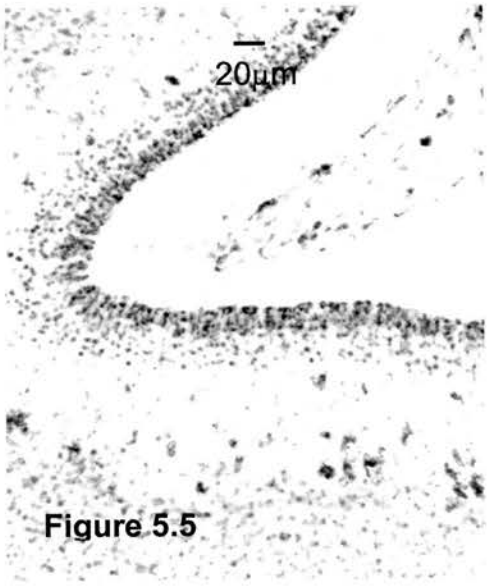
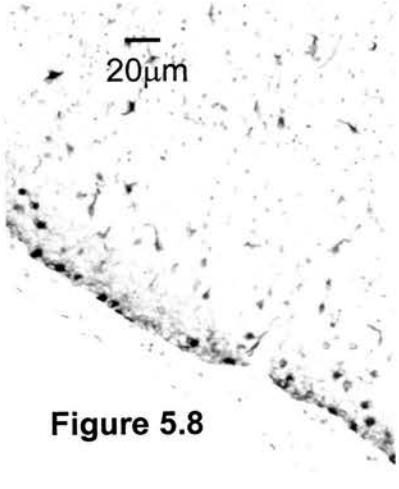


Figure 5.5

Figure 5.6

Figure 5.7



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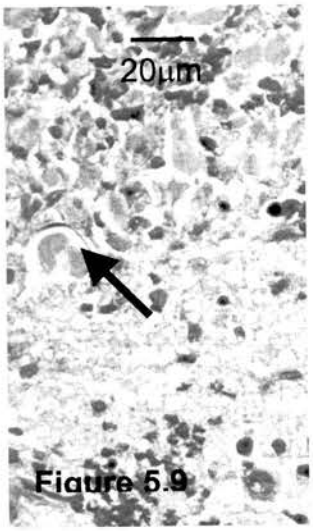

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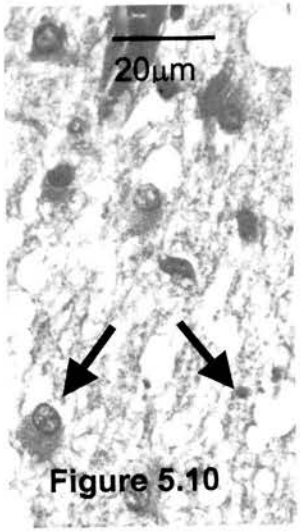

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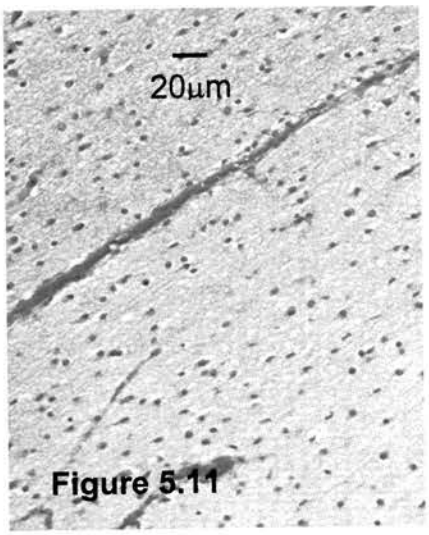

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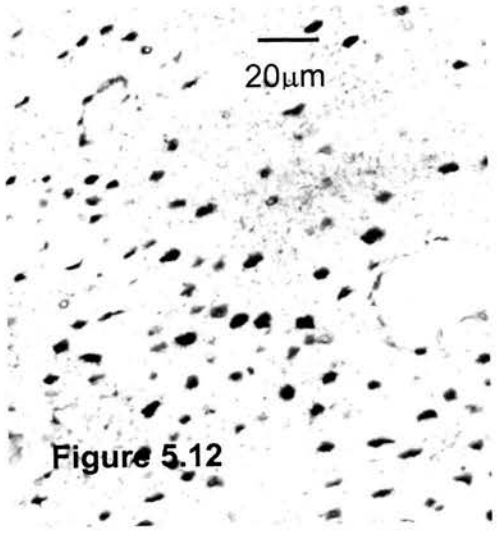

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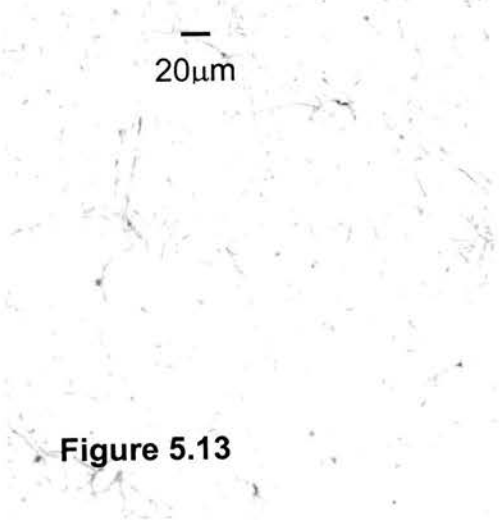


Figure 5.13

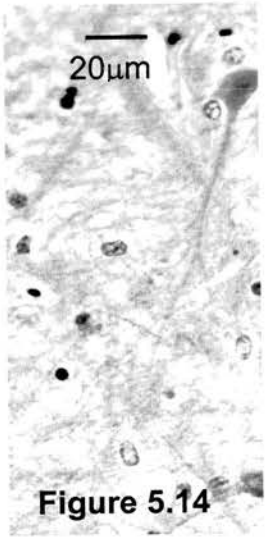


Figure 5.14

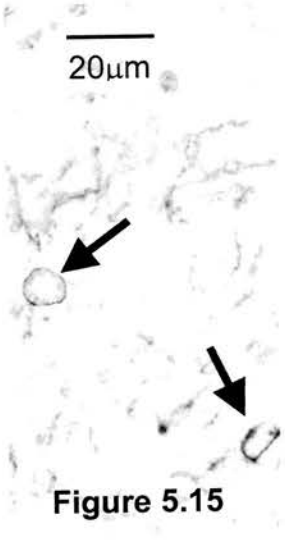
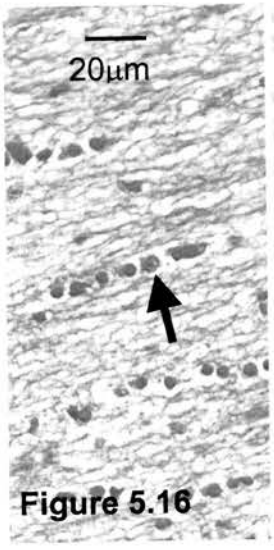


Figure 5.15



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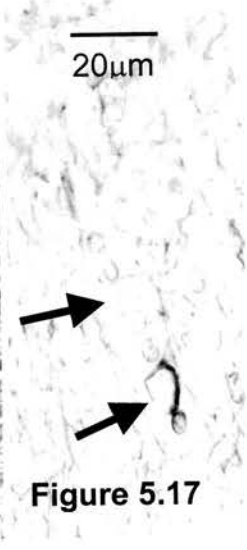

Figure 5.17

3. Estimation of prelabour brain damage

In those cases where brain damage was present, a conclusion as to whether this was likely to be of prelabour origin could be achieved only in infants dying ≤ 3 days from the onset of labour. This was based on the presence of abnormalities thought to first appear approximately three days after brain injury. There is no absolute certainty regarding the time needed for the different responses to become visible (Table 5.3) but the presence of accumulations of macrophages and/or prominent astrocytic hyperplasia in human white or grey matter is generally assumed to require 3 days or more (Table 5.2).

Fifty-nine infants died at or before 3 days from the onset of labour, 43% of those with a full clinical dataset. Of these 59 infants, 46 were asphyxiated (BA) and 13 were born in a good condition (noBA). Twenty-one of the BA group and 11 of the noBA group were preterm (Figure1, Chapter 1). Table 5.5 shows the prevalence of neuropathological abnormalities in these 59 infants. This table has been further subdivided according to whether encephalopathy was one of the features of asphyxia or not.

Damage occurring before the onset of labour was observed in 27 (39%) of the entire cohort and almost exclusively in those who were asphyxiated at birth. Twenty-six (57%) of the asphyxiated group had evidence suggestive of prelabour brain damage (PBD) compared to 1 (8%) of the non-asphyxiated

Table 5.5. Histological evidence of brain damage, including putative prelabour damage, in 59 neonates dying ≤3 days from the onset of labour

	BA Encephalopathy n=10 (17%)		BA No Encephalopathy n=36 (61%)		No BA n=13 (22%)	
	Mature n=8	Preterm n=2	Mature n=13	Preterm n=23	Mature n=2	Preterm n=11
Neuronal Eosinophilia	8 (100%)	1	5 (38%)	4 (17%)	0	4 (36%)
Neuronal Karyorrhexis	8 (100%)	1	1 (8%)	4 (17%)	0	0
Grey Matter Infarcts	0	0	0	0	0	0
White Matter Gliosis*	7 (88%)	1	4 (31%)	9 (39%)	1	0
Grey Matter Gliosis*	5 (63%)	1	0	1 (4%)	0	0
Metabolic astrocytosis	3(38%)	1	1	2(9%)	0	0
Microglial Upregulation	6 (75%)	1	2 (15%)	9 (39%)	0	1 (9%)
Macrophages*	7 (88%)	1	1 (8%)	8 (35%)	0	0
Haemorrhage, Fresh	4 (50%)	0	6 (46%)	11 (48%)	0	5 (45%)
Haemosiderin Deposits	0	0	0	0	0	0
Mineral Deposits*	1 (13%)	0	1 (8%)	6 (26%)	1	1 (9%)
Postnatal damage only	0	1	5	7	1	7
No brain damage	0	0	3	4	0	4
*Estimated Prelabour Brain Damage	8 (100%)	1	5 (38%)	12 (52%)	1	0
*Estimated Prelabour Brain Damage	26 (57%)				1 (8%)	

group – a highly significant difference – $p<0.005$. It is important to note that of the 27 infants judged to have suffered prelabour brain damage, only four of them had survived for more than 2 days, six had survived between 1 and 2 days and all the rest (65%) had survived for less than 1 day from the onset of labour.

4. Correlation of Neuropathology Findings with Clinical Features

Neuronal eosinophilia was no more common in asphyxiated infants (23/53, 43%) than in non-asphyxiated infants (7/17, 41%, $p=0.87$) (Table 5.4). However this feature was detected more frequently in mature (16/26, 62%) than preterm infants (14/44, 32%, $p=0.015$). In contrast with neuronal eosinophilia, neuronal karyorrhexes were significantly increased in asphyxiated infants (19/53, 36%) compared with non-asphyxiated infants (0/17, 0%, $p <0.01$) and were also more prevalent in mature (11/26, 42%) than in preterm infants (8/44, 18%, $p=0.08$) although not significantly so. These observations remained consistent in the subgroup of 59 infants dying less than 3 days old (Table 5.5). In both mature and preterm infants, the asphyxiated infants were more likely to show brain damage than the non-asphyxiated, although brain damage was not universally present in asphyxiated infants (Tables 5.4 and 5.5, Figure 1). Some infants showed evidence of ongoing brain damage with recent events such as neuronal eosinophilia and fresh haemorrhage superimposed on older lesions including established infarcts, macrophage accumulation including cells laden with haemosiderin, extensive micromineralisation and white matter gliosis. Infants with no evidence of asphyxia at birth (mostly preterm infants) were

more likely than asphyxiated infants to appear virtually normal on neuropathological examination, and such changes as were present, including haemorrhage and neuronal eosinophilia, appeared to be recent apart from two mature infants who displayed prominent gliosis.

Encephalopathic infants (Table 5.5) showed a particularly high prevalence of neuronal eosinophilia and karyorrhexes (9/10, 90%). White matter damage was significantly more common in encephalopathic (9/10, 90%) than in non-encephalopathic asphyxiated (13/36, 36%) and non-asphyxiated (1/13, 8%) neonates ($p=0.001$). Encephalopathy was significantly associated with white matter gliosis ($p=0.001$), with macrophage reactions ($p=0.002$) and with metabolic astrocytosis ($p=0.024$) but not with mineral deposits. The minor differences in white matter micromineralisation between asphyxiated and non-asphyxiated infants was not significant. Table 5.5 also highlights the fact that many of the brains of non-encephalopathic asphyxiated infants were apparently undamaged before the onset of labour and that even by the time of death in the postnatal period, 31% of mature and 13% of preterm asphyxiated infants in this subgroup showed no histological abnormality. Although the non-asphyxiated infants appeared to be more prone to postnatal or intrapartum damage, this difference was not significant ($p=0.059$).

Prelabour brain damage tended to be found more often in mature infants compared to immature infants (14/23, 61% vs 13/36, 36%, $p=0.063$). Unsurprisingly, the preterm infants were more susceptible to damage of

recent and likely postnatal origin than were mature infants.

5. Prelabour brain damage and the signs of birth asphyxia

Table 5.6 shows the pathology of prelabour brain damage related to the criteria used for birth asphyxia. Although the strongest clinical association with the features of prelabour damage is the development of a neonatal encephalopathy following a low pH and a poor Apgar score at 5 mins, it is of note that of the 21 infants who had only a low Apgar score and then died and had a post mortem, 8 showed brain damage.

Table 5.6. Features of asphyxia and presence of putative prelabour brain damage

	Full term			Preterm		
	Total		PBD	Total		PBD
	Clinical	PM*	at PM	Clinical	PM*	at PM
Single feature of asphyxia						
Apgar ≤5 at 5 mins	9	7	3	35	14	5
Cord pH < 7.1	0	0	0	1	0	0
1st pH < 7.1	1	1	1	8	5	3
NNE	1	0	0	0	0	0
2 features of asphyxia						
Apgar and low pH	7	5	1	9	4	4
Apgar and NNE	2	0	0	1	0	0
Low pH and NNE	0	0	0	1	0	0
3 features of asphyxia						
Low pH, low Apgar and NNE	11	8	8	4	2	1
TOTAL	31	21	13	59	25	13

NOTE: * A total of 47 infants that died at 3days or less of age = PM, but 1 FT infant with prenatal damage had no asphyxia

PBD- prelabour brain damage, NNE- neonatal encephalopathy, PM- post mortem

All eight infants died within two hours of life, before acid-base studies were performed. By this evidence, a low Apgar score was the sole clinical indicator of PBD in 3 of 13 of mature infants and in 5 of 13 of preterm infants. Only 16 mature and 19 preterm infants of 137 infants survived to 12 hours and remained non paralysed; of these, 14 mature and 6 preterm infants had an encephalopathy. Looked at another way, 14 full term infants had NNE. Eight of these had a post mortem and all had evidence of PBD. Only 6 PT infants had NNE. Of the two who had a post mortem, only 1 had PBD.

Table 5.7. Clinical cause of death in 59 infants surviving ≤ 3days with signs of asphyxia* and presence of prelabour brain damage

	Birth asphyxia		Prelabour brain damage	
	BA n=46	NoBA n=13	PBD n=27	NoPBD n=32
Intrapartum hypoxic-ischemic injury	20 Apg= 7 pH=1 Apg+pH=2 Apg,pH,HIE=10	0	12 Apg=3 Apg,pH,HIE=9	8
Respiratory disease in preterm infant	10 Apg=7 pH=2 Apg+pH=1	5	5 Apg=3 pH=1 Apg+pH=1	10
Intracranial bleed	2 Apg=1 Apg+pH=1	1	1 Apg+pH=1	2
Infection	2 pH=2	4	1 pH=1	5
Congenital anomaly: tracheal atresia, diaphragmatic hernia and renal agenesis	3 Apg=2 Apg+pH=1	1	1 Apg=1	3
Other: hydrops, pulmonary hypoplasia	2 Apg=1 pH=1	0	1 Apg=1	1
Unexplained	7 Apg=3 pH=1 Apg+pH=3	2	6 Apg=2 pH=1 Apg+pH=2 NoBA=1	3

* Note: See page 19 for definitions of asphyxia

Infants with congenital anomalies and preterm infants who died from respiratory complications were more likely to appear in poor condition at birth but did not ultimately show evidence of prelabour brain damage.

6. Clinical Factors Associated with Prelabour Brain Damage (PBD)

Sociodemographic, pregnancy, delivery and postnatal characteristics of infants with putative prelabour brain damage are described case by case in Table 5.8. Details are given of neuropathological findings.

Table 5.8. Clinical and neuropathological features in neonatal deaths

Prenatal brain damage (Cases 1-27)

Case (NNE =E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
1 (E)	42(+)	1. Normal 2. Abnormal CTG, IOL over 4d, ruptured uterus 3. Apgar 4^5, pH 6.91, Seizures, isoelectric EEG, cerebral oedema on USS, died 32h	32 hours	Cerebral oedema; neuronal eosinophilia & karyorrhexis; microglial activation & focal macrophage infiltration in WM.
2	42 (-)	1. Amniocentesis for low AFP, loss FM 36,41/40, PIH 2. Abnormal CTG 3. Good condition, Apgar 10^5, collapse at 1h, died 5h	5 hours	Diffuse WM gliosis; amphophilic globules.
3	40(+)	1. Normal 2. Meconium, abnormal CTG 3. Apgar 1^5, died<1h	10 hours	Fresh microhaemorrhages; WM gliosis & focal WM damage.
4	40(+)	1. Normal 2. Meconium 3. Unexpected condition, Apgar 5^5, pH 7.04, died 13h	18 hours	Cerebral oedema; focal WM gliosis & microglial activation.
5 (E)	40(+)	1. Normal 2. Meconium, abnormal CTG, ruptured uterus 3. Apgar $0^1 0^5$, pH 6.8, HIE 3, died 15hours	28 hours	Cerebral oedema; fresh microhaemorrhages, neuronal eosinophilia & karyorrhexis; microglial activation; WM gliosis & damage.
6 (E)	40(+)	1. Normal 2. PROM, meconium, abnormal CTG, infection 3. Apgar 1^5, pH 6.86, HIE 3, isoelectric EEG, seizures, died 42h	61 hours	Neuronal eosinophilia & karyorrhexis; WM damage; microglial activation & macrophage accumulation; focally gliotic WM.
7 (E)	40(+)	1. Previous NND 2. Unexpected poor condition 3. Apgar 7^5, pH 6.94, abnormal tone, poor respiratory drive, died 26h	39 hours	Gangliosidosis – GM1; WM gliosis.

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
8 (E)	40(+)	1. Normal 2. Abnormal CTG, cord prolapse 3. Apgar $0^{1}2^{5}$, pH 6.79, HIE 3, cerebral oedema on USS, isoelectric EEG, died 14h	21 hours	Cerebral oedema; neuronal eosinophilia; WM gliosis & amphophilic globules.
9	40(+)	1. APH 6,29wks 2. Cord haemorrhage 3. Apgar 0^{5}, died at 1h	2 hours	Focal grey matter gliosis; microglial activation; focal macrophage accumulation & WM damage.
10	39(+)	1. Essential HT 2. Meconium, abnormal CTG 3. Apgar $0^{1}0^{5}$, died<1h	3 hours	WM diffuse microglial activation & macrophage accumulation; focal gliosis of white matter.
11 (E)	39(+)	1. Loss FM 32,39wks, essential HT 2. No labour, meconium, abn CTG, fetomaternal bleed 3. Apgar 4^{5}, pH 7.06, HIE grade 3, died 14h	14 hours	Neuronal eosinophilia & karyorrhexis; grey matter gliosis; microglial activation and macrophage accumulation.
12 (E)	38(+)	1. Unbooked, severe PIH 2. No labour, abn CTG, multiple placental infarctions 3. Apgar $0^{1}2^{5}$, pH 6.57, seizures, HIE grade 3, bilateral echogenicity on USS, died 17h	17 hours	Cerebral oedema, neuronal eosinophilia & karyorrhexis; WM gliosis & macrophage accumulation.
13 (E)	38(+)	1. Loss fetal movements 38/40 2. No labour, abnormal CTG, fetomaternal bleed 3. Apgar $0^{1}0^{5}$, pH 6.8, severe HIE, died 43h	43 hours	Germinal matrix haemorrhage; neuronal eosinophilia & karyorrhexis; WM damage & gliosis; microglial activation & macrophage accumulation.
14 (E)	37(+)	1. Smoking 2. No Labour, abnormal CTG, uterine rupture 3. Apgar $0^{1}0^{5}$, pH 6.69, isoelectric EEG, HIE 3, died 35h	35 hours	Cerebral oedema; neuronal eosinophilia & karyorrhexis; gliosis grey matter; microglial activation & macrophage accumulation.
15 (E)	36(+)	1.Smoking, multiple pregnancy, APH 30-36wks 2.Green vaginal discharge, complex shoulder presentation, abnormal CTG 3. Apgar $0^{1}3^{5}$, pH 6.9, HIE, abnormal background EEG, died 45h	53 hours	Cerebral oedema, neuronal eosinophilia & karyorrhexis; microglial activation & macrophage infiltration; WM gliosis.

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
16	36(+)	1.Smoking, previous anencephalic infant, oligohydramnios, IUGR 2.Breech, meconium 3.Apgar 0^5, died <1h	4 hours	Micromineralisation; WM damage & macrophage accumulation.
17	35(+)	1. Known duodenal atresia, antenatal steroids, ROM 35wks, IUGR 2. Meconium, abnormal CTG 3. Tracheal atresia, oesophageal-pulmonary fistula, Apgar 4^5, pH 6.8, died 11h	11 hours	Cerebral oedema; WM gliosis & microglial activation.
18	35(+)	1. Previous SB, unbooked, massive fetal ascites on USS 2. No labour, meconium, abnormal CTG 3. Apgar 1^5, pH 6.81, abnormal neurology, died 8h	8 hours	Mineralised neurons in basal ganglia; fresh microhaemorrhages; neuronal karyorrhexis; WM gliosis & macrophages.
19	32(+)	1. Known duodenal atresia, amnioreduction, polyhydramnios, antenatal steroids, severe PIH2 2. No labour, abnormal CTG 3. Apgar $0^1 0^5$, pH 6.9, died 1h	1 hour	Cerebral oedema; micromineralisation; focal white matter damage; WM gliosis.
20	28(+)	1. Twin preg, antenatal steroids, hydropic 1^{st} twin 2. Breech, meconium 3. Apgar 1^5, died 2h	7 hours	Microhaemorrhages; basal ganglia micromineralisation; WM damage & gliosis; focal macrophage accumulation.
21	28(+)	1. Smoking, oligohydramnios, IUGR, antenatal steroids 2. No labour, abnormal CTG, breech 3. Apgar 9^5, pH7.09, Abnormal neurology, USS showed IVH, PVL, died 70h	70 hours	Germinal matrix haemorrhage with thrombosed vessels; WM infarction & gliosis; neuronal eosinophilia & karyorrhexis; macrophage accumulation.
22	27(+)	1. Previous SB 25wks, antenatal steroids, oligohydramnios, IUGR 2. No labour, meconium, abnormal CTG 3. Apgar 4^5, died <1h	0.5 hours	Fresh microhaemorrhages; neuronal eosinophilia; focal grey matter gliosis; macrophage accumulation in grey & WM.

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
23	26(+)	1. Smoking, low AFP, oligohydramnios, APH 9/40, grade 3 placenta praevia, ROM 26/40, suspected infection, bicornuate uterus 2. PROM, breech, abnormal CTG 3. Apgar 7^{5}, pH 7.02, died 11h	29 hours	Microhaemorrhages; focal microglial activation & macrophage infiltration of WM.
24	25(+)	1. Severe maternal varicella, heavily sedated and ventilated, antenatal steroids 2. Breech, delivered unexpectedly in adult ITU 3. Apgar $0^{1}4^{5}$, pH 6.9, seizures, severe PVH, died 51h	51 hours	Neuronal eosinophilia & karyorrhexis; germinal matrix haemorrhage; focal microglial activation & macrophage accumulation.
25	25(+)	1. High AFP, severe oligohydramnios, IUGR, APH at 16, 20, 25 wks, ROM 17wks 2. PROM, breech 3. Apgar 1^{5}, died 2h	6 hours	Germinal matrix haemorrhage; neuronal eosinophilia; WM gliosis; microglial activation & macrophage infiltration.
26	24(+)	1. Unbooked, IUGR 2. Breech 3. Apgar 2^{5}, died<1h	2 hours	Focal macrophage accumulation & gliosis in grey matter.
27	24(+)	1.Oligohydramnios, APH 13/40, ROM 21/40, anemia <9g/dl, suspected infection 2. PROM 3. Apgar 8^{5}, pH 7.08, IVH, died 12h	13 hours	Germinal matrix haemorrhages; neuronal eosinophilia; focal WM damage & microglial activation; grey matter gliosis.

Infants with postnatal damage (n=21)

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
28	42 (+)	1. smoker, Graves disease, pernicious anemia 2. induced , forceps delivery, normal CTG 3. Apgar 3^5, full CPR, died 1 hour	10	Slight patchy haemorrhage in basal ganglia
29	41 (+)	1. smoker, previous fetal anomaly and TOP, anemia 2. E Coli on HVS, SVD 3. Apgar 5^5, pH 6.7, likely sepsis	14	Focal slight haemorrhage in central white matter, slight focal neuronal eosinophilia in cortex
30	40 (+)	1. smoker, hypertension 2. forceps delivery, meconium 3. cord pH 6.6, Apgar 0^5, multiorgan failure and NNE	34	Widespread white and grey matter haemorrhage, neuronal eosinophilia
31	39 (+)	1. anemia, PIH 2. induced, shoulder dystocia, tight nuchal cord 3. Apgar 2^5 , pH 7.46, full CPR. Collapse at 6 hrs, massive umbilical haemorrhage.	41	Patchy perivascular haemorrhage
32	38 (+)	1. multiple pregnancy 2. El CS for twins 3. HR at 1 min but Apgar 0^5 , CPR 35 mins	0	Congestion and very occasional perivascular haemorrhages
33	38 (-)	1. infertility, previous SB and NNDs 2. SVD 3. Apgar 10^5 died in cot 48hrs, GBS sepsis	52	Haemorrhage in central white matter, neuronal eosinophilia in hippocampal neurones
34	36 (+)	1. late booker, polyhydramnios 2. induced 3. tracheal atresia, pH 6.8 Apgar 4^5 died 2hrs	6	Haemorrhage in subependymal zone
35	36 (-)	1. infertility, loss FM 34 and 36 wks 2. SVD, normal CTG 3. Apgar 9^5 , died in cot 44hrs	48	Focal (patchy) haemorrhage in central white matter, recent hypoxia (neuronal eosinophilia)
36	31 (-)	1. smoker, abn FAS 2. breech vaginal delivery 3. Apgar 7^5, renal agenesis	41	Patchy white matter haemorrhage and haemorrhage in germinal matrix

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
37	30 (-)	1. incr trisomy risk, amnio, oligohydramnios, ROM 28wk 2. infection, abn CTG, prelabour CS 3. Apgar 9^5, pH 7.2, E Coli sepsis, RDS, pneumothorax	17	Slight haemorrhage in central white matter , occasional cortical neuronal eosinophilia
38	29 (+)	1. smoker, late booker 2. ROM 72hrs, cord prolapse, prelabour CS 3. Apg 6^5, pH 6.9, hypoplastic lungs	4	Small germinal matrix haemorrhages, occasional neuronal hypoxia (eosinophilia) in hippocampal neurones
39	28 (-)	1. twin preg, anemia, PIH, hyperemesis, plac previa 2. EmCS prelabour 3. Apg 9^5, pH 7.5, RDS, pneumothorax	66	Recent hypoxia (neuronal shrinkage and eosinophilia)
40	27 (-)	1. twin preg, amnioreduction, TTTS, IUGR, PIH, hyperemesis, flu 12wks, loss FM 26 wks, ROM 27wks 2. breech vaginal 3. Apg 8^5, pH 7.19, renal failure, hypoplastic lungs	52	Intraventricular haemorrhage and extensive germinal matrix haemorrhage, neuronal shrinkage and eosinophilia
41 (E)	25 (+)	1. APH 23wks, UTI and PV discharge, ROM 27 wks 2. prolapsed arm and cord, assisted breech 3. Apgar 3^5, cord pH 7.04, poor resp drive, ? seizures	18	Neuronal eosinophilia in hippocampus
42	25 (+)	1. smoker, ROM 25 wks 2. suspected intrapartum infection 3. Apgar 4^5, pH 7.21, massive bilateral IVH	10	Slight germinal matrix haemorrhage, neuronal eosinophilia in hippocampus and cerebellar cortex
43	25 (-)	1. mitral valve disease, anemia, APH 24 wks 2. GBS on HVS, unwell, pyrexial, SVD 3. Apgar 8^5, pH 7.38, massive pulmonary hge	36	Haemorrhage and neuronal eosinophilia in hippocampal grey matter
44	24 (+)	1. Low AFP 2. offensive liquor, SVD 3. Apg 1^5, full CPR, died 18mins	2	Germinal matrix haemorrhage
45	24 (+)	1. normal 2. SVD 3. Apgar 4^5, full CPR, died 35 mins	5	Fresh germinal matrix and intraventricular haemorrhages

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
46	24 (+)	1. infertility, ROM 24 wks 2. APH, breech delivery, offensive liquor 3. Apg 5^5, pH 7.19, died 2hrs	7	Patchy haemorrhage in central white matter, neuronal eosinophilia
47	24 (+)	1. smoker, late booker, APH 12 wks, flu illness 20wks 2. EmCS in labour for arm presentation 3. Apg 3^5, poor response to resus, died 20 mins	5	Slight focal haemorrhage in germinal matrix
48	24 (-)	1. late booker, anemia, oligo, recurrent APHs, PROM 2. breech vaginal 3. Apg 6^5, severe RDS	9	Slight germinal matrix haemorrhage and haemorrhages in central white matter

Infants with no brain damage (n=11)

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
49	42 (+)	1. normal 2. Ventouse FTP, meconium, decelerations 3. Apgar 1^5, cord pH 6.7, full CPR, died 38 mins	12	nil
50	40 (+)	1. smoker 2. massive APH in labour, prolonged brady, asystole 3. Apgar 0^5, cord pH 7.22, full CPR, HR 10 mins	2	nil
51	38 (+)	1. polyhydramnios 2. SVD CTG normal 3. Apgar 4^5, pH 6.8, died 8 hours, ?hypoplastic lungs	14	nil

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
52	35(-)	1. amnio for mat age, anemia, hyperemesis, APH 13wks, loss FM 32wks, ROM 34wks, poor weight gain 2. EmCS poor CTG, offensive liquor 3. Apg 9^5, pH 7.19, coliform sepsis	8	nil
53	32(-)	1. smoker, UTI 32 wks 2. vag delivery, breech, pyrexia 3. Apg 8^5, pH 7.16, died hypoxemia, sepsis	13	nil
54	33 (+)	1. smoker, loss FM 33wks, poor wt gain 3rd trim 2. EmCS prelabour fetal brady 3. Apgar 0^5, cord pH 7.15, full CPR, died at 5mins	0	nil
55	27 (+)	1. smoker, previous NND, ROM 21wks 2. massive APH, prelabour EmCS 3. Apg 8^5, pH 7.05, hypoplastic lungs, RDS	8	nil
56	26 (-)	1. late booker, APH 26 wks 2. offensive liquor, growth anaerobes, SVD 3. Apgar 10^5, pH 7.14, massive pulmonary hge	52	nil
57	25 (+)	1. smoker ROM 26 wks 2. breech, cervix clamped down on head pre-delivery 3. Apgar 0^5, baby lived mins only	1	nil
58	25 (+)	1. amnio due t incr trisomy risk, APH 24wks, PROM 10d 2. pyrexial, Em CS for fetal distress in labour 3. Apgar 9^5, pH 7.06, died pseudomonas sepsis	12	nil
59	25(-)	1. smoker 2. breech delivery into toilet, no obvious labour 3. Apgar 7^5, pH 7.24, severe IVH	64	nil

Infants surviving 3-7 days (n=11)

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hours of labour + hours of survival	Neuropathological Features
60 (E)	42 (+)	1. loss FM 24 wks 2. EmCS fetal distress, meconium, normal CTG 3. Apgar 6^5, cord pH 7.11, HIE 3, abn head CT scan	187	Large fresh cortical haemorrhage, neovascularisation, cortical infarct, macrophages, pontosubicular necrosis, hippocampus karyorrhexis, white matter gliosis, basal ganglia gliosis, pontine neuronal loss
61	41(-)	1. normal 2. induced, ventouse 3. Apgar 9^5, scalp pH 7.35, collapse 80h, full CPR, NNE	116	Recent hypoxia (neuronal eosinophilia), central white matter gliosis, mineralising foci (small perivascular but frequent) in central white matter
62 (E)	39 (+)	1. anemia 2. failed Ventouse x3, then forceps 3. Apgar 4^5, pH 7.14, massive ICH, subaponeurotic bleed	82	Fresh haemorrhage in germinal matrix, white matter damage – gliosis and macrophages; pontosubicular necrosis, cerebellar karyorrhectic neurones, focal acute meningitis
63 (E)	36 (+)	1. multiple, amnio for TTTS, anemia, IUGR, HELLP 2. fetal distress, brady, decelerations CS 3. Apgar 3^5, pH 7.26, HIE 3	82	Neuronal eosinophilia in hippocampus, central white matter gliosis and macrophage accumulation, fresh and old (haemosiderin positive) haemorrhages in the germinal matrix, cerebellar cortical hypoplasia
64	34 (-)	1. amnio for trisomy risk, IUGR, hyperemesis, Gr 4 placenta previa, PV discharge 2. Em CS prelabour 3. Apgar 9^5, cord pH 7.36, wt 1.25kg, died in cot at 86h, full CPR unsuccessful	86	Fresh haemorrhages in central white matter, neuronal eosinophilia in hippocampus and cerebellar cortex

Case (NNE=E)	Gestation (asphyxia at birth)	Clinical Features	Total hrs of labour hours of survival	Neuropathological Features
65 (E)	29 (+)	1. anemia, APH 29wks 2. assisted breech, brady for 29mins 3. Apgar 5^5, pH 6.7, HIE, pulm hge, ICHs	82	Severe neuronal hypoxia in hippocampus, fresh haemorrhage in central white matter, germinal matrix and IV haemorrhage, recent + older hypoxia, brain stem neurones+cerebellar cortex central white matter gliosis +macrophages
66 (E)	27 (+)	1. smoker, amnio for incr trisomy risk, IUGR, AEDF, PIH 2. EmCS in labour fetal brady 3. Apgar 6^5, pH 6.71, HIE 3, pulm hge, ICH	135	Focal cortical infarction macrophage accumulation +neovascularisation, central white matter gliosis, large germinal matrix haemorrhage + IVH. Brain stem haemorrhage, cerebellar cortical atrophy
67	27 (+)	1. unbooked, severe PIH 2. SVD at home into toilet 3. Apgar 2^5, pH 7.06, PIE, multiorgan failure	131	Central white matter gliosis and focal macrophage accumulation. Maturing small haemorrhage in cerebellar cortex
68	27 (-)	1. smoker, NIDDM, UTI 18 wks 2. SVD 3. Apgar 8^5, pH 7.36, massive pulmonary hge	73	Neuronal eosinophilia in hippocampus and cerebellar cortex
69	26 (+)	1. smoker, heavy alcohol, late booker, anemia, APH 12 wks, recurrent abdo trauma 2. EmCS for placental abruption, fetal brady 3. Apgar 3, pH 7.23, massive pulm hge, seizures	164	Germinal matrix, central WM and IVH, pontosubicular necrosis, white matter damage+gliosis, macrophages in pons.
70	25 (-)	1. maternal varicella 25 wks in ICU, heavy sedation 2. unexpected delivery, twins 3. Apgar 9, pH 7.38, suspected seizures, RDS	111	Neuronal eosinophilia & karyorrhexis; germinal matrix haemorrhage; focal microglial & macrophage accumulation.

Key 1. pregnancy features
2. labour and delivery features
3. resuscitation and neonatal features

ROM-rupture of membranes, PROM- ruptured membranes >24hours, HT- hypertension, IOL- induction of labour, IUGR- intrauterine growth retardation, PVH- periventricular haemorrhage, APH- antepartum haemorrhage, FM- fetal movements, SB- stillbirth, AFP- serum alpha fetoprotein, WM- white matter

A careful comparison was made of the pregnancies leading to the births of infants with features of prelabour damage (PBD, n=27) compared with those without such damage (noPBD, n=32), table 5.8. Unmarried status was common overall but was significantly more common in the PBD group (24 vs 21, p=0.036). Fewer mothers in the PBD group received antibiotics in pregnancy (1 vs 8, p=0.031), more had emergency Caesarean section (17 vs 9, p=0.007) for CTG abnormalities (18 vs 8, p=0.005) and more had meconium stained amniotic fluid (11 vs 3, p= 0.005). The Apgar score was 0 at 1 minute of age in 33% of those with PBD, significantly more likely than in the noPBD group (9 vs 2, p=0.008) and the former group were heavier and more mature (2526 grams vs 1824 grams, p=0.033, and 34.6 vs 31.2 weeks gestation, p=0.051 respectively). The PBD group was more likely to be ventilated after birth for a poor respiratory drive (8 vs 3, p=0.037) and although both groups had initial acidosis, this was commoner in the PBD group (6.90 vs 7.16, p=0.02). The time to spontaneous respiration was longer (5 mins vs 1 min, p=0.009) and the 5 minute Apgar score was correspondingly less good (2 vs 5, p=0.021) in the PBD group. Reflecting the larger birthweight and more mature status, the PBD group had a higher first blood pressure (46 mmHg vs 36 mmHg, p=0.019) and was less likely to receive surfactant (4 vs 13, p=0.024). The time to death however was similar in the two groups (12 hours vs 7 hours, p=0.416).

Table 5.9. Clinical features associated with prelabour brain damage (PBD)

	PBD n=27	NoPBD n=32	Sig
Unmarried#	24(89)	21(66)	**0.036**
Smoker#	5(19)	13(41)	0.080
Multiple gestation#	2(7)	3(9)	1.000
Pregnancy-induced hypertension#	3(11)	3(9)	1.000
Antibiotics in pregnancy or labour#	1(4)	8(25)	**0.031**
Infection prior to or during labour#	2(7)	8(25)	0.092
Oligohydramnios#	6(22)	3(9)	0.275
Polyhydramnios#	2(7)	3(9)	1.000
Complicated pregnancy#	20(74)	22(69)	0.653
Caesarean section in labour#	17(63)	9(28)	**0.007**
CTG abnormalities#	18/23(78)	8/18(38)	**0.003**
Meconium stained liquor#	11(41)	3(9)	**0.005**
Apgar 0 at 1minute#	9(33)	2(6)	**0.008**
First pH^	6.9(6.8,7.1)	7.16(6.8,7.24)	**0.020**
5 minute Apgar score^	2(0,4)	5(2,8)	**0.021**
T to spontaneous respirations (min) ^	5(1,15)	1(0,4)	**0.009**
Birth asphyxia#	26(96)	20(62)	**0.002**
Birth weight (g) *	2526(1344)	1824(1120)	**0.033**
Gestation (wks) *	34.6(6.5)	31.2(6.5)	**0.051**
Small for gestational age#	4(15)	1(3)	0.169
Male sex#	12(44)	22(69)	0.060
First mean blood pressure (mmHg)*	46(3)	36(27)	**0.019**
Poor respiratory drive#	8(44)	3(14)	**0.037**
Surfactant received#	4(14)	13(42)	**0.024**
Abnormal neurological examination ≥ 6 hours of age#	12(44)	4(13)	**0.006**
Neonatal encephalopathy ≥12h age#	9(33)	1(3)	**0.004**
Age at death (h) ^	12(1,32)	7(1,26)	0.416

* mean, standard deviation, p value (independent samples t-test)
# number, percentage, 2-tailed significance (Chi-square test)
^ median, interquartile range, asymptotic 2-tailed significance(Mann-Whitney U test)

No differences in sociodemographic or pregnancy factors were identified on comparison of the encephalopathic and non-encephalopathic asphyxiated groups, but CTG abnormalities were present in 80% of the former group and in only 43% of the latter group (p=0.04).

7. The placenta

The placenta was available for examination in 41 cases (59% of those having a post-mortem examination). In 7 cases there was histological evidence of infection and in 33 cases there was none. All of the 7 infected placentas came from infants delivered prematurely. In 2 (25 and 27 weeks gestation) the inflammation was focal and in 4 it was more generalized (at 24, 24, 30 and 35 weeks gestation). The placenta of an additional baby, born at 41 weeks gestation, showed focal acute deciduitis without inflammation of the placenta, membranes or cord. Only 2 of these infants had evidence of prelabour brain damage. Thus there was no concordance of placental and brain pathology.

Discussion

This aim of this study was to describe the neuropathology in a geographically defined cohort of early neonatal deaths and to seek associations with antepartum and intrapartum events as well as with the infant's condition at birth and during life. Previous studies reporting the neuropathology of perinatal death have been hospital-based, have reflected the experience of specialist centres or have selected patients according to gestational age or disease process. This study aimed to provide population-based information from Scottish neonatal deaths occurring over a two year period. The only exclusions were infants with chromosomal abnormalities and with abnormalities of the cardiovascular and central nervous systems because these might themselves lead to neuropathological changes. Seventy cases were reviewed with extensive neuropathology.

Birth asphyxia criteria

Although intrapartum hypoxia manifesting as birth asphyxia is uncommon and in decline[43], it is still viewed as a potentially preventable cause of death or damage often with expensive medico-legal implications. Yet there is much evidence that neurodevelopmental delay and cerebral palsy are associated with birth asphyxia in only a minority of cases and also that the majority of birth asphyxiated infants do not manifest developmental delay or cerebral palsy[87;90]. The study definition of 'birth asphyxia' has been discussed previously (Chapter

3). This study used broad inclusion criteria for the diagnosis of birth asphyxia to ensure that no cases were missed. This liberal classification may have led to the inclusion of infants whose depressed condition was due to other factors such as extreme immaturity, sepsis or metabolic disease, including case 7 (Table 5.7) with gangliosidosis GM1. Evaluation of the individual clinical features of asphyxia in relation to neuropathological abnormality was performed later.

The Neuropathology

Fifty-one percent of the 137 eligible infants had detailed neuropathological investigation. The range of neuropathological abnormalities resembles those reported in previous studies [20-23;26-29;32;232;232;235;236;239;248;252;292;319]although the prevalence of grey matter and neuronal damage is generally higher than reported elsewhere. In general, many studies report findings of preterm infants or stillbirths where white matter damage has predominated. Terplan alone, in a large study of perinatal deaths from 1967, reports similar incidences of neuronal damage among neonatal deaths (table 1)[320]. Both necrosis and apoptosis have been implicated in perinatal neuronal loss[321]. Unequivocal signs of cell death are more easily detected in the differentiated neurons of the mature infant brain and were particularly prevalent in asphyxiated infants who suffered seizures. In only very few of the present cases were all neurons affected even within a single target area of the brain. Specific cell surface receptors may confer these differing levels of vulnerability, as well as forming the basis of perinatal patterns of neuronal involvement, as in pontosubicular necrosis[291]. The reactions

of the immature brain to hypoxic/ischaemic and other injury differ from those of the adult brain. The white matter appears to be particularly vulnerable in the perinatal period and both white matter infarction and the presence of axonal swellings have been described previously[290;292] although this latter feature has not been widely recognised.

Brain injury originating before the onset of labour was observed in 46% of the entire cohort, 57% of those with birth asphyxia and 90% of those with neonatal encephalopathy. Such brain damage was commoner in full term than preterm infants. These figures are higher than reported in previous studies of neonatal deaths[23;26;30;32;33;146;248;251;254]. Studies of stillborn infants generally report a higher incidence of established brain damage[23;234;235;252;253;322] than those of live births although this was not the case in the stillborn cohort of the Scottish Perinatal Neuropathology Study where the percentage with pre-existing damage was 35%[323].

The higher incidence of prelabour brain damage in mature infants may result from these infants dying from the consequences of the injury itself. Preterm infants on the other hand, may be delivered following acute maternal conditions, such as pre-eclampsia or infection and then succumb to injury during the delivery or in the postnatal period due to prematurity-related problems. Haemorrhagic lesions observed in this study were all acute and appeared to have occurred postnatally. In no infant were haemosiderin-laden macrophages seen.

Haemorrhage was no more common in infants with prelabour brain damage. The classical lesions of periventricular leucomalacia were not observed in this study. These cysts, representing focal areas of necrosis commonly clustered around the posterior horns of the lateral ventricles, generally take around 6 days to become apparent[324], and their absence in this study may be due to the short survival time of the infants.

Neuropathological considerations in interpretation of results

Judgements about neuropathological abnormalities are more difficult in the preterm than in the brain at term. The glial response, though present, is less marked in younger gestations and becomes more pronounced towards full term. Although it is possible that such responses may not have been as developed within the more immature noBA group, there were many immature infants within the birth asphyxia cohort who exhibited marked astrocytic and microglial changes. In fact, 30-40% of asphyxiated preterm infants showed gliosis and an increase in inflammatory cells. This in accordance with Gilles and co-workers[27] who found white matter astrocytosis in 33% of very low birth weight infants.

Abnormal proliferation of astrocytes may be difficult to differentiate from the 'myelination gliosis' of the normal developing brain. Myelination occurs in the white matter of the cerebral hemispheres of the human brain from the end of the second trimester and is characterised by proliferation and enlargement of oligodendrocytes and astrocytes. Immature glia and immature migrating neurons

may also be difficult to distinguish from reactive astrocytes. All of these cells can be differentiated from the astrocytic hyperplasia seen in response to injury by the demonstration of glial fibrillary acidic protein (GFAP) in the cytoplasm of reactive glia. This immunocytochemical technique was used to avoid confusion with myelination gliosis and other immature glia. This study also found that some astrocytes, found to be markedly reactive on GFAP immunocytochemistry, were not easily seen on routine staining. It is concluded that a meaningful assessment of gliosis in the neonatal brain requires GFAP immunocytochemistry. The significance of the apparent Alzheimer type II astrocytosis present in a number of cases is unclear but it has been remarked upon previously in immature brains[291]. It may represent the result of metabolic upset and in this study was more prevalent in infants with brain damage.

Cell death is an integral part of the development of the central nervous system and up to 50% of neurones die by programmed cell death (apoptosis) before maturity, due to limiting trophic support from the target tissues they innervate. The classical sign of apoptosis, the karyorrhectic nucleus, has been recognised by pathologists for many years[325]. Apoptosis also appears to be the preferred mode of death in the developing brain following a variety of insults. Cells at an earlier stage of development are likely to die by apoptosis rather than primary necrosis, which is usual in cells which are differentiated. Although our understanding of why one type of cell death is favoured over another is limited, it is possible that milder insults are more likely to result in apoptotic cell death

than severe insults. For the purposes of this study, nuclear karyorrhexis was regarded as abnormal, if large numbers of cells within a tissue were involved or if accompanied by other post-hypoxic cellular responses.

Despite these considerations, comparison of the asphyxiated infants and those not apparently suffering from birth asphyxia shows clear differences in terms of neuropathological changes. Examination confined to infants who died within 3 days of the start of labour, and the separation of the asphyxiated group into those with and without neonatal encephalopathy, identifies a spectrum of damage. Unsurprisingly, the mature infants who died after displaying neonatal encephalopathy are most likely to show neuropathological changes. All of the brains in this study were carefully examined in order to determine whether any damage could have occurred before the onset of labour. If it is accepted that features such as focal or diffuse astrocytic hyperplasia and parenchymal macrophage accumulation are cellular reactions which require three days to become established, a majority of infants with features of BA sustained brain damage prenatally, including all 8 of the FT encephalopathic group. What is uncertain is how old such damage is, but the background of apparently normal brain development suggests that the insult was sustained not long before labour commenced. It is harder to draw conclusions about the preterm infants in this study but the absence of neuropathological changes in virtually all the mature and most of the preterm infants who did not display asphyxia is reassuring.

The difficulties associated with interpreting these neuropathological findings and attributing timing of onset to them is not underestimated. Every abnormality has been included Table 5.7, whether focal or diffuse, recent or old, but it is accepted that interpretation is subjective. Some observations on perinatal brain necrosis have assumed that the injury has arisen from a single insult. However, the results of this study and reports by previous authors[24;237;239;240;292;308] show that brain lesions of varying ages may occur in the same infant. Therefore the possibility of multiple or repetitive injuries has to be taken into account when dating lesions and ascribing pathogenesis. Difficulties with timing the abnormalities arise from the fact that experimental studies are not possible in human infants although classic studies were able to relate pathology findings to major clinical incidents[32;292]. Some experts also comment that the results of animal work may not be directly relevant to the human situation[32] although the timing of an insult may be far more accurate[292]. A number of the classic studies were conducted before cell specific immunocytochemistry became available although this is not the case for more recent papers. Earlier papers may not have always included, or added, the duration of labour as a factor in timing, and interpretation may be hampered by longer survival. It is noted that the infants with a history of encephalopathy had survived for more than one day in most instances. It is conceded that seizure activity might possibly induce and accelerate some of the changes seen in the brains of such infants but the presence of diffuse astrocytosis in other infants who had survived very few hours and who died with no evidence of seizure activity reinforces the

possibility of prenatal origin. A more secure evidence base for timing neuropathological events awaits the evolution of new markers of cell damage and irreversible cell death. The clinical significance of some lesions described such as diffuse astrocytosis, and in particular their contribution to the cause of death, remains uncertain.

Correlation of Clinical Factors and Neuropathology

This study identified few factors which predicted the incidence of prelabour brain damage. Damage occurring before the onset of labour was commoner in more mature and heavier infants, who were more likely to be delivered by emergency caesarean section. Abnormal CTG, meconium staining of liquor, asystole at birth, acidosis and prolonged depression of spontaneous respirations are all common in the presence of intrapartum asphyxia yet were found to be predictive for prelabour brain damage. Grafe studied the brains of 98 perinatal deaths and found meconium staining of the placenta to be associated with white matter necrosis in the brain[235]. It has been suggested that meconium in the liquor may cause vasoconstriction of the umbilical vessels although this is difficult to substantiate following delivery[166].

An abnormal CTG was the only clinical factor differentiating the asphyxiated infants who displayed encephalopathy and neuropathological abnormality from those who did not. Abnormal heart rate recordings have previously been described in fetuses with central nervous system lesions and malformations[99;326]

and a persistent non-reactive fetal heart rate tracing has been described in neurologically impaired infants who had clinical evidence of compromise occurring prior to the onset of labour[327]. Infants with severe encephalopathy show a lack of heart rate variability during life[328].

Infants who went on to develop classical signs of hypoxic-ischemic encephalopathy were far more likely to show signs of prelabour brain damage. As discussed previously, it is possible that seizure activity may induce or accelerate post-injury changes but even infants without seizures who had an abnormal early neurological examination at 6 hours were more likely to have established neuropathological lesions. Neurological abnormality in these infants may be the result of previously sustained damage. In many infants, acute change such as cerebral oedema and haemorrhage co-existed with older lesions. The possibility arises that infants who have been damaged before the onset of labour, may tolerate even a normal labour poorly, manifesting signs of fetal distress and sustaining further damage at relatively ordinary levels of hypoxia.

Intrauterine growth restriction, polyhydramnios and oligohydramnios have previously been associated with established brain damage [27;30;32;234] but no similar associations were found in this study. This may have been due to the small number of cases studied with these complications. In the context of fetal brain damage, polyhydramnios is often attributed to impairment of fetal swallowing and oligohydramnios to renal dysfunction following the asphyxial

insult. In this study, abnormalities of liquor volume may have gone undetected due to the presumed short interval between brain injury and the onset of labour.

Recently the presence of prenatal infection has been linked to white matter damage[145;146;329]. This study found no support for this association and in fact infection during labour was commoner in those infants without established damage. The best diagnosis of intrauterine infection is the presence of histological placental inflammation or funisitis. Chorioamnionitis usually results in expeditious delivery of the fetus and any neuropathological changes, resulting either directly from intrauterine infection or more probably from the ensuing fetal inflammatory response[330] may take days to develop[329], and may exceed the survival time of many of our infants. Despite the intention to collect placentas from all infants who died, in practice this was poorly achieved and examination was limited to 59% of infants undergoing post mortem. A more complete analysis may have yielded additional information.

Implications for Surviving Infants

It is possible that the neuropathological findings reported here represent the most severe end of a spectrum of perinatal brain damage resulting in a fatal outcome while infants surviving perinatal asphyxia might show lesser degrees of similar pathology. However the possibility also exists that dead infants and survivors represent two completely different groups in terms of both causation and pathology. It is also possible that a similar proportion of normally delivered

infants have exactly the same changes. Recent neuroimaging studies of neonates surviving with encephalopathy, with or without seizures, have a bearing on these questions. A large study by Cowan and colleagues[33] concluded on the basis of MRI examination performed in the first two weeks of life that brain damage in mature infants presenting with newborn encephalopathy was most often acute and of perinatal onset particularly in an encephalopathic group without seizures. Very few infants in that study displayed evidence of prenatal brain damage on 1-1.5 Tesla sequencing. Neuropathological corroboration was achieved in very few cases. Three of 21 infants with HIE (14%), who had undergone detailed neuropathology designed to establish the incidence of prenatal damage, had such changes in the brain. In the absence of immunocytochemical investigation of gliosis and brain macrophage accumulation in all deaths, their conclusions about the prevalence of prenatal abnormalities may be an underestimate. Higher resolution MRI may be required to detect early changes. The difficulties surrounding timing of lesions have been discussed within our own study in which conclusions regarding presence or absence of prenatal brain damage were confined to infants who died less than 3 days from the onset of labour and based on neuropathological examination rather than imaging. Against a background of normal brain development, the cerebral insult was likely to have been sustained only shortly before the onset of labour, possibly even precipitating the onset of labour. Evidence of ongoing neuronal damage was also present within this series, not dissimilar from the findings in Cowan's study, but this was frequently in addition to the damage

identified as occurring pre-labour within the constraints of current knowledge.

The question arises as to whether the established changes observed in our cohort translate to significant functional consequences in survivors. There is little doubt that some infants who exhibited widespread, severe changes may have developed neurological sequelae if they had survived. However more localised lesions or milder gliotic change may affect functionally unimportant parts of the brain or may simply not result in sufficient neuronal death to cause neurological impairment. It is well-established that abnormality of the internal capsule on MRI has a high sensitivity and specificity for later neurological impairment following hypoxic-ischemic encephalopathy[331]. Infants without such abnormality do well. Although MRI is poorly sensitive for histological change[302;303] it may be that some damage undetectable by MRI has little clinical consequence. On the other hand, MRI of the brain may appear normal in up to a quarter of patients with cerebral palsy[332]. In such obvious functional impairment, histological damage must surely exist. Newer MRI technology with diffusion weighted imaging and higher strength magnetic fields may improve detection of perinatal brain injury. More detailed topographical analysis may indicate the significance of the brain injury observed within this cohort.

It might be expected that brain damage in survivors would be less extensive and severe than in those with a fatal outcome. Whether the brain damage observed in our study represents the result of persisting or repeated insult, or the onset of a potentially reversible cascade accruing from a single insult, is uncertain. A

multistep pathological process might present opportunities for intervention to limit further brain damage.

There is increasing evidence to suggest that combined insults may have synergistic effects in causing brain injury. Early differentiating oligodendroglia in culture indicate a particular vulnerability to combined glucose and oxygen deprivation but not to either alone[333]. Combined exposure to endotoxin and a hypoxic-ischemic insult in a neonatal rat model results in extensive brain damage that cannot be explained by simple additive effects and suggests synergy between ischemia and inflammation[334]. This work supports the finding that combined intrapartum infection and evidence of hypoxic-ischemia dramatically increases the risk of spastic cerebral palsy compared to hypoxia-ischemia alone (OR 78)[164]. The possibility that infants with antenatal damage may experience further compromise during labour, suggests that the early identification of such a group with subsequent avoidance of labour might be neuroprotective. Studies of neonatal asphyxia and cerebral palsy have failed to show a protective effect of Caesarean section[335]. This may be in part because distinction is generally not made between elective prelabour and emergency in-labour CS. However, Badawi and colleagues found that elective caesarean section was associated with a odds ratio of 0.17 for neonatal encephalopathy in the Western Australian case-control study[59]. Others have shown a protective effect of prelabour CS for PVL in preterm infants in the context of chorioamnionitis (OR 0.15)[336].

The fact that a significant proportion of clinically asphyxiated infants display no evidence of brain damage, and that infants who are not asphyxiated at birth frequently display only recent postnatal damage, offers hope for a good clinical outcome if such infants could be identified and "rescued" by medical intervention. This study has demonstrated that the current battery of pregnancy and labour-associated investigations remain blunt instruments in accurately predicting the arrival of a prenatally brain damaged infant. Future work must address the development of methods for detecting antepartum damage so that optimal management of these vulnerable fetuses can be planned. Further evidence is also required regarding evolution of cellular reactions in the developing brain. The findings in this study support the notion that the birth of a compromised "asphyxiated" encephalopathic infant is not necessarily the result of a mismanaged labour or of a lack of vigilance in pregnancy.

Summary

In 1862, William John Little, physician to the London Orthopaedic Hospital presented a paper describing the contribution of prolonged and difficult labour to the condition of spastic diplegia[337]. From this time onwards, cerebral palsy became synonymous with 'birth asphyxia' despite attempts by Sigmund Freud in the late 1890s to attribute cerebral palsy to damage occurring prior to labour[338].

Such pervasive opinion about the intrapartum origin of cerebral palsy has continued despite large epidemiological studies published over the last 15 years indicating that a minority of cases of cerebral palsy are secondary to intrapartum influences. In fact these studies suggest that the majority of childhood neuromotor impairment originates prior to the onset of labour[10;87] These findings have been supported by observation of antenatally occurring brain injury at post mortem and on imaging of the fetus and the newborn [32;146;252;290;301].

Despite this evidence, obstetric malpractice claims have soared over the last 50 years such that the obstetric work force is declining throughout the developed world. In the United States of America, 76% of obstetricians report being sued at least once and one in seven no longer practice in the field [339]. In some areas

of the country due to workforce shortages, many women are no longer under the care of an obstetrician, but are cared for by birth attendants only. In the United Kingdom, 1800 new cases of cerebral palsy are diagnosed each year. Of these, one third will result in litigation and one third of these claims will be awarded damages. Cerebral palsy constitutes 5% of all medical negligence claims within the NHS but accounts for 60% of all payments, amounting to £451 million in the year 2001/2 [340;341].

The Scottish Perinatal Neuropathology Study was designed to investigate the clinical factors associated with asphyxia-related death and to determine the prevalence of antenatal brain injury occurring within a population of early neonatal deaths and stillborn infants. It sought to investigate the clinical factors associated with prenatal brain damage in an attempt to explain the pathogenesis.

Results from this study have shown that adverse features such as antepartum haemorrhage, anaemia and intrauterine growth retardation often complicate pregnancies resulting in early neonatal death. A high incidence of previous pregnancy loss and infertility was common as were mothers to be unmarried or to smoke. No factors predicted the birth of an infant with asphyxia apart from CTG abnormalities and meconium staining of the liquor.

Seventy newborn infants underwent detailed neuropathology. Evidence of prelabour damage was observed in nearly half of a group of infants surviving for less than three days. This damage was more common in asphyxiated infants. Clinical associations of such established damage were few. Indicators of fetal distress such as cardiotocograph abnormalities and meconium staining predicted prelabour damage, as did a depressed condition at birth. Nearly all infants who demonstrated a neonatal encephalopathy had evidence of brain damage which had occurred prior to the onset of labour.

These results have relevance to both perinatal medicine and paediatric neurodevelopment. Obstetricians strive to eliminate morbidity and mortality during pregnancy and the perinatal period. Many have developed high risk clinics for the identification and surveillance of vulnerable fetuses. General improvements in antenatal and intrapartum care have led to important reductions in the perinatal mortality rate. Despite this, the results of this work show that damage may occur undetected during fetal life and without obvious warning. Current instruments for fetal surveillance are at present not sensitive enough to detect such fetal injury and new diagnostic techniques are needed to assess brain development, detect early brain injury and to monitor interventions aimed at minimizing brain injury. Although routine ultrasound imaging in pregnancy now occurs throughout the developed world, newer technology such as 4 dimensional (real time) fetal imaging may eventually provide in depth information about the normality of fetal behaviour. It is possible that measurements of the quantity

and quality of fetal movement may allow assessment of neurological damage if present. Fetal MRI has already provided insights into normal neurodevelopment including myelination and neuronal migration, and has documented a spectrum of malformation and injury to the fetal brain[301;342]. Newer research techniques such as functional MRI[343] and MR spectroscopy[344] may provide information about metabolism at both regional and cellular levels following fetal hypoxic-ischemic brain injury.

Identification of fetal brain injury raises both obstetric and neonatal management issues. The counselling of a mother whose fetus has sustained such injury must be informed by accurate prognostic information. The plasticity of the developing brain may influence prediction of later neurodevelopmental sequelae. Avoidance of further injury may mean avoidance of labour or expeditious delivery, both of which may have significant morbidity for the mother and infant.

Current neonatal neuroprotective strategies such as brain cooling rely on a window of opportunity following primary brain injury in which to prevent secondary cell death. A fetus who has been damaged some time before labour may not benefit from such an intervention. Preliminary results from the hypothermia trials suggest that hypothermia may not be beneficial to those infants with severe encephalopathy[345]. Within this Scottish study, 90% of those with severe encephalopathy showed evidence of damage occurring prior to the

onset of labour. It is likely that secondary cell death would have been well established within this group of infants. It is possible that the resistance of some infants with severe encephalopathy to the neuroprotective effects of hypothermia may be due to the advanced state of energy failure and cell death within these damaged brains. Accurate detection of pre-existing brain injury may influence application of such an intervention in the future.

The detection of brain injury is not sufficient. Preventative strategies can only be developed through an understanding of the pathogenesis. Recent interest has focused on the role of infection in white matter injury[329;330;346]. Evidence suggests that the fetal response is more detrimental than the direct effect of the pathogen[330]. The role of maternal pyrexia may also be significant[151]. Emerging concerns about the synergistic effects of hypoxia and infection may modify intrapartum management[334].

This study found no support for the role of infection in fetal brain damage and indeed identified no factors to explain the brain injury observed. Adverse features in the maternal history were common compared to all Scottish maternities but affected infants with damage as often as those without. Individual vulnerability may predispose a particular infant to suffer damage at a level of hypoxia that another infant may tolerate unharmed.

This study found a high prevalence of antenatally occurring brain injury among asphyxiated infants who die in the neonatal period. It is unclear whether these results are applicable to survivors of perinatal asphyxia or whether these infants who die represent a different group altogether. The majority of infants with signs of birth asphyxia escape their insult unscathed as do many infants with hypoxic-ischaemic encephalopathy. A normal neurodevelopmental examination at 18 months was found in over half of a recent cohort of infants with moderate or severe encephalopathy[11]. Those infants with encephalopathy who eventually die may be a group in whom effects of antenatal and intrapartum injury have combined to make extrauterine life impossible while those with a less severe or an acute brief insult may make a full recovery. It may however be simplistic to imagine a spectrum of sequelae resulting from a spectrum of injury and antenatal brain damage observed histologically may be of a different pathoaetiology to that resulting in cerebral palsy.

Investigation of the pathogenesis of brain injury and disease in adults has revcaled that an individual's genetic profile may influence response and recovery. In particular the apolipoprotein E gene has received much interest due to its strong association with Alzheimer's disease and atherosclerosis. The ε4 allele in particular is a risk factor for Alzheimer's disease and poor outcome following traumatic brain injury and cardiac bypass surgery[189;190;197]. A small number of studies in early life suggest the ε4 genotype may be advantageous for

fetal survival and childhood cognitive performance[40;212;213]. This project sought to determine differences in the prevalence of APOE genotype in a population of perinatal deaths and investigate any relationship with brain injury.

Healthy newborn infants were found to have an increase in the prevalence of the ε4 allele compared to both perinatal deaths and to adults supporting the possibility for an advantageous role of this gene in early development. Antagonistic pleiotropic effects of the ε4 allele may be responsible for beneficial effects in childhood but later adverse effects on cognitive decline. Although ε4 has been associated with neurological injury and deterioration in later life, no such association was found with the presence of brain injury in these perinatal subjects.

Further work is needed to clarify the role of APOE in perinatal mortality, through linkage disequilibrium and/or whole genomic studies. With regard to brain injury, ApoE has important functions in both neuronal repair and synaptic remodeling[39;188]. Although ApoE may not affect the occurrence of brain injury as such, it may have an influence on longterm neurodevelopmental outcome. Genetic studies of survivors of perinatal brain injury may help to establish such an association if present.

It is the aim of all who work in perinatal medicine to prevent maternal and

infant mortality and morbidity. The results presented in this thesis demonstrate that brain injury does occur in utero and responsibility must follow to prevent such damage or at least to develop interventions to limit further injury. Furthermore, 'fetal distress' during labour may not always represent the presence of potentially damaging acute asphyxia but instead may signify an already damaged fetus whose tolerance for a normal labour has already been compromised.

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Appendix 1

Scottish delivery units in the Scottish Perinatal Neuropathology Study

Simpson Memorial Maternity Pavilion, Royal Infirmary of Edinburgh
Eastern General Hospital, Seafield Rd, Edinburgh
St John's Hospital, Howden Rd, Livingston, West Lothian
Borders General Hospital, Melrose, Roxburghshire
Falkirk and District General Hospital, Majors Loan, Falkirk, Central
Stirling Royal Infirmary, Livilands gate, Stirling, Central
Perth Royal Infirmary, Perth, Tayside
Ninewells Hospital, Dundee, Tayside
Aberdeen Maternity Hospital, Cornhill Rd, Aberdeen, Grampian
Raigmore Hospital, Inverness, Highland
Vale of Leven Hospital, Alexandria, Strathclyde
Inverclyde Royal Hospital, Greenock, Strathclyde
Ayrshire Central Hospital, Kilwinning Rd, Irvine, Strathclyde
Cresswell Maternity Hospital, Dumfries, Strathclyde
Southern General Hospital, Govan Rd, Glasgow
Glasgow Royal Maternity Hospital, Rottenrow, Glasgow
Rutherglen Maternity Hospital, Rutherglen, Glasgow
Queen Mother's Hospital, Yorkhill, Glasgow
Royal Alexandra Hospital, Corsebar Rd, Paisley, Strathclyde
Forth Park Hospital, Kirkcaldy, Fife
Bellshill Maternity Hospital, Bellshill, Strathclyde
Law Hospital, Carluke, Strathclyde

Appendix 2
Clinical Dataset for The Scottish Perinatal Neuropathology Study

Maternal Details

Age (y)

married- yes, no

social class- professional, trade/clerical, unskilled manual, housewife, unemployed, student, missing

smoker- yes, no

other recreational drug abuse- intravenous, oral, inhaled drugs

abdominal surgery- yes, no

uterine surgery- yes, no

number of previous pregnancies

number of previous pregnancy losses

number of previous terminations of pregnancy

blood group- O Neg, O Pos, A Neg, A Pos, AB Neg, AB Pos, B Neg, B Pos

Antenatal Details

booking status- unbooked, booked after 16 weeks, booked at/before 16 weeks

assisted conception- ovulation induction, IVF, GIFT, AID, ICSI

weight at booking (kg)

systolic blood pressure at booking (mmHg)

diastolic blood pressure at booking (mmHg)

multiple pregnancy- yes, no

amniocentesis performed- yes, no

reason for amniocentesis performed- increased trisomy risk on screening, maternal age, maternal request, amnioreduction

serum screening- normal, not performed, increased trisomy risk, increased NTD risk

rubella immune- yes, no

abnormal fetal anomaly scan- yes, no, not performed

medications in pregnancy- nil or vitamins only, antibiotics, sedatives, antihypertensives, antiepileptics, insulin, steroids, combination

anemia- nil, <9g/dl

oligohydramnios- yes, no

polyhydramnios- yes, no

hyperemesis requiring hospitalisation- yes, no

Grade 3/ Grade 4 placenta praevia- yes, no

antepartum haemorrhage, first trimester- yes, no

antepartum haemorrhage, second or third trimester- yes, no

loss of fetal movements (gestation)- yes, no

pyrexial illness with flu symptoms incl urinary tract infection (gestation)- yes, no

pyrexial illness without flu symptoms, incl urinary tract infection (gestation)- yes, no

diabetes- nil, pregnancy associated diet-controlled, pregnancy associated insulin-controlled, non-pregnancy associated insulin-dependent diabetes mellitus

premature rupture of membranes (gestation) <35 weeks- yes, no

intrauterine infection with isolated pathogen (gestation)- yes, no

abdominal trauma (gestation)- yes, no

essential hypertension- yes, no

pregnancy-induced hypertension- diastolic 20mmHg above booking or 90 mmHg (+/- proteinuria, +/- oedema), eclampsia

failure to gain weight in pregnancy- nil, 2nd trimester, 3rd trimester, throughout pregnancy

Intrapartum Details

initiation of labour- spontaneous, induced, no labour

length of first stage (h, min)

length of second stage (h, min)

duration of ruptured membranes- at delivery, < 24hours, >24 hours

delivered at home- yes, no

malpresentation of baby- yes, no

mode of delivery- spontaneous, forceps/Ventouse, emergency CS prelabour, emergency CS in labour, elective CS

meconium stained liquor- yes, no

CTG abnormalities- yes, no

CTG abnormalities- details

epidural- yes, no

general anaesthetic- yes, no

cord prolapse- yes, no

pyrexial in labour >38C- yes, no

intrapartum infection- details incl pathogen, white cell count, C reactive protein
excessive bleeding in labour- yes, no
baby with weight < 3rd centile- yes, no
weight of baby (kg)
occipitofrontal circumference of baby (cm)
gestation of baby (wks)
gestation of baby < 37 weeks- yes, no
sex of baby- male or female

Newborn Details

Apgar score at 1 min
Apgar score at 5mins
Apgar score at 10 mins
resuscitation required- nil, intubation only, full resuscitation including cardiac massage and drugs
time to spontaneous respirations > 10 mins- yes, no
admitted to SCBU- yes, no
initial pH
initial pH <7.1- yes, no
grade of HIE- nil, 1, 2, 3
age at death (h)
renal impairment- yes, no (defined as Creatinine >120, Hematuria, proteinuria, oliguria >24hours)
inotropic requirement- yes, no
first mean arterial BP- mmHg
abnormal coagulation screen- yes, no
hypoglycemia- yes, no
hyperglycemia- yes, no
main respiratory problem- poor drive, RDS, PPHN, pneumothorax, pulmonary haemorrhage, hypoplastic lungs, pleural effusions
seizures- yes, no, unsure
cranial USS results- details
EEG results-details
CT scan results- details
Medications during life- details

Appendix 3
Protocol for Neuropathological Specimens

From 30 weeks gestation onwards (20 blocks):

R and L frontal parasagittal

R and L parietal parasagittal

R and L parietal convexity

R and L basal ganglia at level of mamillary bodies

R and L thalamus

R and L hippocampus at level of lateral geniculate body

R and L occipital

Midbrain

Pons

Medulla

R and L cerebellar hemispheres (including dentate nucleus) and vermis

From 24-30 weeks gestation (14 blocks):

R frontal

R and L hemispheres- level of basal ganglia

R and L hemispheres- level of thalamus

L occipital

Midbrain

Pons

Medulla

R and L cerebellar hemispheres (including dentate nucleus) and vermis

Appendix 4
Publications

- Becher JC, Bell JE, Keeling JW, McIntosh N, Wyatt B. The Scottish Perinatal Neuropathology Study- clinico-pathological correlation in early neonatal deaths. *Arch Dis Child Fetal Neonatal Ed* 2004;89(5):F399-407

- Becher JC, Bell JE, McIntosh N, Keeling JW. Distribution of apolipoprotein E alleles in a Scottish healthy newborn population. *Biol Neonate.* 2005; 88(3):164-167

- Bell JE, Becher JC, Wyatt B, Keeling JW, McIntosh N. Brain damage and axonal injury in a Scottish cohort of neonatal deaths. *Brain.* 2005;128:1070-1081

- Becher JC, Keeling JW McIntosh N, Wyatt B, Bell JE. The distribution of apolipoprotein E alleles in Scottish perinatal deaths. *J Med Genetics.* In Press

Consent has been obtained from joint authors and from publishers of all above journals to include copies of each paper as appended.

ORIGINAL ARTICLE

The Scottish perinatal neuropathology study: clinicopathological correlation in early neonatal deaths

J C Becher, J E Bell, J W Keeling, N McIntosh, B Wyatt

Arch Dis Child Fetal Neonatal Ed 2004;**89**:F399–F407. doi: 10.1136/adc.2003.037606

The supplementary tables can be found at http://adc.bmjjournals.com/supplemental/.

See end of article for authors' affiliations

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Background: A proportion of neonatal deaths from asphyxia have been shown to be associated with pre-existing brain injury.
Objectives: (*a*) To compare the epidemiology of infants displaying signs of birth asphyxia with those not showing signs; (*b*) to examine the neuropathology and determine if possible the timing of brain insult comparing asphyxiated with non-asphyxiated infants; (*c*) to compare the clinical features of those born with birth asphyxia with and without pre-labour damage.
Methods: Over a two year period, all 22 Scottish delivery units collected clinical details on early neonatal deaths. Requests for post mortem included separate requests for detailed neuropathological examination of the brain. Infants were classified into two groups: birth asphyxia and non-birth asphyxia. Clinicopathological correlation was used to attempt to define the time of brain insult.
Results: Detailed clinical data were available on 137 of 174 early neonatal deaths that met the inclusion criteria. Seventy of 88 parents who had agreed to post mortem examination consented to a detailed examination of additional samples from the brain; in 53 of these cases the infant was born in an asphyxiated condition. All asphyxiated and encephalopathic infants, 38% of mature and 52% of preterm infants with features of birth asphyxia but without encephalopathy, and only one of 12 infants without any signs of birth asphyxia showed damage consistent with onset before the start of labour.
Conclusions: In a large proportion of neonatal deaths, brain injury predates the onset of labour. This is more common in infants born in an asphyxiated condition.

The three major causes of neonatal death are lethal malformations, prematurity, and birth asphyxia.[1] Whereas the general public considers major malformations and premature birth as unavoidable mischance, birth asphyxia implies a lack of care in labour. Although birth asphyxia is classically linked to intrapartum hypoxia-ischaemia in full term infants, often proceeding to a neonatal encephalopathy—the so-called hypoxic-ischaemic encephalopathy, a proportion of preterm babies are also born in a neurologically depressed condition almost certainly related to poor oxygenation in labour. Asphyxia is acknowledged to be an imprecise term, but is still used regularly by the profession and parents. It may be implied by one or more of the following features: a low Apgar score[2–7]; a baby who is difficult to resuscitate; metabolic acidosis in either the cord[5 6 8 9] or early neonatal blood samples; the development of neonatal encephalopathy.[10–12] A history of these particular features may be sought retrospectively if an infant goes on to develop neurodevelopmental delay. None of these indicators, when applied prospectively to infants born in poor condition, has good sensitivity, specificity, or predictive value for neurodevelopmental delay or disability, although in full term infants the development of neonatal encephalopathy is more specific.[13] It is clear that perinatal asphyxia is not likely to be an important factor in the development of every case of neonatal encephalopathy or in most cases of cerebral palsy.[14–19] This view has been endorsed by a statement from the International CP Task Force.[20 21]

Obstetric care has seen dramatic changes over the last few decades. Most changes have contributed to the steadily falling stillbirth and neonatal death rates.[1 22–25] However, despite better clinical care and widespread use of fetal monitoring and fetal blood sampling, full term infants continue to be born in a neurologically depressed condition. Such infants cause considerable distress to parents and staff. They contribute both to early neonatal mortality and to the pool of children who display later neurodevelopmental disability with cerebral palsy. Although in some cases obstetric risk factors can be identified, affected children also result from pregnancies and labours that, even when scrutinised critically, appear to be normal.

Litigation for perceived perinatal mismanagement is increasingly common, particularly in relation to infants born in a neurologically depressed condition—usually manifested by a poor Apgar score—and often reflexly labelled birth asphyxia. Some recent anecdotal reports and small series of infants born in poor condition have shown neuropathological abnormality at autopsy that must have preceded the onset of labour. These generally represent the collected experience of specialist referral centres[26–30] or focus on a particular age group of infants—for example, those born preterm.[31–35] Only rarely do the neuropathological studies include correlation with clinical factors.[36–38] Further insights into cliniconeuropathological correlation in the first weeks of life are now being achieved by neuroimaging.[39] This Scottish study was set up to identify neuropathological abnormalities in a population cohort of perinatal deaths and to explore the relation between clinical features and pathological findings. We report here the findings in the neonatal deaths.

The specific aims of this paper are to:

- review the epidemiology (sociodemographic, antenatal, and perinatal factors) of the early neonatal deaths overall

and to compare infants who displayed signs of birth asphyxia with those who did not;
- investigate the neuropathological status in those infants in whom a post mortem was authorised, and to determine whether lesions could be of prenatal origin;
- determine if infants who have pre-existing brain damage are, when born alive, more likely to be born in an asphyxiated condition;
- compare the antepartum and intrapartum course of early neonatal deaths of infants born with birth asphyxia with and without pre-existing damage.

METHODS

Study setting and patients

The Scottish perinatal neuropathology study was a prospective observational and experimental study involving all 22 delivery units within Scotland. Patients were recruited during a two year period for each centre. The study started in January 1996, and recruitment of cases was completed by January 1999. The base study considered all perinatal deaths of infants who were $\geq$ 24 weeks gestation at birth and $\leq$ 7 days at time of death delivered in Scotland over the two year period. This paper concerns the epidemiology and neuropathology of the liveborn subset of the study cohort. The stillborn infants presented somewhat different features and will be reported on separately.

Infants with central nervous system or cardiac malformations, major chromosomal abnormalities, or central nervous system infection were excluded because it was felt that the neuropathological changes associated with such conditions might interfere with the interpretation of any changes superimposed by perinatal insult.

Figure 1 lists how the cohort of 692 qualifying perinatal deaths was reduced by various exclusions through the 221 liveborn infants to the 70 infants from whom the brain was available for examination in this study. These 70 infants were classified according to whether they displayed birth asphyxia (BA group) or not (noBA group). Analysis of those who died three days or less after the onset of labour allowed identification of pathological features likely to have predated labour and birth. Placentas were available for histological examination from 41 of the 70 infants.

Ethical and consent procedures

Before the start of the study, each delivery unit obtained approval from their local research ethics committee to approach appropriate parents. As different units received ethical permission at slightly varying times, the spread of data collection was three years, although it was two years for each individual centre. Cases were enrolled at the time of post mortem request by the clinician responsible for the care of the infant during life. A detailed clinical dataset was collected on all infants regardless of enrolment status. The purpose of the study was explained to parents. Signed consent was obtained for autopsy, and on a separate consent form, if authorised also for extended neuropathological research studies on the brain.

Clinical details

For each case a detailed questionnaire was completed by specially trained midwives or other local staff who recorded a battery of clinical information and the results of investigations relating to each pregnancy, labour, delivery, and neonatal course. This was entered into a central database (SPSS) by the study clinical coordinator (JCB). Information on the intrapartum cardiotocograph (CTG) was recorded if available.

Diagnosis of asphyxia

No test is available to accurately diagnose clinically important intrapartum asphyxia. The CTG is notorious for its poor predictive value.[40 41] As one of the principle aims of the study was to determine if infants with pre-existing brain damage are predisposed to neurological depression at birth which might be labelled as birth asphyxia, we used fairly broad inclusion criteria.

- An Apgar score at five minutes of $\leq$ 5.0; this is the traditional assessment and it is widely recognised that a low five minute Apgar score has an association, although weak, with both neonatal death and morbidity in surviving infants.[42]
- A cord or initial blood pH of $<$ 7.1; obstetric epidemiology has shown that a scalp pH of less than 7.25 is abnormal and delivery is indicated if less than 7.2.[40 41] The relation between scalp and cord pH is good with a sensitivity of 93%.[43] However, the neonate is rarely difficult to resuscitate unless the cord pH is less than 7.0. We arbitrarily chose an intermediate level (pH $<$ 7.1) as indicating some degree of birth asphyxia in this group of early neonatal deaths. Recognising the limitations, we also used (in the absence of a cord pH) a first blood gas with a pH less than 7.1 to indicate asphyxia.
- The presence of grade 2/3 neonatal encephalopathy. This is widely accepted as having a closer association with significant birth asphyxia and long term neurodevelopmental disability.[13 44] The grading of encephalopathy used was that of Sarnat and Sarnat.[45]

Because of the diverse clinical circumstances, not all criteria were available for assessment in each case. Infants who displayed at least one of these criteria were classified as showing clinical evidence of birth asphyxia (BA group). If none of these criteria were present, the infant was included in the non-asphyxiated group (noBA).

Pathological examination

Autopsies

Autopsies were conducted in six Scottish centres, and the brain was retained in fixative for later examination. In the south east of Scotland, the fixed brains were examined in the Department of Neuropathology at the Western General Hospital, Edinburgh. Elsewhere they were sampled locally according to a previously agreed protocol. Up to 20 representative paraffin embedded blocks were prepared in each case from all areas of the cerebrum (including temporal hippocampus), and from the basal ganglia and thalami, midbrain, pons, medulla, vermis, and cerebellar hemispheres. These blocks were collected centrally for review and further investigation in Edinburgh. Paraffin sections were stained routinely with haematoxylin and eosin and luxol fast blue/cresyl violet (myelin). Selected sections were investigated immunocytochemically for astrocytic status, using an antibody to glial fibrillary acidic protein and for microglia/macrophages (antibodies to CD68 and MHCII) or stained with Perls Prussian blue stain (haemosiderin). The neuropathological appearances in grey and white matter were assessed independently in all cases by two observers (JEB and BW), who were initially blind to the clinical history. Selected cases were also reviewed by JWK. Recorded neuropathological features included neuronal eosinophilia and karyorrhexis, astrocytic hyperplasia, activated microglia and accumulation of macrophages, haemorrhage (recent and older), vascular responses, and foci of mineralisation and of infarction. The neuropathological features were then correlated with the gestational and postnatal age of the infant and

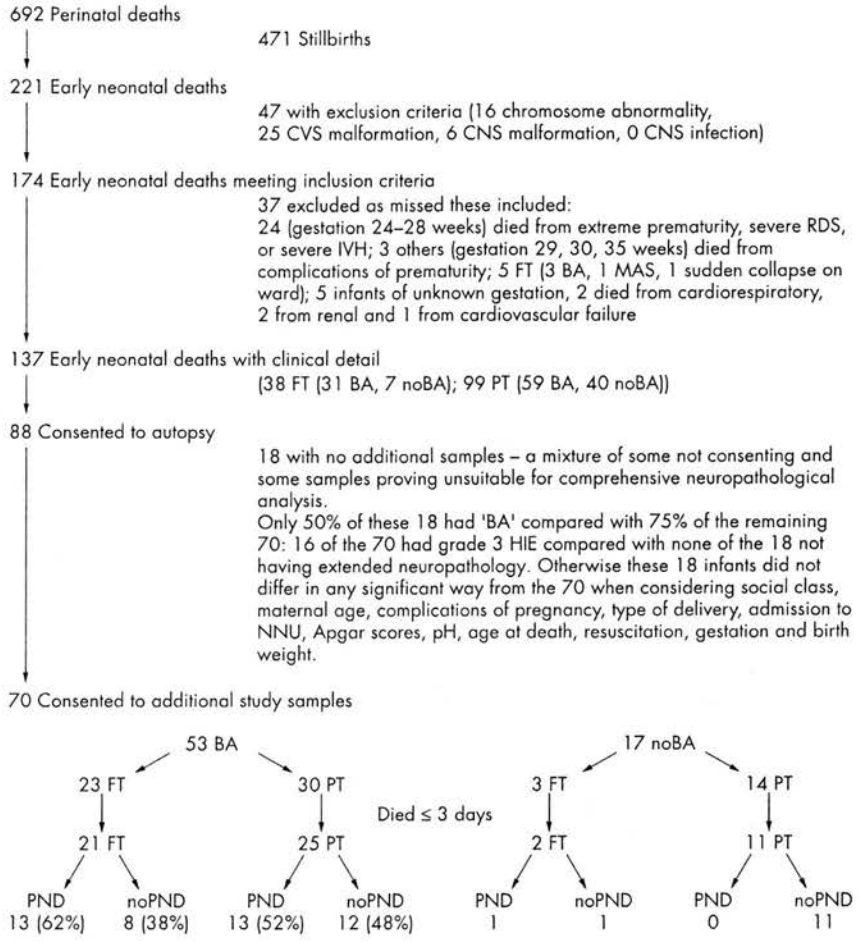

Figure 1 The Scottish perinatal deaths cohort. FT, full term; RDS, respiratory distress syndrome; PT, preterm < 37 weeks; IVH, intraventricular haemorrhage; BA, birth asphyxia; MAS, meconium aspiration syndrome; noBA, no birth asphyxia; HIE, hypoxic-ischaemic encephalopathy; PND, prenatal brain damage; NNU, neonatal unit; noPND, no prenatal brain damage; CVS, cardiovascular system; CNS, central nervous system.

with the criteria of birth asphyxia, in combination and individually.

A judgment of whether the damage dated from before the onset of labour, and was therefore prenatal, was based in part on the presence of patently mature lesions such as established infarcts, previous haemorrhage, or extensive mineralisation. However, these features were present in the minority of brain damaged infants. More diffuse features such as definite macrophage infiltration/accumulation and/or prominent reactive astrocytic hyperplasia in white matter are thought to develop over a period of more than three days (table 1). We estimated that the presence or absence of prenatal brain damage could only be determined reliably in infants who died at ≤ 3 days of age (n = 59).

Placenta

The placenta, cord, and membranes were examined macroscopically, and cord length, placental measurements, and trimmed weight were recorded. Any abnormality was described. Histological samples were taken to include a cross section of the umbilical cord, one strip of membranes (adjacent to the hole through which the baby was delivered, if identifiable), and two blocks of placenta with both fetal and maternal surfaces. Blocks and slides from the placenta and adnexa were submitted for central review in Edinburgh (JK). Histological evidence of infection, specifically chorioamnionitis in the extraplacental membranes or chorionic plate and funisitis, were recorded, as was villitis if generalised.

Statistical analysis

Data were recorded in SPSS. Descriptive statistics were used to examine the prevalence of clinical variables. The χ^2 test with Yates correction (or Fisher's exact test where sample size was less than 20) was used to compare categorical variables, and the unpaired t test or Mann-Whitney U test to compare the difference in continuous variables. Significance was assumed at $p < 0.05$, but we recognise that a large number of tests were performed, and some positive results at this level may have occurred by chance. As the epidemiology was performed on observational data, we leave the reader to consider the implications at this level rather than apply a correction such as that of Bonferroni. The statistical comparison of the pathology of asphyxiated and non-asphyxiated infants was made using χ^2 tests with Yates' correction.

Table 1 Timing of injury to the central nervous system after cerebral insult

Pathological feature	Timing of onset after injury	References
Neuronal eosinophilia	6–24 hours	60–62
Neuronal karyorrhexis	12–48 hours	61, 63–65
Infarcts—necrosis	3–8 hours	60, 65, 66
Infarcts—cavitation	14–42 days	65–68
White matter gliosis	3–11 days	31, 32, 58, 60, 61, 65, 67, 69, 70
Grey matter gliosis	3–5 days	30, 63, 68, 71
Microglial upregulation	3 hours–3 days	60, 61, 66, 67, 71
Macrophage infiltration	3–7 days	63, 65–68
Fresh haemorrhage	Minutes	67
Haemosiderin deposits	2–3 days	27, 67, 72
Mineralisation	3–14 days	58, 60, 65, 67

RESULTS

Population and study cohort

Of the 692 deaths in the two years of the study, 221 were early neonatal deaths corresponding to the estimated early neonatal death rate of 2.5/1000 live births in Scotland.

Of the 137 deaths analysed (fig 1), 90 were classified as BA and 47 as noBA according to our liberal definition. Table 2 shows how they met the criteria for birth asphyxia. Most infants died from the effects of prematurity, congenital anomalies, or "anoxia". The causes of death included one case each of GM1 gangliosidosis, laryngeal atresia, and diaphragmatic hernia, all of which may have contributed to the clinical picture of asphyxia. Twenty out of 137 (15%) pregnancies studied were twin (19) or triplet (one). Complications of pregnancy were common, in particular, oligohydramnios (20%), intrauterine growth restriction (14%), premature rupture of membranes (23%), and second or third trimester antepartum haemorrhage (29%). Although abnormal serum screening for α fetoprotein and human choriogonadotrophin occurred in 14 pregnancies, in only five of these was amniocentesis carried out. The other six amniocenteses were performed for amnioreduction (five) or at maternal request (one). Of 62 cases of fetal anomaly scan, 21 were abnormal (including multiple abnormalities). Two infants were conceived following induction by ovulation stimulating drugs, and two by in vitro fertilisation. Emergency caesarean section took place in 57 (42%) deliveries, of which 21 were performed before the onset of labour and 36 were intrapartum. Two infants were born by elective caesarean section, one because of a previous caesarean section and the other because it was a twin pregnancy. There was no excess over the expected proportion of early neonatal deaths delivered out of hours (2100–0900 and weekends; 61%). An abnormal infection screen was found in 15 of the total group (group B *Streptococcus* (seven), coliforms (four), *Staphylococcus aureus* (two), others (two)), of which eight were thought to have died from overwhelming sepsis (three group B *Streptococcus*; one group A *Streptococcus*; two coliforms; one *Pseudomonas*, and one unidentified).

Seventy neonates were fully enrolled in this study with their parents agreeing to both an autopsy and the extended brain sampling. Of these 70, 53 were thought to be asphyxiated (BA group; 23 mature ($\geq$ 37 weeks) and 30 preterm (24–36 weeks) infants), and 17 did not appear to be asphyxiated (noBA group; three mature ($\geq$ 37 weeks) and 14 preterm (24–36 weeks) infants) (fig 1). The mature infants lived for between 15 minutes and seven days, with only three surviving for more than three days. The preterm infants lived for between five minutes and 6.8 days, with only eight infants surviving for more than three days. The ratio of asphyxiated (77%) to non-asphyxiated (23%) was slightly skewed in the group of autopsied infants towards asphyxiated cases when compared with the whole cohort of liveborn infants included in the detailed epidemiological survey (n = 137; 66% asphyxiated, 34% non-asphyxiated).

Clinical comparison of BA and noBA cohorts

Detailed supplementary tables can be found at http://adc.bmjjournals.com/supplemental/. Briefly, the mothers were comparable for age, weight, height, social class, marital status, parity, and all other factors examined (supplementary table 1). Mothers of infants who were born in an asphyxiated state were less likely to have received steroids during pregnancy (20% *v* 36%, p = 0.036). Hyperemesis (8% *v* 23%, p = 0.013), placenta praevia (2% *v* 11%, p = 0.037), intrauterine growth retardation (10% *v* 23% p = 0.066), and pyrexia or flu-like illness during pregnancy (6% *v* 17%, p = 0.061) were less common in the BA cohort (supplementary table 2). Markers of fetal distress (supplementary table 3), such as meconium staining and cardiotocograph (CTG) abnormalities,[40] were significantly more prevalent in the BA cohort (26% *v* 11%, p = 0.040; 59% *v* 33%, p = 0.004). Intrapartum infection, indicated by positive vaginal swabs, maternal pyrexia, increased white cell count, or increased C reactive protein, occurred in 12 cases (*Escherichia coli* and other coliforms, group B *Streptococcus*, and *Staphylococcus aureus*) but was not more common in the BA group. Malpresentation was less common in the BA cohort (30% *v* 45%, p = 0.087).

The noBA cohort were of younger gestation (29 *v* 32, p = 0.017), lighter, and had a smaller head circumference (supplementary table 4). The BA cohort, who had lower Apgar scores, required more resuscitation as a result. Eighteen (20%) infants in the BA group were asystolic at birth. Infants in the BA cohort were more likely to die early compared with those in the noBA cohort (10.3 h *v* 43 h, p = 0.002). Of 137 infants, only 106 were admitted to a neonatal unit. Of the remaining 31 infants, five were born in good condition and died suddenly and unexpectedly: three were found dead in their cots on the postnatal ward after transfer from the labour ward, and two suffered a sudden acute deterioration in the labour ward after a normal delivery. Twenty four infants had severe birth asphyxia,

Table 2 Clinical features of birth asphyxia in 137 early neonatal deaths

Features of asphyxia	Full term	Preterm
Total number of infants	38	99
Single feature only		
Apgar $\leq$ 5 at 5 min	9	35
Cord pH < 7.1	0	1
1st pH < 7.1	1	8
NNE	1	0
Two features		
Low Apgar and low pH	7	9
Low Apgar and NNE	2	1
Low pH and NNE	0	1
Three features		
Low pH, low Apgar, and NNE	11	4
Total with some indication of asphyxia	31 (82%)	59 (60%)

All infants had a five minute Apgar score. Only 12 full term infants and 11 preterm infants had cord pH measured. An additional 22 full term infants and 35 preterm infants had the pH measured on arrival in the local neonatal unit. 16 full term infants at 12 hours of age were not paralysed, and 14 of these had features of an encephalopathy. 19 preterm infants remained alive and non-paralysed at 12 hours; six had an encephalopathy.
NNE, Neonatal encephalopathy.

and, although they had signs of life at or shortly after birth, they could not be resuscitated sufficiently to move them to the neonatal unit. One extremely premature infant (a triplet) was given only compassionate care.

Clinical details of infants admitted to a neonatal unit were often limited because of early death (supplementary table 5). Within the first hour of birth, infants in the BA group had a considerably lower initial arterial blood pH (6.96 *v* 7.25, $p < 0.001$). In survivors of more than 12 hours, those in the BA group were more likely to have renal dysfunction (55% *v* 24%, $p = 0.029$) and to require assisted ventilation for poor respiratory drive (33% *v* 3%, $p < 0.001$). The noBA cohort, in keeping with their shorter gestation, had a greater incidence of respiratory distress syndrome and were more likely to have received exogenous surfactant (73% *v* 40%, $p = 0.001$) and to have muscular paralysis (36% *v* 14%, $p = 0.011$). In infants who survived for longer than 12 hours, abnormal neurology was documented in 83% of the BA cohort compared with 20% of the noBA cohort ($p < 0.001$). Seizures were significantly more common (19%) in the BA than in the no BA group (3%) ($p < 0.027$), but many infants ($n = 30$) were treated prophylactically with anticonvulsants or paralysis, and many ($n = 61$) died before seizures might have been expected. The groups were comparable for other features of systemic dysfunction, in particular coagulopathies, necrotising enterocolitis, cardiovascular instability, and glucose homoeostasis.

Neuropathological findings and identification of prenatal brain damage

Eighty eight infants underwent autopsy, and 70 parents authorised the additional samples required for this research study (fig 1). Table 3 shows the prevalence of neuropathological abnormalities in these 70 infants, classified into the BA and noBA groups (53 *v* 17) and according to their gestation (mature *v* preterm). Table 4 shows similar data for the infants aged 3 days and less. A detailed table of clinicopathological correlation for each infant in the group of 27 with putative prenatal brain damage has been provided for the interested reader (supplementary table 6). In table 4, the BA group has been further subdivided according to whether encephalopathy was one of the features of birth asphyxia.

In both mature and preterm infants, the asphyxiated infants were more likely to show brain damage than the non-asphyxiated, although brain damage was not universally present in asphyxiated infants (tables 3 and 4). Some infants showed evidence of continuing brain damage, with recent events such as neuronal eosinophilia and fresh haemorrhage superimposed on older lesions including established infarcts, macrophage accumulation including cells laden with haemosiderin, extensive micromineralisation, and white matter gliosis. Infants with no evidence of asphyxia at birth (mostly preterm infants) were more likely than asphyxiated infants to appear virtually normal on neuropathological examination, and such changes as were present, including haemorrhage and neuronal eosinophilia, appeared to be recent except in two mature infants who displayed prominent gliosis.

In cases in which brain damage was present, a conclusion as to whether this was likely to be of prenatal origin could be achieved only in infants who died at $\leqslant$ 3 days of age. This was based on the presence of abnormalities thought to first appear about three days after brain injury. There is no absolute certainty about the time needed for the different responses to become visible (table 1), but the presence of accumulations of macrophages and/or prominent astrocytic hyperplasia in human white or grey matter is generally assumed to require three days or more. Evidence from the literature for this timing is presented in more detail in supplementary table 7. It is important to note that, of the 27 infants judged to have suffered prenatal brain damage, only four had survived for more than two days, six had survived one to two days, and all the rest (65%) had survived for less than one day from the onset of labour. On this basis, 26 (57%) of the asphyxiated group had evidence suggesting prenatal brain damage compared with one (8%) of the non-asphyxiated group, a highly significant difference ($p < 0.005$) (table 4).

Table 4 also shows that infants in the BA group who were encephalopathic displayed a particularly high prevalence of brain damage. Nine of 10 infants in this group showed macrophages or gliosis, or both, together with other confirmatory signs of continuing damage such as neuronal karyorrhexis and eosinophilia. Table 4 also highlights the fact that many of the brains of non-encephalopathic asphyxiated infants were apparently undamaged prenatally and that even by the time of death in the postnatal period, 31% of mature and 13% of preterm asphyxiated infants in this subgroup had apparently normal brains. Although the non-asphyxiated infants appeared to be more prone to postnatal or intrapartum damage, this difference was not significant ($p < 0.059$). Unsurprisingly, the preterm infants were more susceptible to damage of recent, and therefore probably, postnatal origin than were mature infants.

Clinical factors associated with prenatal brain damage

A careful comparison was made of the pregnancies leading to the births of infants with features of pre-labour damage (PND group, $n = 27$) compared with those without such damage (noPND group, $n = 32$). Fewer mothers in the PND group received antibiotics in pregnancy (1 *v* 8, $p = 0.031$), more had caesarean section (17 *v* 10, $p = 0.015$) and emergency caesarean section (17 *v* 9, $p = 0.007$) for CTG abnormalities (18 *v* 8, $p = 0.005$), and more had meconium present in the amniotic fluid (11 *v* 3, $p = 0.005$). The Apgar score was 0 at birth in 33% of the PND group, significantly more than in the noPND group (9 *v* 2, $p = 0.008$), and the former group were heavier and more mature (2526 *v* 1824 g, $p = 0.033$, and 34.6 *v* 31.2 weeks gestation, $p = 0.051$ respectively). The PND group were more likely to be ventilated after birth for a poor respiratory drive (8 *v* 3, $p = 0.037$), and, although both groups were acidotic, had a more acidic first pH (6.90 *v* 7.08, $p = 0.022$). The time to spontaneous respiration was longer (5 *v* 1 minute, $p = 0.009$), and the five minute Apgar score was correspondingly less good (2 *v* 5, $p = 0.021$). Reflecting the larger birth weight and more mature status, they had a higher first blood pressure (46 *v* 36 mm Hg, $p = 0.019$) and were less likely to receive surfactant (4 *v* 13, $p = 0.024$). The time to death, however, was similar in the two groups (12 *v* 7 hours, $p = 0.42$).

No differences in sociodemographic or pregnancy factors were identified between the encephalopathic and non-encephalopathic asphyxiated groups, but CTG abnormalities were present in 80% of the former group and in only 43% of the latter group ($p < 0.04$).

Prenatal damage and the signs of birth asphyxia

Table 5 shows the pathology of prenatal brain damage related to the criteria we used for birth asphyxia. Although the strongest clinical association with the features of pre-labour damage is the development of a neonatal encephalopathy after a low pH and a poor Apgar score at five minutes, it is of note that, of the 22 infants who had only a low Apgar score and then died and had a post mortem examination, 11 showed brain damage. By this evidence, a low Apgar score was the sole clinical indicator of prenatal damage in three of

Table 3 Histological evidence of brain damage in 70 neonates

	BA group (n = 53; asphyxiated infants)		NoBA group (n = 17; non-asphyxiated infants)	
Pathological feature	Mature (n = 23)	Preterm (n = 30)	Mature (n = 3)	Preterm (n = 14)
Neuronal eosinophilia	14 (61)	9 (30)	2	5 (36)
Neuronal karyorrhexis	11 (48)	8 (27)	0	0 (0)
Grey matter infarcts	1 (4)	3 (10)	0	0 (0)
White matter gliosis	11 (48)	14 (47)	2	0 (0)
Grey matter gliosis	7 (30)	5 (17)	1	0 (0)
Microglial upregulation	9 (39)	14 (47)	1	1 (7)
Macrophages	9 (39)	14 (47)	0	0 (0)
Fresh haemorrhage	11 (48)	19 (63)	0	8 (57)
Haemosiderin deposits	0 (0)	1 (3)	0	0 (0)
Mineral deposits	2 (9)	8 (27)	2	1 (7)

Values in parentheses are percentages.
Mature, ≥37 weeks; preterm, 24–36 weeks.

13 mature infants and in eight of 13 preterm infants. Only 16 mature and 19 preterm infants in the PND group survived to 12 hours and remained non-paralysed; of these, 14 mature and six preterm infants had an encephalopathy. Looked at another way, 14 full term infants had clinical neonatal encephalopathy. Eight of these had a post mortem examination, and all had evidence of prenatal damage. Only six preterm infants had neonatal encephalopathy. Two of these had a post mortem, and only one had evidence of prenatal damage.

The placenta

In 41 cases (59% of those who had a post mortem examination), a placenta was available for examination. In seven cases, there was histological evidence of infection, and in 33 cases there was none. All of the seven infected placentas came from infants delivered prematurely. In two (25 and 27 weeks gestation), the inflammation was focal, and in four it was more generalised (at 24, 24, 30, and 35 weeks gestation). The placenta of an additional baby, born at 41 weeks gestation, showed focal acute deciduitis without inflammation of the placenta, membranes, or cord. Only two of these infants had evidence of prenatal brain damage. Thus there was virtually no concordance of placental and brain pathology.

DISCUSSION

Early neonatal deaths

A major aim of this study was to determine the neuropathology in a geographically defined cohort of early neonatal deaths and to seek associations with events in the mother's pregnancy, labour, and delivery, and with the infant's condition at birth and during the period before death. We excluded infants with chromosomal abnormalities and with abnormalities of the cardiovascular and central nervous systems because these might themselves lead to neuropathological changes. We were able to review 137 cases with carefully documented clinical detail, and 70 with extensive neuropathology.

Birth asphyxia criteria

Although intrapartum hypoxia manifesting as birth asphyxia is uncommon and in decline,[46] it is still viewed as a potentially preventable cause of death or damage often with expensive medicolegal implications. Yet there is much evidence that neurodevelopmental delay and cerebral palsy are associated with birth asphyxia in only a minority of cases and also that most birth asphyxiated infants do not manifest developmental delay or cerebral palsy.[47 48] We used broad inclusion criteria for the diagnosis of birth asphyxia to ensure that we missed no cases and were able to evaluate the individual clinical features of asphyxia in relation to neuropathological abnormality. This may have led to the inclusion of infants whose poor condition was due to other factors such as sepsis and/or metabolic disease, including case 10 (supplementary table 6) with gangliosidosis GM1. Two thirds of our cohort of 137 infants were born in a poor condition. We used the clinical finding of depression at birth manifested by Apgar scores or fetal/neonatal acidosis as a marker of an acute intrapartum event leading to birth asphyxia. An assessment for neonatal encephalopathy in

Table 4 Histological evidence of brain damage, including putative prenatal damage, in 59 neonates aged 3 days or less

	Encephalopathic		No encephalopathy		No encephalopathy	
	BA group (n = 10; 17%)		BA group (n = 36; 61%)		BA group (n = 13; 22%)	
Pathological feature	Mature (n = 8)	Preterm (n = 2)	Mature (n = 13)	Preterm (n = 23)	Mature (n = 2)	Preterm (n = 11)
Neuronal eosinophilia	8 (100)	1	5 (38)	4 (17)	0	4 (36)
Neuronal karyorrhexis	8 (100)	1	1 (8)	4 (17)	0	0
Grey matter infarcts	0	0	0	0	0	0
White matter gliosis*	7 (88)	1	4 (31)	9 (39)	1	0
Grey matter gliosis*	5 (63)	1	0	1 (4)	0	0
Microglial upregulation	6 (75)	1	2 (15)	9 (39)	0	1 (9)
Macrophages*	7 (88)	1	1 (8)	8 (35)	0	0
Fresh haemorrhage	4 (50)	0	6 (46)	11 (48)	0	5 (45)
Haemosiderin deposits	0	0	0	0	0	0
Mineral deposits	1 (13)	0	1 (8)	6 (26)	1	1 (9)
*Estimated prenatal brain damage	8 (100)	1	5 (38)	12 (52)	1	0

Values in parentheses are percentages.
Mature, ≥37 weeks; preterm, 24–36 weeks.

Table 5 Features of asphyxia and presence of putative prenatal damage (PND)

	Full term			Preterm		
	Total		PND	Total		PND
Features of asphyxia	Clinical	PM	at PM	Clinical	PM	at PM
Single feature only						
Apgar ⩽ 5 at 5 min	9	7	3	35	15	8
Cord pH < 7.1	0	0	0	1	0	0
1st pH < 7.1	1	1	1	8	5	2
NNE	1	0	0	0	0	0
Two features						
Apgar and low pH	7	5	1	9	3	2
Apgar and NNE	2	0	0	1	0	0
Low pH and NNE	0	0	0	1	0	0
Three features						
Low pH, low Apgar and NNE	11	8	8	4	2	1
Total	31	21	13	59	25	13

One full term infant with prenatal damage had no asphyxia.
PM, Total of 47 infants who died at 3 days or less of age. NNE, Neonatal encephalopathy.

combination with these markers would have been more specific,[21] but many of our infants died within hours of delivery, and a record of neurological examination was not always obtained. In addition, the administration of muscle relaxants to a fifth of our population precluded such an assessment. Finally, 70% of our group were preterm and thus unlikely to exhibit the classical signs of neonatal encephalopathy.

Clinical

The epidemiological background of this cohort is similar to other recent studies from the developed world.[42 49–51] Analysis of the maternal sociodemographic information and the detailed data from the pregnancy did not identify any reliable predictors for birth asphyxia or for neuropathological abnormalities. Significant placenta praevia and hyperemesis were protective against asphyxia in general, possibly because these mothers were more intensively monitored. Even taking neonatal encephalopathy in isolation as a marker for prenatal asphyxia, no differences were identified between encephalopathic and non-encephalopathic asphyxiated infants in the pregnancy or sociodemographic factors monitored in this study. A history of pyrexia or flu-like illness in pregnancy has previously been found to be associated with neonatal encephalopathy.[51] Our series did not show this association, and pyrexia was more common in pregnancies that resulted in non-asphyxiated infants. Intrauterine growth restriction has previously been strongly associated with neonatal encephalopathy[49 50 52] and affected 14% of our population, although not just the asphyxiated infants.

CTG abnormalities are common and are poorly predictive of fetal acidosis.[53] Both CTG abnormalities and meconium staining of liquor were more common in infants with prenatal damage in this study, and CTG abnormalities proved to be the only difference between the encephalopathic BA and non-encephalopathic BA groups (80% *v* 43%, $p < 0.04$). Randomised trials have shown that, although monitoring of fetal heart rate can reduce the numbers of neonatal seizures, there is no change in the incidence of long term neurological damage,[54] suggesting that some fetal heart rate abnormalities may reflect prior compromise. Although meconium staining alone has a high false positive rate,[55] it is associated with increased perinatal mortality and morbidity.[56] It has been hypothesised that intra-amniotic meconium may cause vasoconstriction of the umbilical vessels[57] inducing fetal hypoxia-ischaemia. This is difficult to substantiate after delivery.

Neuropathology

About half (51%) of the 137 eligible infants had detailed neuropathological investigation. The range of neuropathological abnormalities resembles those reported in previous studies,[28–35 58 60 61 63–69 73] although the prevalence of neuronal damage and damage to the grey matter is higher than elsewhere. Judgments about neuropathological abnormalities are more difficult in the preterm than in the term brain. Despite these difficulties, comparison of the asphyxiated infants and those not apparently suffering from birth asphyxia shows clear differences in terms of neuropathological changes. Examination confined to infants who died within three days of the start of labour, and separation of the asphyxiated group into those with and without neonatal encephalopathy, identifies a spectrum of damage. Unsurprisingly, the mature infants who died after displaying neonatal encephalopathy are most likely to show neuropathological changes. All of the brains in this study were carefully examined to determine whether any damage could have occurred before the onset of labour. If it is accepted that features such as focal or diffuse astrocytic hyperplasia and parenchymal macrophage accumulation are cellular reactions that require three days to become established, we may conclude that most infants with features of birth asphyxia had sustained brain damage prenatally, including all eight of the full term encephalopathic group. We are unable to the establish the age of the damage, but the background of apparently normal brain development suggests that the insult was sustained not long before the start of labour. It is harder to draw conclusions about the preterm infants in this study, but the absence of neuropathological changes in virtually all the mature and most of the preterm infants who did not display asphyxia is reassuring.

We do not underestimate the difficulty of interpreting these neuropathological findings and attributing the time of onset. Every abnormality has been included in supplementary table 6, whether focal or diffuse, recent or old, but we accept that interpretation is subjective. Establishing the timing is difficult because experimental studies are not possible in human infants, although classic studies were able to relate pathology findings to major clinical incidents.[60 67] Some experts also comment that the results of animal work may not be directly relevant to the human situation.[60 67] Immunocytochemical investigation is mandatory to separate reactive astrocytosis from myelination gliosis. A number of the classic studies were conducted before cell specific immunocytochemistry became available, although this is not the case for more recent papers. Earlier papers may not

have always included, or added, the duration of labour as a factor in timing, and interpretation may be hampered by longer survival. It is noted that the infants with a history of encephalopathy had survived for more than one day in most instances. We concede that seizure activity may induce and accelerate some of the changes seen in the brains of such infants, but the presence of diffuse astrocytosis in other infants who had survived very few hours and who died with no evidence of seizure activity reinforces the possibility of prenatal origin. A more secure evidence base for timing neuropathological events awaits the evolution of new markers of cell damage and irreversible cell death. The clinical significance of some of the lesions described such as diffuse astrocytosis, and in particular their contribution to the cause of death, remains uncertain.

Correlation of clinical factors and neuropathology

This study has failed to identify any pointers that would predict the birth of a compromised infant. Abnormal CTG, and meconium staining of liquor were the only predictive factors for birth asphyxia or prenatal brain damage. Previous studies have reported an association between oligohydramnios and prenatal brain damage possibly related to impaired blood flow in the umbilical cord. Abnormal CTG was the only clinical factor differentiating the asphyxiated infants who displayed encephalopathy and neuropathological abnormality from those who did not. Recently the presence of prenatal infection has been linked to brain damage. We found no support for this association.

Implications for surviving infants

It is possible that the neuropathological findings reported here represent the most severe end of a spectrum of perinatal brain damage resulting in a fatal outcome, while infants surviving perinatal asphyxia might show lesser degrees of similar pathology. However, the possibility also exists that dead infants and survivors represent two completely different groups in terms of both causation and pathology. Recent neuroimaging studies of surviving neonates with encephalopathy, with or without seizures, have a bearing on these questions. A large study by Cowan *et al*[16] concluded, on the basis of magnetic resonance imaging performed in the first two weeks of life, that brain damage in mature infants with neonatal encephalopathy was most often acute and of perinatal onset particularly in an encephalopathic group without seizures. Very few infants in that study displayed evidence of prenatal brain damage on magnetic resonance imaging. Neuropathological corroboration was achieved in very few cases. In the absence of immunocytochemical investigation of gliosis and brain macrophage accumulation in all deaths, their conclusions about the prevalence of prenatal abnormalities may be an underestimate. We have discussed the difficulty of timing the lesion in our own study, in which conclusions on the presence or absence of prenatal brain damage were confined to infants who died less than three days after the onset of labour and based on neuropathological examination rather than imaging. We suggest that the cerebral insult was probably sustained only shortly before the onset of labour (even possibly precipitating the onset of labour). Evidence of continuing neuronal damage was also present in our series, not dissimilar to the findings of Cowan *et al*, but this was often in addition to the damage identified as occurring before labour within the constraints of current knowledge. It might be expected that brain damage in survivors would be less extensive and severe than in those with a fatal outcome. Whether the brain damage observed in our study represents the result of persisting or repeated insult, or the onset of a potentially reversible cascade accruing from a single insult, is uncertain. A multistep pathological process might present opportunities for intervention to limit further brain damage.

The fact that a significant proportion of clinically asphyxiated infants display no evidence of brain damage, and that infants who are not asphyxiated at birth often display only recent postnatal damage, offers hope for a good clinical outcome if such infants could be identified and "rescued" by medical intervention. This study shows that the current battery of investigations associated with pregnancy and labour remain blunt instruments in accurately predicting the arrival of an asphyxiated and prenatally brain damaged infant. Future work must address the development of methods for detecting antepartum damage so that optimal management of these vulnerable fetuses can be planned. Further evidence is also required on evolution of cellular reactions in the developing brain. The findings in this study support the notion that the birth of a compromised "asphyxiated" encephalopathic infant is not necessarily the result of a mismanaged labour nor the lack of vigilance in pregnancy.

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Original Paper

Biology of the Neonate

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Distribution of Apolipoprotein E Alleles in a Scottish Healthy Newborn Population

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Key Words
Newborn infants · Apolipoprotein E · Alleles · Genotypes

Abstract
The different alleles of the human apolipoprotein E polymorphism, ApoE ε2, ε3, ε4, have important implications for systemic lipid metabolism, immunological function and for the brain in maintenance and in response to injury. Few studies have focussed on their role in early life. The ApoE alleles and genotypes were ascertained in the cord blood of 371 full-term and normal Scottish newborn infants using PCR methodology. The results were compared to previously published data for Scottish adults in late middle age. There was a marginally significant over-representation of ε4 and under-representation of ε3 alleles in healthy infants as compared with adults. Inspection of the individual genotypes confirms the over-representation of ApoE 4/4 and 2/4 with a reduction in ApoE 2/3 and 3/3 when compared with Scottish adults. Although these results may have occurred by chance, the ApoE ε4 allele may confer an increased risk of premature death.

Introduction

Apolipoprotein E (ApoE) is a normal component of the very-low-density lipoprotein (VLDP) fraction in plasma in humans and many animal species, with an important role in lipid uptake and binding to specific lipoprotein receptors and in cholesterol metabolism. ApoE polymorphism was demonstrated by isoelectric focussing and absence of certain isoforms was associated with hyperlipidaemia III [1]. After initial confusion, the genetic control of ApoE subtypes was narrowed down to a single locus on chromosome 19 with three alleles [2], giving rise to six phenotypes.

Much of the early clinical interest in ApoE related to hyperlipidaemia, atherosclerosis and risk of coronary artery disease [3]. The relative frequencies of the common alleles in many populations were calculated from samples from blood donors or healthy volunteers. The frequency of the ε3 allele in Caucasians is remarkably similar irrespective of country of domicile, although there are differences in ε2 and ε4 frequencies between some of the groups studied [3].

Although the age range of the normal or control subjects is recorded in many studies, there are few where results are stratified by age and only a single French study [4] where a comparison was made between children and parents, which showed no significant differences between the two groups. Herrmann et al. [5] examined the plasma

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apolipoprotein levels in German newborns and related these to the ApoE phenotype.

The importance of ApoE in neurodevelopment and neurological disease, particularly Alzheimer's disease, has emerged during the last decade [6–8]. The relationship of different alleles to the response to head trauma [9] and ischaemic brain injury [10, 11] has been described more recently.

Our interest in a potential role for ApoE in the development of prenatal and perinatal brain injury prompted us to examine the relative frequency of ApoE alleles in healthy Scottish newborns and compare the results with data previously published for healthy Scottish adults [12], providing a baseline for further studies.

Material and Methods

This study was approved by the Lothian Research Ethics Committee (LREC 1998/6/53).

Healthy liveborn infants: Blood spots were collected on Guthrie cards from umbilical cord blood samples obtained randomly from placentas in the delivery unit in the Royal Infirmary of Edinburgh. All infants were full-term infants with birth weights greater than 2 kg. No maternal or infant demographics were collected. However, none of the infants gave cause for concern and all left hospital when due to do so. The samples were collected anonymously and stored before analysis in batches. Four hundred and eight blood spots were obtained.

Healthy adults: Data previously published on ApoE distribution in 400 healthy Scottish adults between the ages of 45 and 60 with a mean age of 53 ± 0.22, who were born in North East Scotland, were used for comparison with healthy newborn infants in this study [12]. This adult sample was randomly selected from General Practitioner lists.

Two 2-mm holes were punched from each blood spot into a 0.3-ml, 96-well PCR rack using a clean Harris micro-punch (Lab Sales, Cambridge UK, Catalogue No. WB10-0007). 200 µl of FTA reagent (Lab Sales Catalogue No. WE12-0204) was added and the rack placed in a shaker for 5 min at room temperature to remove impurities. Washing was performed three times leaving bound DNA on the card. The treated card was washed twice with Tris-buffered EDTA. The card was allowed to dry thoroughly, overnight, at room temperature. The ApoE PCR methodology was adapted from Hixson and Vernier [13] and is detailed below.

An initial DNA denaturing at 95°C for 2 min was followed by 30 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 1 min and a final extension of 72°C for 5 min. The PCR reaction mixture contained 34.55 µl double-distilled H_2O, 5 µl 10× PCR buffer (Invitrogen), 1.8 µl 50 m*M* $MgCl_2$, 8 µl 1.25 m*M* dNTPs, 20 pmol of each primer, 1.25 U taq DNA polymerase (Invitrogen). The customised primers were MWG (Biotech) and are shown below:

Downstream primer: ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A

Upstream primer: TCC AAG GAG CTG CAG GCG GCG CA

Table 1. Comparison of observed allele distribution with Hardy-Weinberg expectation

	E3 homozygotes	E3 heterozygotes	No E3	Total
Healthy newborns				
Numbers observed	192	139	31	362
Hardy-Weinberg expectation	188	146	28	362
Adults				
Numbers observed	232	152	16	400
Hardy-Weinberg expectation	237	142	21	400

10 µl of each PCR reaction was run on a 2% agarose gel and visualised with ethidium bromide under UV light. The resultant PCR product was 227 bp in size. This was digested overnight with 1 µl *Hha*I restriction digest enzyme (New England Biolabs) at 37°C. Samples were then run on a 4% Metaphor agarose gel, visualised with ethidium bromide under UV light and photographed.

The genotype for each sample was determined from the migration pattern of bands in the gel. The number of alleles in each group was compared with published data for the Scottish adult population using the χ^2 test for trend.

Results

Unambiguous results were obtained from 371 of 408 samples. The failure of satisfactory analysis of the remaining 37 samples was attributed to the extraction of insufficient DNA from the blood spot or the degradation of DNA in those blood spots stored for more than 12 months.

Table 1 shows the frequency of ApoE genotypes in each population sample compared with the Hardy-Weinberg distribution. For each population, departure from the Hardy-Weinberg equilibrium is trivial and not statistically significant (table 1).

The distribution and values of alleles in normal healthy babies is compared with published data from healthy adults in a Scottish population [12] and comparison of the genotypes within the two populations are shown in table 2. The χ^2 for trend of the alleles shows a marginally significant over-representation of ε4 and less ε3 in healthy infants as compared with adults, and inspection of the individual genotypes (table 2) confirms the over-representation of 4/4 and 2/4 with a reduction in 2/3 and 3/3 when compared with Scottish adults.

Table 2. Contingency table comparing normal Scottish adults [12] and healthy Scottish newborn infants

Cohort	ε2	ε3	ε4	Total
Alleles				
Adults (n = 400)	66 (8%)	616 (77%)	118 (15%)	800
Newborn (n = 371)	63 (8%)	538 (72%)	141 (19%)	742
χ^2 for trend: ε2 p = 0.93, ε3 p = 0.049, ε4 p= 0.024				

	2/2	3/3	4/4	2/3	2/4	3/4
Apo E genotypes						
Adults	2	233	4	51	11	99
Newborn	2	199	15	44	15	96
χ^2 p = 0.109 overall						

Discussion

Interest in ApoE initially concentrated on its importance in lipoprotein and cholesterol metabolism [14]. It has become apparent that ApoE has more diverse roles in development and in reaction to insults, not only directly in the central nervous system but also indirectly through its modulation of immune responses [8]. The three isoforms of ApoE differ by only a single amino acid substitution at one of two sites [3], but these apparently small differences alter their role in metabolism enormously [8].

ApoE is strongly expressed within the brain particularly in astrocytes [15] where ready availability of lipids and cholesterol is essential for maintenance and repair of cell membranes [16]. The relative frequency of different alleles of ApoE has been calculated for many population groups. The ε3 allele is the commonest in all published studies. In most populations, the next commonest is ε4. Amongst Caucasians, whose allele frequency has been studied in many countries, the relative frequency of alleles is remarkably similar, apart from Finnish subjects. All studies in Caucasians have shown an ε3 frequency of <0.79, lower than the frequency amongst American Indians, Chinese and Japanese (>0.82). There are, however, differences in ε4 and ε2 distribution between geographically separated Caucasian groups, most notably a significant reduction in ε2 frequency with an excess of ε4 in a Finnish population [3].

Few investigators have looked at the effect of age on ApoE allele frequency. No difference in the distribution of ApoE alleles was shown between young and old [17], nor between parents and children [4]. Herrmann et al. [5] examined 199 German newborns and found an excess of ApoE ε3 and ε2 with reduced prevalence of ε4, a result which contrasts with the present findings.

Our study of healthy newborns from the South East of Scotland was carried out to provide baseline information for planned studies of ApoE status of defined groups of babies dying in the prenatal and infant periods. We have demonstrated an excess of the ε4 allele and a corresponding decrease in the ε3 allele amongst 371 healthy babies at birth when compared with allele frequencies amongst 400 healthy Scottish adults. Although this population of adults was a historical cohort, the distribution of allele frequencies is similar to other adult Caucasian populations reported in the literature [3]. Davignon et al. [3] reviewed the published data and found that in 5,805 Caucasians the ApoE allele frequency was 0.08 for ε2, 0.77 for ε3 and 0.15 for ε4, a distribution which is precisely the same as that reported for the Scottish adult population used in this study. The decline in ApoE ε4 between birth and late middle age in the Scottish population may reflect an increased mortality of ε4 carriers between these ages. Possession of the ε4 allele in adults is strongly associated with coronary heart disease [3], an important cause of morbidity and premature mortality amongst the Scottish adult population.

Our findings support the suggestions of Zetterberg et al. [18] that the ε4 allele may exert a protective effect on the developing embryo or fetus resulting in an excess of e4 at birth. Wright et al. [19] describe improved neurodevelopmental scores in children with ApoE ε4 allele at the age of 2 years. Other studies suggest that the ε4 allele may be associated with improved cognitive performance and higher educational achievement in early adulthood [20, 21]. This is in contrast to the well-described adverse effects of the e4 allele on memory function and the predisposition to dementia in later adulthood [7, 22]. Natural selection may favour genes conferring short-term benefits at the cost of deterioration in later life. Antagonistic pleiotropic effects of the ApoE ε4 allele may be responsible for protective effects early in life but deleterious effects in late adulthood. It may be relevant that the high cholesterol levels associated with the ε4 allele do not become apparent until children are aged at least 3 years [23, 24] in keeping with delayed appearance of allele-associated deleterious effects.

ApoE has wider relevance than merely its influence on lipid and cholesterol metabolism [8] and these other effects should not be ignored. Mahley and Rall [8] have speculated about a role for ε4 in reducing susceptibility to infection and it is well recognised that infection of the

gestation sac is a common cause of spontaneous delivery in the late second and early third trimester of pregnancy [25–27], although fetal infection is a lesser, but nevertheless important, cause of fetal wastage. The appreciation that ApoE is involved in some intracellular signalling pathways and may have a role in apoptosis [8] offers up a wide range of targets for influencing embryonic development.

Another approach to the investigation of a potential advantage of ε4 to the embryo or fetus is to investigate ApoE allele status at different gestations in the context of the presence or absence of specific pathological disorders, both generalised and organ specific. Such investigations may further expand our understanding of the diverse roles of ApoE and of the specific mechanisms involved.

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Brain damage and axonal injury in a Scottish cohort of neonatal deaths

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Summary

Despite the clinical and medicolegal significance attached to perinatal asphyxia, the neuropathological basis of this condition remains obscure. There are very few studies in the literature which correlate the pathological findings in neonatal brains with detailed epidemiological data, and none which are population based. In a Scotland-wide study of neonatal deaths, 70 brains have been examined. On the basis of glial and macrophage reactions, we previously identified infants with putative antepartum brain damage in this cohort and have related these reactions to signs of birth asphyxia. The present study explores the extent of neuronal/axonal injury in these infants since this is likely to be the basis for neurological deficits in surviving infants. We have also investigated these brains for β-amyloid precursor protein (βAPP) positivity to determine whether this is a useful marker of neuronal injury in neonates. Neuronal eosinophilia and karyorrhexes were detected in 43% and 27% of the cohort, respectively; maximally in the subiculum and ventral pons, but often present elsewhere. White matter damage was detected in 24% of cases but without classic cystic lesions of periventricular leucomalacia. βAPP positivity was present in neuronal soma in 52% of cases and, in axons, in 27% of cases, and was seen from as early as 25-weeks gestation. Axonal bulbs were clearly delineated by βAPP positivity and were usually located in the cerebral white matter and internal capsule, and infrequently in the brain stem. Although white matter damage and βAPP axonal positivity were often detected in the same cases ($P = 0.034$), these features also occurred independently of each other. Both neuronal karyorrhexes and white matter βAPP positivity were significantly correlated with the features of birth asphyxia, particularly a history of seizures. Immunocytochemistry for both βAPP and glial fibrillary acidic protein proved useful in detecting neuropathological features which escaped detection on routine examination, particularly in preterm infants. The presence together of recent and older damage in individual brains suggests that there is an ongoing neuronal response to cerebral insults. We find that βAPP is a useful marker of white matter damage in the neonatal brain. Immunopositivity for βAPP in these circumstances is not attributable to inflicted or accidental trauma. While birth related trauma cannot be ruled out, hypoxia/ischaemia is a likely cause in these infants. However, the exact pathogenesis of neuronal/axonal injury in the neonatal brain remains unclear.

Keywords: neonatal death; neuropathology; amyloid precursor protein; axonal injury; antepartum brain damage

Abbreviations: βAPP = β amyloid precursor protein; GFAP = glial fibrillary acidic protein; H&E = haematoxylin and eosin

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Introduction

The origins of childhood neurological deficits are still poorly understood. Complications of pregnancy and adverse peripartum events are of uncertain predictive value. Perinatal asphyxia in the majority of cases is not followed either by

neonatal encephalopathy or by cerebral palsy (Hall, 1989; Nelson, 1989; Nelson and Leviton, 1991; Blair and Stanley, 1993; Yudkin *et al.*, 1995; Edwards and Nelson, 1998) and there is international consensus (the International Cerebral Palsy Task Force) that encephalopathy and cerebral palsy are infrequently caused by perinatal asphyxia (Bakketeig, 1999; MacLennan, 1999). However preterm delivery is a recognized risk factor for such conditions (Murphy *et al.*, 1995, 1997; Wood *et al.*, 2000).

Major cohort studies of neonatal encephalopathies and related clinical conditions have shown that the associative factors are surprisingly diverse (Badawi *et al.*, 1998*a*, *b*). However, these studies seldom include data on neuropathological findings of the deceased infants. On the other hand, reported perinatal neuropathological studies usually represent the collected experience of specialist referral centres (Gilles and Murphy, 1969; Rorke, 1982; Levene *et al.*, 1985; Pape and Wigglesworth, 1989; Squier and Keeling, 1991; Marin-Padilla, 1996, 1997, 1999), or focus on particular age groups such as preterm infants (Skullerud and Westre, 1986; Leviton and Paneth, 1990; Paneth *et al.*, 1990; Golden *et al.*, 1997; Gilles *et al.*, 1998). Only a small number of studies have investigated the clinical correlates of identified neuropathological features (Ellis *et al.*, 1988; Mito *et al.*, 1993; Gaffney *et al.*, 1994). Although in recent years neuroimaging has proved to be a powerful tool for studying the extent and location of lesions in the perinatal brain (Cowan *et al.*, 2003), neuropathological examination may be more sensitive for defining the nature of lesions. There is a complete dearth of studies designed to match detailed neuropathology with equally detailed demographic data in unselected groups of neonates.

The Scottish Perinatal Neuropathology Study was set up to investigate these issues and, in particular, to establish the prevalence of brain damage in a national cohort of perinatal deaths, including stillbirths and early neonatal deaths from 24 weeks gestation to 1 week of postnatal age, occurring during a 2 year period in the late 1990s. We recently reported that evidence of antepartum brain damage in the neonates in this cohort was more prevalent in those displaying signs of birth asphyxia than in non-asphyxiated infants (57% versus 8%), particularly in those who had suffered seizures (90%) ($P < 0.005$) (Becher *et al.*, 2004). The assessment of brain damage in these neonatal deaths was based mainly on the detection of glial and microglial/macrophage responses. However, the important question of neuronal damage was not explored in our previous study.

In the present study, we further investigated this group of neonates to determine the prevalence of neuronal damage, and particularly of axonal injury, in relation to reactive changes in supporting cells and to the clinical history. One reliable marker of axonal injury, β amyloid precursor protein (βAPP), has rarely been investigated in the human infant brain other than in medicolegal cases (Arai *et al.*, 1995; Baiden-Amissah *et al.*, 1998; Geddes *et al.*, 2001*a*, *b*; Reichard *et al.*, 2003). It is recognized that both traumatic and hypoxic axonal injury is associated with βAPP positivity (Geddes *et al.*, 2001*a*, *b*). In this study, we examined 70 neonatal brains for the presence of βAPP positivity in relation to other evidence of brain damage and to the clinical history.

Material and methods

In the Scottish Perinatal Neuropathology Study, we set out to examine the brain in all neonatal deaths (24 weeks gestation to 1 week postnatal) occurring during a two-year period in Scotland. Infants with chromosomal, cardiac and CNS abnormalities and with CNS infections were excluded. Post-mortem examination was achieved in 51% of the eligible cases (n = 137 in total, 70 brains examined). These 70 infants were divided into those who had died within 3 days of the onset of labour (n = 59) and those who had died between 3 and 7 days (n = 11). The three-day cut-off point was selected because changes such as macrophage accumulation and astrocytic hyperplasia are thought to require 3 days to become evident, from which we inferred an antepartum origin for these reactions.

Cases were co-ordinated from all 22 Scottish obstetric units through obstetric, paediatric, paediatric pathology and neuropathology colleagues. Consent for research use was obtained from the parents and the study was approved in each centre by the local research ethics committee.

Clinico-epidemiological data was gathered for each case. Up to 20 paraffin blocks were prepared from each brain according to a standard protocol and included representative samples from the cerebrum and hippocampus, basal ganglia and thalami, midbrain, pons, medulla, vermis and cerebellar hemispheres. Sections were examined after staining with haematoxylin and eosin (H&E), and with antibodies to glial fibrillary acidic protein (GFAP) (Dakocytomation Ltd; Ely, UK; diluted 1:1000, with trypsin pretreatment at 37°C for 20 min), the macrophage marker CD68 (Dako; diluted 1:200, with microwave antigen retrieval) and βAPP (Chemicon Europe Ltd; Chandlers Ford, UK; monoclonal clone 22C11, diluted 1:100, with microwave antigen retrieval).

Sections were assessed independently by two observers (J.E.B. and B.W.) without reference initially to the clinical history or to the results of the other staining procedures. Neuronal damage was assessed in routinely stained preparations by evidence of neuronal eosinophilia and/or karyorrhexis as well as the presence of axonal swellings in the white matter. The presence or otherwise of astrocytic hyperplasia, macrophage accumulation and microglial upregulation as well as βAPP positivity in neuronal soma and axons were scored as positive or negative for each case. Concordance between the two observers was achieved in >90% of cases and discrepant cases were resolved at a multiheaded microscope and by further review (J.K.). The localization and patterns of βAPP immunopositivity was correlated with gestation, clinical history and evidence of glial and macrophage reactions, as well as with evidence of neuronal and white matter damage assessed on routine (H&E) staining throughout the brain.

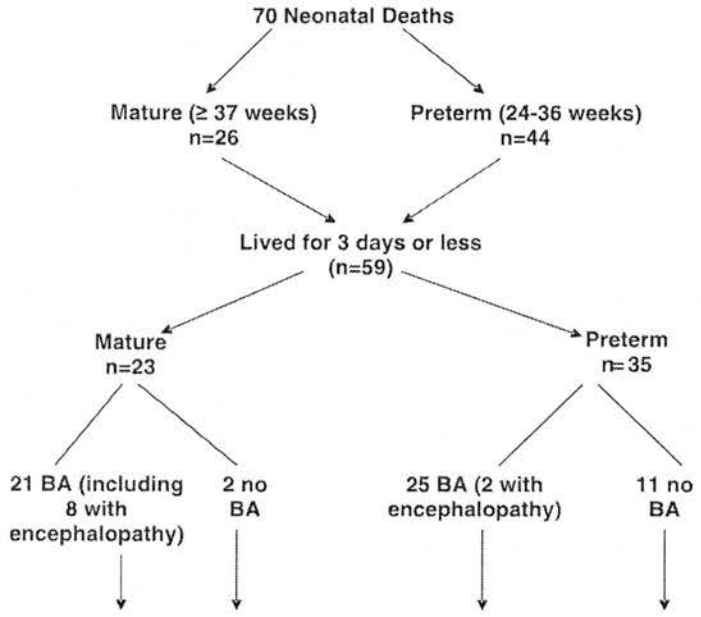
Statistical analysis was performed using the χ^2 test (Fisher's exact test where sample size was <20).

Results

Clinical details

Of the 70 neonates enrolled in this study, 26 were mature infants (⩾37 weeks of gestation) and 44 were preterm (24–36 weeks) (Fig. 1). Thirty-eight infants were delivered by Caesarean section, performed as an elective procedure in one case and as an emergency in 37 (before the onset of labour in half of these and usually precipitated by cardiotocograph

CORRELATION OF NEUROPATHOLOGICAL FINDINGS AND BIRTH ASPHYXIA IN NEONATES DYING WITHIN 3 DAYS OF THE ONSET OF LABOUR*



Gliosis	12 (57%)	1	11 (44%)	0 (0%)
Microglia/ macrophages	11 (52%)	0	10 (40%)	0 (0%)
Eosinophilia	13 (62%)	0	5 (20%)	4 (36%)
Karyorrhexis	9 (43%)	0	5 (20 %)	0 (0%)

*3 DAY CUT OFF TO ALLOW ASSESSMENT OF PUTATIVE ANTEPARTUM BRAIN DAMAGE
BA – BIRTH ASPHYXIA

Fig. 1 Cohort of neonatal deaths.

abnormalities). The mature infants lived for between 15 min and 7 days with only three surviving for >3 days. The preterm infants lived for between 5 min and 5.5 days, with only eight infants surviving for >3 days. Neonatal encephalopathy was observed in 10 mature and five preterm infants. Most infants died of the effects of prematurity, or of congenital anomalies other than those of the CNS (which were excluded from the study), or of 'anoxia'. Fifty-three of the 70 neonates displayed one or more clinical signs of perinatal asphyxia, including 15 with seizures (10 of these infants died aged ≤3 days), while 17 did not appear to be asphyxiated. Perinatal asphyxia was defined in this study by the presence of one or more of the following: (i) an Apgar score of ≤5.0 at 5 min; (ii) a cord or initial blood pH of <7.1; and/or (iii) grade 2/3 encephalopathy. Signs of birth asphyxia were associated with a higher prevalence of antepartum brain damage (57% versus 8%, $P < 0.005$), but were not predictive in all cases. Oligohydramnios and meconium staining of the amniotic fluid were also associated with antepartum brain damage, but there was no concordance with placental pathology. A detailed analysis of these and other clinicoepidemiological factors, which may have contributed to neonatal death and to antepartum brain damage in this cohort, has been published previously (Becher *et al.*, 2004).

Routine neuropathological findings

Assessment of neuronal damage was first undertaken in sections stained with H&E. Neuronal eosinophilia (Fig. 2A), marking recent onset hypoxic change, was observed in some or all differentiated nerve cell bodies in the cerebral cortex, basal ganglia, brain stem, particularly in the ventral pons and inferior olive, and in the cerebellar dentate nuclei and Purkinje cell layer. Karyorrhectic neuronal nuclei (Fig. 2B) were observed in a similar distribution, but additionally in the dentate gyrus of the hippocampus and in the cerebellar granular layer in occasional cases. If neuronal karyorrhexes were only present in very small numbers, they were considered indistinguishable from the normal neuronal drop out known to

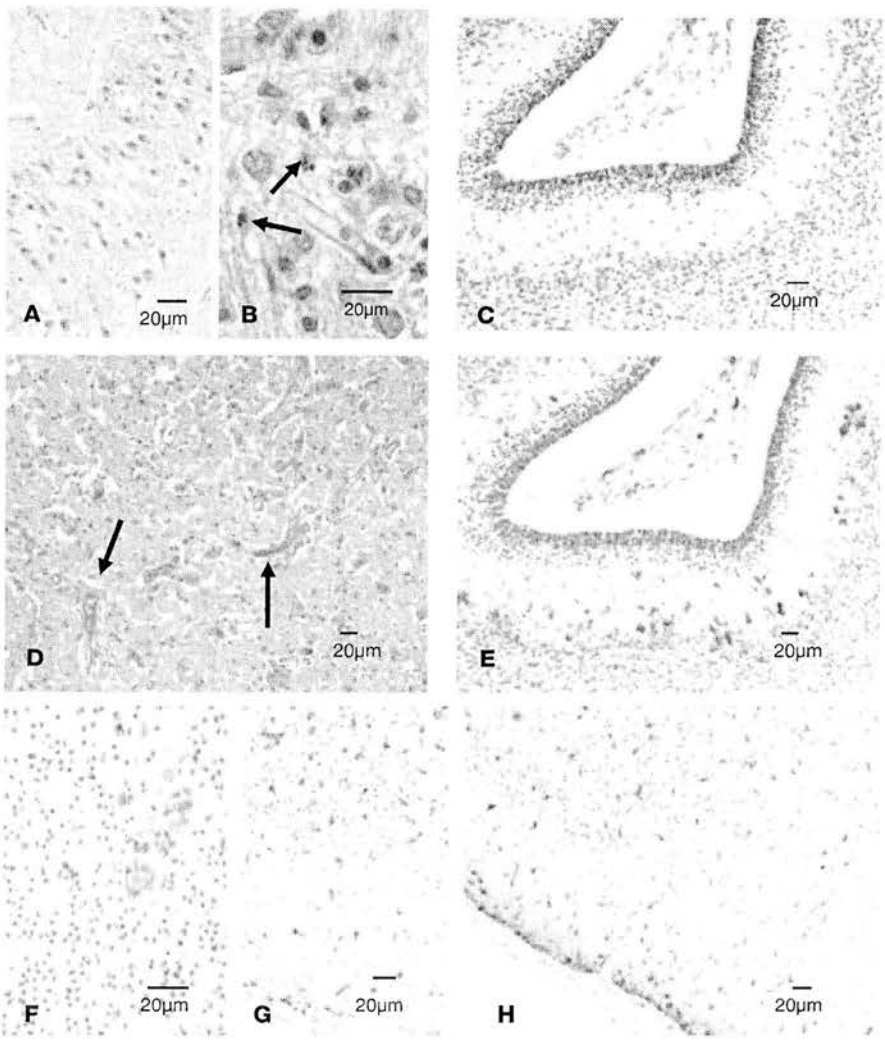


Fig. 2 Evidence of brain damage in grey matter. (**A**) Eosinophilic hippocampal neurons from a 41-week gestation infant who survived for 1 day. H&E staining. (**B**) Pontine neurons in a 39-week gestation infant who survived <1 day. Karyorrhectic neurons are shown (arrows) between more nearly normal neurons. H&E staining. (**C**) Cerebellar cortex from a 38-week gestation infant who survived just under 2 days. Very few Purkinje cells survive at the interface between molecular and internal granular layers. H&E staining. (**D**) Cerebral cortex from a 42-week gestation infant who survived 7 days and who displayed widespread cortical necrosis. Surviving cortex showed neovascularization (arrows) and the tissue is beginning to disintegrate. H&E staining. (**E**) The cerebellar cortex shown in (**C**) stained for microglia/macrophages using an antibody to CD68. Macrophages are concentrated along the former Purkinje cell layer. (**F**) Cerebral cortex from a 26-week gestation infant who survived for 40 min. A focal infarct, marked by CD68 positive macrophages, is shown in the cortex. (**G**) More generalized cortical CD68-positive macrophage infiltration is present in the cortex of the infant shown in (**D**). (**H**) A GFAP-positive cortical glial response is also present in the case illustrated in (**D**) and (**G**).

occur during development and were disregarded. Even when present in large numbers, the changes affected only a subpopulation of neurons in the neocortex, but were more numerous and concentrated in the subiculum of the temporal lobe (spreading to involve the entorhinal cortex and the neighbouring sectors of the cornu ammonis) and in the ventral pons. The most severely affected cases showed loss of most Purkinje cells (Fig. 2C). Neuronal eosinophilia was identified in 43% of the cohort and karyorrhexes in 27%. Occasional infants showed cortical infarction with prominent neovascularization and perivascular fibrosis (Fig. 2D). Figures 2E–G show focal and diffuse macrophage responses in the context of neuronal loss, not all of which had been suspected on routine staining (Fig. 2F). Cortical damage also results in a conspicuous glial response (Fig. 2H). Case-by-case glial and macrophage reactions are shown in Table 1.

Evidence of white matter damage in routinely stained sections was present in 24% of cases, ranging from eosinophilic homogenization of the neuropil to areas of frank infarction, in relation to which axonal retraction bulbs (Fig. 3A) were

identified in three cases and with more widespread surrounding white matter damage. Moderate numbers of karyorrhectic glial nuclei were identified within damaged white matter, accompanied by macrophage infiltration and astrocytic hyperplasia (Fig. 3B). Even in apparently normal white matter (Fig. 3C), unexpected astrocytic hyperplasia was sometimes revealed by GFAP immunostaining (Fig. 3D). In contrast, Fig. 3E shows normal white matter astrocytic

Table 1 *Pattern of βAPP positivity in 70 neonatal deaths, correlated with glial and macrophage reactions*

(A) Infants dying within 3 days (n = 59)

Cases with antepartum damage (n = 27)	Gestation (asphyxia at birth)	βAPP neurons	βAPP axons	βAPP mineralizing foci	Metabolic astrocytosis	Reactive astrocytosis	Macrophage reactions
1 (E)	41 (+)	+	+	−	−	+	+
2 (E)	41 (+)	+	+	−	+	+	+
3 (E)	40 (+)	+	−	−	−	+	+
4 (E)	40 (+)	+	−	+	−	+	+
5 (E)	39 (+)	+	+ +	−	−	+	+
6 (E)	38 (+)	+	−	−	−	+	−
7 (E)	38 (+)	+	+	−	+	+	+
8 (E)	37 (+)	+	−	−	+	+	+
9 (E)	36 (+)	+	−	−	+	+	+
10	40 (+)	+	+	−	−	+	+
11	36 (+)	+	−	+ +	−	+	+
12	35 (+)	+	−	−	−	+	+
13	36 (+)	±	+ +	+	−	+	+
14	32 (+)	+	±	+	−	+	−
15	28 (+)	−	−	+	+	+	+
16	28 (+)	+	−	−	−	+	+
17	27 (+)	+	−	−	±	+	+
18	25 (+)	−	+	+	−	±	+
19	26 (+)	−	−	−	−	−	+
20	24 (+)	−	−	−	−	+	−
21	40 (+)	−	−	−	−	+	±
22	40 (+)	−	−	−	−	+	+
23	26 (+)	−	−	−	−	−	+
24	25 (+)	+	−	−	−	−	+
25	42 (−)	+	−	+	−	+	−
26	40 (+)	−	+	−	−	+	−
27	40 (+)	+	−	−	+	+	−
Cases with postnatal damage (n = 21)	**Gestation (asphyxia at birth)**	**βAPP neurons**	**βAPP axons**	**βAPP mineralizing foci**	**Metabolic astrocytosis**	**Reactive astrocytosis**	**Macrophage reactions**
28	24 (+)	−	−	−	−	−	−
29	36 (−)	+	−	−	−	−	−
30	28 (−)	±	−	−	−	−	−
31	24 (+)	±	−	−	−	−	−
32	39 (+)	+	−	−	−	−	−
33	31 (−)	+	−	+	−	−	−
34	24 (+)	−	−	−	−	−	−
35	24 (+)	−	−	−	−	−	−
36	36 (+)	−	−	−	−	−	−
37	40 (+)	+	+	−	−	−	−
38	25 (−)	−	−	−	−	−	−
39	42 (+)	±	−	−	−	−	−
40	38 (−)	−	−	−	−	−	−
41	27 (−)	−	−	−	−	−	−
42	29 (+)	+	−	−	−	−	−
43 (E)	25 (+)	−	−	+	−	−	−
44	41 (+)	−	−	−	−	−	−
45	30 (−)	NA	NA	NA	−	−	−
46	25 (+)	−	+	−	+	−	−
47	24 (−)	−	−	−	−	−	−
48	38 (+)	−	−	+	−	−	−

Table 1 *Continued*

Cases with no damage (n = 11)	Gestation (asphyxia at birth)	βAPP neurons	βAPP axons	βAPP mineralizing foci	Metabolic astrocytosis	Reactive astrocytosis	Macrophage reactions
49	40 (+)	−	+	−	−	−	−
50	32 (−)	+	−	−	−	−	−
51	35 (−)	−	−	−	−	−	−
52	25 (+)	−	−	−	−	−	−
53	25 (+)	−	−	−	−	−	−
54	27 (+)	−	−	+	−	−	−
55	42 (+)	−	−	−	−	−	−
56	30 (+)	−	−	−	−	−	−
57	26 (−)	−	−	−	−	−	−
58	38 (+)	±	−	−	−	−	−
59	25 (−)	+	−	−	−	−	−

(B) Infants dying at 4–7 days (n = 11)

Damaged and undamaged cases (n = 11)	Gestation (asphyxia at birth)	βAPP neurons	βAPP axons	βAPP mineralizing foci	Metabolic astrocytosis	Reactive astrocytosis	Macrophage reactions
60	41 (−)	+	+	+	+	+	−
61	27 (+)	+	−	−	−	+	+
62	25 (−)	−	+	−	−	−	−
63 (E)	27 (+)	−	+	+	−	+	+
64 (E)	42 (+)	+	+	−	−	++	++
65	27 (−)	−	−	−	−	−	−
66 (E)	36 (+)	+	−	++	−	+	+
67 (E)	29 (+)	+	−	+	−	+	+
68 (E)	39 (+)	+	+	−	−	+	+
69	34 (−)	−	+	−	−	−	−
70	26 (+)	+	++	−	−	+	+

E = infants displaying encephalopathy; NA = blocks returned to source hospital before βAPP staining was performed.
−, negative; ±, occasional positive cells or features across the section; +, moderate number of positive cells or features across this section; ++, very numerous positive cells or features across this section.

immunoreactivity in this age group. No foci of cystic periventricular leucomalacia were seen.

Small foci of perivascular mineralization were present in the central white matter in 19% of the total cohort, more commonly in the preterm infants. One case displayed extensive mineralization in the basal ganglia and internal capsule.

In nine cases, glial nuclei in both white and grey matter were enlarged, pale and prominent (Fig. 3F), resembling the Alzheimer type II astrocytes observed in hepatic encephalopathy. Although the cytoplasm of such cells was not generally prominent, they were GFAP positive (Fig. 3G) and, in most of these cases, a reactive astrocytosis was confirmed in the white matter. Some mature neonates showed prominent changes of so-called myelination gliosis (glial cells with small darkly stained nuclei and prominent asymmetric cytoplasm, often arranged in rows) (Fig. 3H), but such cells did not prove to be GFAP reactive in contrast to juxtaposed normal astrocytes (Fig. 3I). Interpretation of such cases sometimes proved difficult on routine staining, especially in the presence of incipient white matter damage, and GFAP staining proved essential for the detection of true astrocytic hyperplasia.

Immunoreactivity for βAPP

βAPP immunopositivity was more prevalent in grey matter (neuronal perikarya, 53%) than in white matter (axons, 27%) (Table 1) Three patterns of βAPP positivity were observed in the cases in this study.

βAPP was detected in neuronal cell bodies in 52% of the study cases; this occurred in both mature and preterm infants (Table 1), varying from global expression in all differentiated neurons to particular subsets of neurons in the cortex (Fig. 4A), basal ganglia, cerebellum or brain stem. The inferior olivary nuclei and neurons in the floor of the fourth ventricle, the dentate nuclei and the Purkinje cells were particularly likely to display βAPP positive neurons. The neurons of the immature cortex in premature babies and the germinal matrix cells and migrating foci were generally negative. Staining of the perikaryon occurred both in morphologically normal cells and in those which proved to be eosinophilic or frankly karyorrhectic on routine staining, although not all karyorrhectic neurons were positive (Fig. 4B). Within the hippocampus, the CA1 sector neurons were occasionally unstained when the rest of the cornu ammonis and the neurons of the subiculum were positive.

Axonal βAPP positivity was detected in 27% of study cases (Table 1) and was seen predominantly in the periventricular

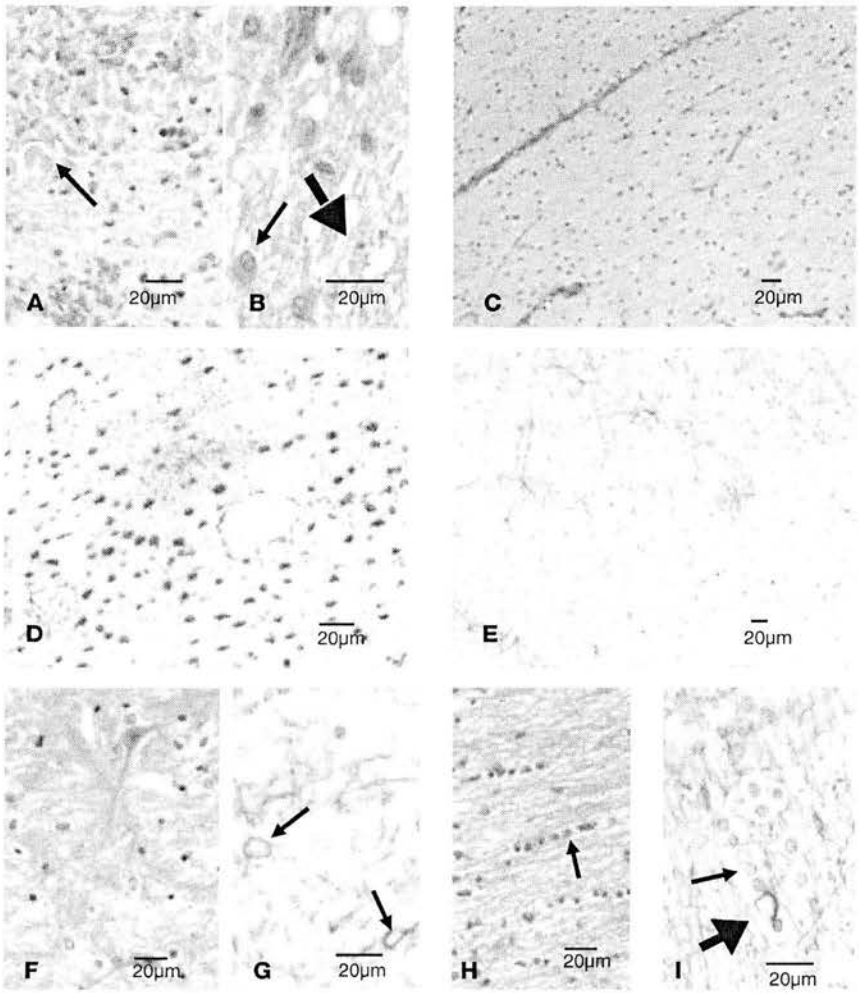


Fig. 3 Evidence of white matter damage and glial/macrophage reactions. (**A**) A focal area of white matter damage, marked by eosinophilic axonal bulbs (arrowed) is seen in a 28-week gestation infant who survived for nearly 3 days. The area of damage was in the proximity of a germinal matrix haemorrhage. H&E staining. (**B**) White matter damage characterized by astrocytic hyperplasia (fine arrow) and glial karyorrhexes (broad arrow) present in a 42-week gestation infant who survived 7 days. H&E staining. (**C**) Survey of apparently unremarkable white matter in a 42-week gestation infant who survived 4 h. H&E staining. (**D**) Immunostaining with an antibody to GFAP reveals reactive astrocytosis in the infant shown in (**C**). (**E**) Immunostaining for GFAP in an infant age- and stage-matched with the infant shown in (**C**) and (**D**) who did not show white matter astrocytic hyperplasia. (**F**) Glial nuclei in the basal ganglia of a 41-week gestation infant who survived for nearly 2 days. Eosinophilic change is seen in neurons within this field and astrocytic nuclei are pale and somewhat irregular. H&E staining (**G**) Immunostaining of the basal ganglia shown in (**F**) reveals that the pale glial nuclei are associated with GFAP positivity (arrows) without displaying cytoplasmic hyperplasia. (**H**) White matter from an infant of 41-weeks gestation who survived for just over 1 day displaying 'myelination' gliosis. Lines of glial nuclei, some with eosinophilic cytoplasm (arrowed) are shown. H&E staining. (**I**) Same case as (**H**) stained for GFAP. While the majority of cells in the glial columns are GFAP negative (fine arrow), there is an occasional interposed GFAP-positive cell (broad arrow).

central white matter of the cerebral hemisphere and the white matter of the cerebellum. Well-demarcated positive axons were particularly common in the internal capsule (Fig. 4C). Staining patterns varied from smudgy irregular patches of positivity in otherwise apparently normal white matter, to clearly demarcated single axons or bundles of axons, often with evidence of axonal beading. Axonal bulbs and varicosities were most numerous in association with areas of infarction or haemorrhage and were strongly positive for βAPP. The brain stem showed only occasional axonal positivity, particularly in the cerebellar peduncles, in contrast with frequent positivity observed in neuronal soma in the stem.

Perivascular and white matter mineralizing foci were strongly positive for βAPP in all the cases in which this feature had been noted in routinely stained sections (15 out of the 70 study cases; 21%) (Fig. 4D), including the case with significant mineralization in the internal capsule (Fig. 4E). These foci were particularly common in the basal ganglia

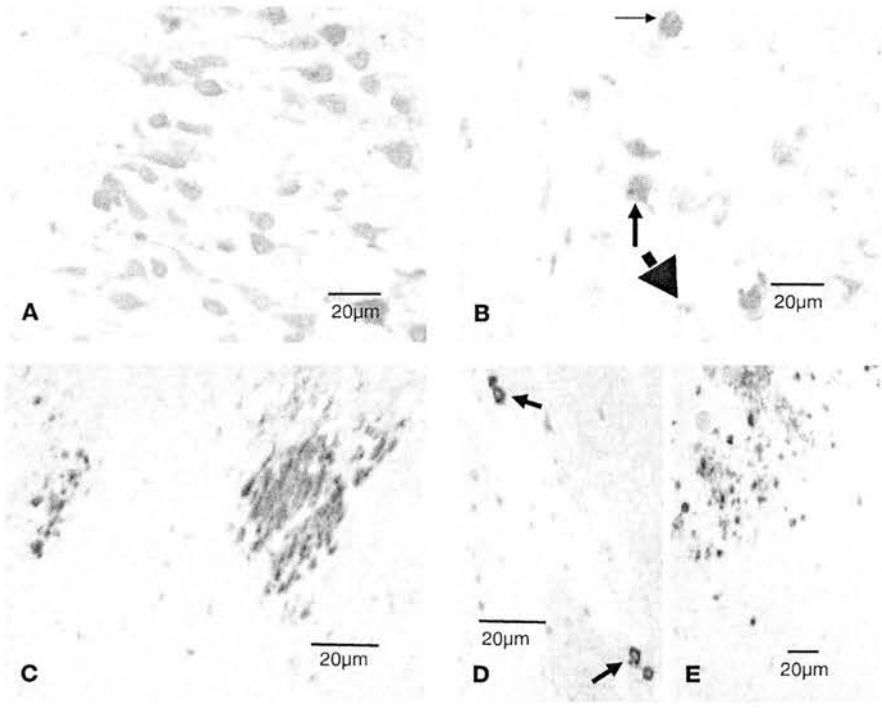


Fig. 4 βAPP immunoreactivity in the neonatal brain. (**A**) Hippocampal neurons from the infant shown in Fig. 2A displaying positivity for βAPP, co-localizing with eosinophilia. (**B**) Pontine neurons from a 39-week gestation infant who survived 14 h and who displayed prominent neuronal karyorrhexes in the ventral pons. Many karyorrhectic neurons are βAPP positive (fine arrow), but an occasional affected neuron is negative (broad arrow). (**C**) Internal capsule from the infant whose pons is illustrated in (**B**). Bundles of βAPP positive axons are seen and some of these display axonal bulbs. (**D**) Perivascular deposits of mineral in the basal ganglia are positive for βAPP in a 41-week gestation infant who survived 4 days. (**E**) Large focus of mineralization in the internal capsule of a 36-week gestation infant who survived 7 h. The mineralized area is strongly positive for βAPP.

and associated white matter. Mineralized foci did not stain positively with antibodies to GFAP and CD68.

Each of these patterns of βAPP positivity was sometimes found in isolation but much more frequently in association with glial and macrophage responses (Table 1). βAPP positivity was found occasionally in neurons in cases in which no other evidence of damage had been detected. Conversely, in five cases displaying microglial/macrophage and/or glial responses suggestive of antepartum damage, no βAPP positivity was detected. Although faint βAPP positivity was noted in reactive astrocytes, glia and blood vessels were generally negative for this marker.

Table 2 summarizes the prevalence of βAPP positivity in different groups of infants in the present study. One case was not available for βAPP staining, but of those available, 37 (52%) showed neuronal and 19 (28%) showed axonal βAPP positivity. The prevalence of βAPP in the 59 infants dying at ≤3 days was significantly related to the timing of damage, being higher in both neuronal bodies ($P = 0.08$) and axons ($P = 0.03$) in infants with antepartum damage. Even some infants with no apparent damage on routine staining showed occasional βAPP positivity (27% in neurons and 9% in axons). In the 11 infants dying between 4 and 7 days of age, 36% (and all of the three infants born at term) showed both neuronal and axonal βAPP positivity. In all groups, preterm infants showed a lower prevalence of βAPP positivity in neurons and axons. Table 2 also shows the declining prevalence of βAPP positivity in neonates with postnatal and no observable damage compared with those displaying antepartum damage. In contrast, the infants aged ≥3 days displayed the highest prevalence of axonal βAPP positivity. White matter damage (24%) and βAPP positivity (27%) were usually present together ($P = 0.034$).

Correlation of neuropathological findings with clinical features

Neuronal eosinophilia was no more common in asphyxiated infants (43%, $n = 53$) than in non-asphyxiated infants (41%, $n = 17$) ($P = 0.87$). However, this feature was detected more frequently in mature (62%, $n = 26$) than preterm (32%, $n = 44$) infants ($P = 0.015$). In contrast with neuronal eosinophilia, neuronal karyorrhexes were significantly increased in asphyxiated infants (36%) compared with non-asphyxiated infants (0%) ($P < 0.01$) and were also more prevalent in mature (27%) than in preterm infants (7%), although not significantly so ($P = 0.08$). Encephalopathic infants showed a particularly high prevalence of neuronal eosinophilia and karyorrhexes

Table 2 *Neuronal βAPP positivity in the cohort of 70 neonatal deaths*

(A) Infants dying within 3 days (n = 59)			
		Localization of βAPP immunopositivity	
		Neurons	Axons
Cases with antepartum damage (n = 27) (70% neuronal, 33% axonal βAPP positivity in mature and preterm taken together)	Mature (n = 14)	11 (79%) (100% in eight encephalopathic infants)	6 (43%) (50% in eight encephalopathic infants)
	Preterm (n = 13)	8 (62%) (positive in one encephalopathic infant)	3 (23%) (negative in one encephalopathic infant)
Cases with postnatal damage (n = 21) (40% neuronal, 10% axonal βAPP positivity in mature and preterm taken together)	Mature (n = 6)	3 (50%)	1 (17%)
	Preterm (n = 14) (one case not available)	5 (36%)	1 (7%)
Cases with no observable damage (n = 11) (27% neuronal, 9% axonal βAPP positivity in mature and preterm taken together)	Mature (n = 3)	1 (33%)	1 (33%)
	Preterm (n = 8)	2 (25%) (negative in one encephalopathic infant)	0 (0%) (negative in one encephalopathic infant)
(B) Infants dying at 4–7 days (n = 11)			
		Localization of βAPP immunopositivity	
		Neurons	Axons
Cases with recent and older damage (n = 11) (64% neuronal, 64% axonal βAPP positivity in mature and preterm taken together)	Mature (n = 3)	3 (100%)	3 (100%)
	Preterm (n = 8)	4 (50%)	4(50%)

(90%). White matter damage was significantly more common in encephalopathic (90%) than in non-encephalopathic asphyxiated (29%) and non-asphyxiated (12%) neonates (P = 0.001). Infants who survived for ⩾3 days had a higher prevalence of white matter damage than those who survived birth by only a few hours (P = 0.03). Encephalopathy was significantly associated with reactive astrocytosis (P = 0.001), with macrophage reactions (P = 0.002) and with metabolic astrocytosis (P = 0.024), but not with deposits of mineral. The minor differences in micromineralization in white matter parenchyma between asphyxiated and non-asphyxiated infants did not amount to significance.

White matter βAPP immunopositivity was confined to the asphyxiated group (12 out of 46 cases) among infants who died at ⩽3 days, but was not most prevalent in the encephalopathic infants. However, somatic neuronal βAPP positivity was significantly more common in encephalopathic infants than in those infants who did not have seizures (P = 0.012) (Table 2). A minority of cases showed no evidence of βAPP positivity despite the focal presence of reactive astrocytosis and/or macrophage infiltration of the white matter, suggesting that damaged neurons may already have been removed in these cases. Conversely in infants with postnatal brain damage, mostly in the form of fresh haemorrhage, neuronal βAPP positivity was present in six cases in the absence of astrocytic or macrophage reactions. Overall, there was a significant correlation between white matter damage observed in routinely stained sections and axonal βAPP positivity (P = 0.034). The prevalence of axonal βAPP positivity following forceps or Caesarean delivery (n = 38) was 37% compared with only 16% in 32 infants delivered normally (P = 0.01).

Discussion

This paper documents the prevalence of neuronal damage and βAPP positivity in a population based cohort of 44 preterm and 26 mature neonatal deaths ascertained in a two-year study. Based on the presence of glial and/or macrophage reactions, we have concluded that brain damage occurred before the onset of labour in 46% of the 59 infants who died at or before 3 days of age (Becher *et al.*, 2004). This assessment of the timing of neuropathological damage was grounded in previous observations in human infants (Norman, 1978; Low *et al.*, 1989; Squier and Keeling, 1991; Del Bigio and Becker, 1994; Wigglesworth and Bridger, 1994; Norenberg, 1994; Squier, 2001; Kinney *et al.*, 2002). However, our previous study failed to identify any pointers which would reliably predict the birth of an infant with antepartum brain damage. We did find an association between brain damage and oligohydramnios, meconium staining of the liquor and an abnormal cardiotocograph, but we found no evidence for an association with prenatal infection or placental inflammation.

This study focuses on neuronal damage. Certain staining and morphological changes (eosinophilia and karyorrhexis) signify irretrievable neuronal death. Most animal studies of timed cerebral insult indicate that neuronal karyorrhexis is visible within 24 h (Ferrer *et al.*, 1994; Ferrer, 1996; Tan *et al.*, 1998) and estimates of the time lapse between insult and karyorrhexis in the human brain are similar (Friede, 1972; Low *et al.*, 1989; Wigglesworth and Bridger, 1994; Squier, 2001). The development of neuronal eosinophilia is thought to require at least 6 h in the rat model (Graeber *et al.*, 2002) and possibly longer in the human infant (Norman, 1978; Low *et al.*, 1989). From these estimates, it is clearly impossible to infer a definite antepartum origin to the neuronal changes in this cohort, in contrast with the glial and macrophage changes reported previously. With respect to eosinophilia, it is as likely that this was a postnatal event as prenatal in infants surviving for more than a few hours. However, more long-standing neuronal damage in the form of karyorrhexis may have commenced before labour in infants who died during the first day of life.

Apart from two cases of minor cortical dysplasia and one of GM1 gangliosidosis, the damage observed in this study was superimposed on otherwise normally developed brains and is similar to that reported in previous studies (Gilles and Murphy, 1969; Friede, 1972; Norman, 1978; Rorke, 1982; Roessmann and Gambetti, 1986; Skullerud and Westre, 1986; Ellis *et al.*, 1988; Low *et al.*, 1989; Leviton and Paneth, 1990; Paneth *et al.*, 1990; Squier and Keeling, 1991; Mito *et al.*, 1993; Del Bigio and Becker, 1994; Gaffney *et al.*, 1994; Norenberg, 1994; Wigglesworth and Bridger, 1994; Marin-Padilla, 1996, 1997, 1999; Golden *et al.*, 1997; Gilles *et al.*, 1998; Squier, 2001; Graeber *et al.*, 2002; Kinney *et al.*, 2002), although the prevalence of grey matter and neuronal damage is higher in the present cases. Both necrosis and apoptosis have been implicated in perinatal neuronal loss (Edwards *et al.*, 1997). Unequivocal signs of cell death are more easily detected in the differentiated neurons of the mature infant brain and were particularly prevalent in asphyxiated babies who suffered seizures. In very few of the present cases were all neurons affected, even within a single target area of the brain. Specific cell surface receptors may confer these differing levels of vulnerability, as well as forming the basis of perinatal patterns of neuronal involvement, as in ponto-subicular necrosis (Kinney and Armstrong, 2002). The reactions of the immature brain to hypoxic/ischaemic and other injury differ from those of the adult brain. The white matter appears to be particularly vulnerable in the perinatal period and both white matter infarction and the presence of axonal swellings have been described previously (Norman, 1978; Squier, 2001), although this latter feature has not been widely recognized.

This study reports the first evaluation of βAPP immunoreactivity in a large series of cases with perinatal brain damage. βAPP is known to be upregulated in neuronal stress and is a recognized marker of traumatic axonal damage (Graham *et al.*, 2000). Geddes *et al.* (2001*a*, *b*) have described a geographic pattern of axonal βAPP staining in the cerebrum and in the lower brain stem, particularly in the cortico-spinal tracts, in babies with non-accidental head injury. Most of this immunopositivity, with the exception of βAPP positive axonal bulbs, was ascribed to hypoxic rather than traumatic injury. Reichard *et al.* (2003) also described βAPP immunopositive bulbs in a series of 29 medicolegal infant cases, not all of whom had sustained traumatic injury. Results in the present study show that βAPP is a very useful marker for axonal injury in neonates as young as 25 weeks gestation. Similar findings were reported in a previous small study (Baiden-Amissah *et al.*, 1998). Inflicted trauma was ruled out in the present cases since virtually all were under close hospital supervision throughout life. The infants displaying βAPP positivity were delivered by a variety of methods including vaginal delivery and, in 19 cases, delivery by emergency Caesarean section, suggesting that axonal injury in these infants originates with antepartum events rather than prolonged birth-related trauma. It is apparent that βAPP staining patterns in the developing brain should be interpreted with caution and that the presence of βAPP positive axonal bulbs should not necessarily be ascribed to a traumatic event.

Since βAPP positivity appears very rapidly after a neuronal insult (Sherriff *et al.*, 1994; Baiden-Amissah *et al.*, 1998; Graham *et al.*, 2000), this feature is unlikely to be of use in ascribing brain damage in the neonate to the antepartum, intrapartum or postnatal period except perhaps in infants who die immediately after birth and when labour has been short. Immunodetection in the neuronal cell body implies upregulation of βAPP expression whereas axonal positivity results from disturbed axonal transport (Sherriff *et al.*, 1994). Uncertainties remain as to the duration and/or reversibility of these phenomena. A study of periventricular leucomalacia revealed that βAPP immunoreactive axons which were detected around early lesions were not present in mature lesions (Arai *et al.*, 1995). Perivascular amphophilic droplets and foci of micromineralization were consistently βAPP positive in the present study, supporting the thesis that these features may result from breakdown of the blood brain barrier (Squier, 2001), since soluble APP is present in the circulation as well as in CSF (Mattson, 1997). βAPP was not generally observed in immature neuronal cell bodies, unlike preterm axons. Overall, βAPP expression was more frequently observed in mature than preterm brains.

With respect to reactive gliosis, some confusion exists in the literature between the so-called myelination gliosis of normal development and the pathological state of reactive astrocytosis (Squier, 2001; Kinney and Armstrong, 2002). The cells of 'myelination gliosis' are reminiscent of reactive astrocytes in that they have eccentric flares of eosinophilic cytoplasm but they are oriented in regular fashion in white matter of undamaged appearance, which is in the process of myelination. In the present study, we have found such cells to be GFAP negative and infer that they are oligodendroglia. In contrast, astrocytes which prove to be markedly reactive on GFAP immunocytochemistry may be inconspicuous on

routine staining. We conclude that a meaningful assessment of gliosis in the neonatal brain requires GFAP immunocytochemistry. The significance of the apparent Alzheimer type II astrocytosis present in a number of cases is unclear, but has been remarked upon previously in perinatal brains (Kinney and Armstrong, 2002). It may represent the result of metabolic upset and, in our series, was more common in infants with damaged brains.

Correlation between βAPP positive axonal injury and glial/macrophage reactions, although significant ($P = 0.034$), was lower than we had anticipated and quite a number of encephalopathic cases with reactive changes did not display white matter βAPP positivity. This may be a sampling phenomenon, since both βAPP reactive axons and gliosis may be focal phenomena.

With respect to pathogenesis of brain damage in the neonate, many of the pathological features noted in this study have been ascribed classically to hypoxic/ischaemic insults (Rivkin and Volpe, 1993). However, consideration should also be given to other possible mechanisms, including systemic infection and inflammation (Pape and Wigglesworth, 1989; Ellis *et al.*, 2000; Wheater and Rennie, 2000)—possibly mediated by pro-inflammatory cytokines (Yoon *et al.*, 1997; Duggan *et al.*, 2001) or by free radicals (Tan *et al.*, 1998; Kinney and Armstrong, 2000). We did not find any evidence of an association between brain damage and intrauterine infection in the Scottish study (Becher *et al.*, 2004). The possibility exists that cytokine upregulation in the brain may be a response to neural damage, rather than its cause. Similarly, some of the observed neuronal changes including βAPP expression may be the result rather than the cause of seizures in encephalopathic infants. However, it should be noted that these cellular changes also occurred in infants who had not had seizures. This study highlights the prevalence of recent onset neuronal damage in both mature and preterm infants often co-present with older lesions, suggesting an ongoing neuronal response to cerebral insults. Unfortunately, the pregnancy-associated clinical features that might predict the likelihood of brain damage remain elusive (Becher *et al.*, 2004) and the exact pathogenesis of neuronal/axonal injury remains unclear in the neonatal brain.

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ORIGINAL ARTICLE

The distribution of apolipoprotein E alleles in Scottish perinatal deaths

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Background: The apolipoprotein E (ApoE) polymorphism has been well studied in the adult human population, in part because the e4 allele is a known risk factor for Alzheimer's disease. Little is known of the distribution of ApoE alleles in newborns, and their association with perinatal brain damage has not been investigated.

Methods: ApoE genotyping was undertaken in a Scottish cohort of perinatal deaths (n = 261), some of whom had prenatal brain damage. The distribution of ApoE alleles in perinatal deaths was compared with that in healthy liveborn infants and in adults in Scotland.

Results: ApoE e2 was over-represented in 251 perinatal deaths (13% *v* 8% in healthy newborns, odds ratio (OR) = 1.63, 95% confidence interval (CI) 1.13 to 2.36 and 13% *v* 8% in adults, OR = 1.67, 95% CI 1.16 to 2.41), both in liveborn and stillborn perinatal deaths. In contrast, the prevalence of ApoE e4 was raised in healthy liveborn infants (19%) compared with stillbirths (13%, OR = 1.59, 95% CI 1.11 to 2.26) and with adults (15%, OR = 1.35, 95% CI 1.04 to 1.76). However, no correlation was found between ApoE genotype and the presence or absence of perinatal brain damage.

Conclusions: This study shows a shift in ApoE allelic distribution in early life compared with adults. The raised prevalence of ApoE e2 associated with perinatal death suggests that this allele is detrimental to pregnancy outcome, whereas ApoE e4 may be less so. However, ApoE genotype did not appear to influence the vulnerability for perinatal hypoxic/ischaemic brain damage, in agreement with findings in adult brains and in animal models.

Apolipoprotein E (ApoE) is a 299 amino acid protein primarily concerned with lipoprotein and cholesterol metabolism. The human ApoE gene is located on chromosome 19 and has three allelic forms, e2, e3, and e4, encoding different isoforms of the ApoE protein.[1] ApoE is expressed at high levels in brain tissue where it is produced mainly by astrocytes, which package ApoE with cholesterol for presentation to neurones.[2 3] ApoE e4 is a well known risk factor for sporadic and familial Alzheimer's disease.[4 5] and is also associated with a poorer outcome following head injury.[6] On the other hand, ApoE e2 has been linked with other central nervous system (CNS) disorders, including sporadic Parkinson's disease.[7]

There is growing evidence that ApoE, lipid metabolism and immune responses are related both systemically and in the CNS.[8 9] Thus ApoE appears to suppress the production by glia of various pro-inflammatory cytokines, including tumour necrosis factor-α, interleukin-1β, and interleukin-6.[10] Accumulating evidence suggests that ApoE e3 is more effective than e4 in neuronal repair mechanisms and the maintenance of synapses and dendrites.[11]

Much of what is known of the role of ApoE in the CNS relates to adult life and older age groups. The influence of ApoE alleles on human development has received little attention despite the importance attached to this polymorphism in relation to brain pathology in later life. We have recently established that there is a high prevalence of prenatal brain damage in Scottish perinatal deaths, both in liveborn and stillborn infants.[12 13] In the present study, we hypothesised that the absence of one or more ApoE e3 alleles might predispose to brain injury in the perinatal period. It is known from population studies that the distribution of ApoE polymorphisms shows geographic and ethnic variations.[14] For this reason we took as our standards for comparison both the data published previously for the adult Scottish population[15] and our own study of the ApoE genotype distribution in Scottish healthy newborns.[16] We report here the ApoE genotype distribution in a geographically matched cohort of perinatal deaths and examine a possible association between brain damage and particular ApoE alleles.

MATERIALS AND METHODS

In a Scotland wide study undertaken in the late 1990s, all perinatal deaths that were at least 24 weeks gestation at birth and <7 days at the time of death were identified over a 2 year period (n = 745). The initial purpose of the study was to investigate the epidemiology and neuropathological status of these infant and fetal deaths and to identify any factors that might predispose to brain damage, particularly of prenatal origin. Neonates and fetuses with malformations of the CNS (n = 12) and of the heart (n = 28), those with major chromosomal abnormalities (n = 28) or with evidence of CNS infection (n = 0), and extremely macerated stillbirths (n = 118) were all excluded from the study. Consent for the study was withheld in 229 cases and 69 deaths were notified only after the close of the study. After these exclusions, 70 liveborn and 191 stillborn infants qualified for this study. Two pairs of twins were enrolled in this study, one pair liveborn and one stillborn. For the purposes of this study, the ApoE result of the second twin was excluded from analysis. In other multiple pregnancies occurring in the study, only one infant of the siblings had died. No sequential sibling pregnancies were entered in this 2 year study. Therefore, this perinatal ApoE study investigated 261 perinatal deaths, each born within a different family in Scotland during a 2 year period, representing 35% of the total perinatal deaths in that time.

Frozen tissue samples were not available. A PCR method for ApoE genotyping, described by Hixson and Vernier,[17] was adapted for analysis of formalin fixed, paraffin embedded

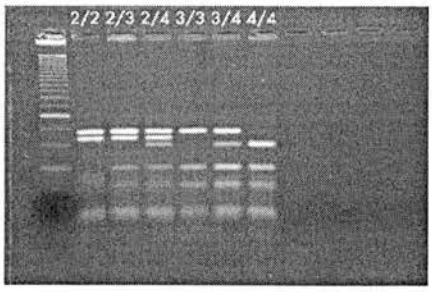

Figure 1 Metaphor gel showing the migration pattern for ApoE genotypes. Band 1 shows the control ladder.

tissue. DNA was extracted in each case from two or three 5 μm sections using a commercial kit (Qiagen DNeasy tissue kit, catalogue no 69506; Qiagem). The eluted DNA solution was added to a master mix solution containing customised primers (MWG Biotech; downstream primer: 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCCA-3', upstream primer: 5'-TCCAAGGAGCTGCAGGCGGCGCA-3'). The master mix also contained 5 μl of 10× PCR buffer, 8 μl of 5 mmol/l nucleotides, 1.5 μl 50 mmol/l MgCl, 1.25 μl *Taq* polymerase (Invitrogen), and 20 pmol of each primer. DNA cycling was run on a Techne Flexigene thermocycler at 95°C for 3 minutes followed by 45 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 2 minutes, with a final extension of 72°C for 8 minutes. The PCR product of 227 bp was visualised with ethidium bromide on a 3% agarose gel. The remaining PCR product was mixed with 1 μl *Hha*I restriction digest enzyme (New England Biolabs) at 37°C overnight, run on a 4% Metaphor gel, and again visualised with ethidium bromide under ultraviolet light.

The genotype for each sample was determined from the migration pattern of bands in the gel. After digestion, fragments of different sizes allow ApoE genotyping (fig 1). ApoE e2/2 is characterised by the presence of two fragments, 91 bp and 81 bp in size. ApoE e4/4 is characterised by a unique 72 bp fragment, while ApoE e3/3 lacks the 81 bp and 72 bp fragments. Heterozygotes have a combination of different fragments. PCR was repeated for cases in which the band pattern was faint or equivocal until a definite result was obtained. In the small number of cases in which paraffin embedded brain tissue failed to yield a result despite repeated extraction, blocks of spleen sometimes gave a positive result, presumably due to shorter periods of formalin fixation.

When the ApoE analyses were complete for the whole cohort, the cases were classified according to whether they were liveborn or stillborn and analysed for association with evidence of brain damage. The ApoE allele distribution for these perinatal deaths was compared with published data for Scottish adults[15] and healthy liveborn infants.[16] The Scottish adults were healthy, late middle aged men and women randomly selected from General Practitioner lists in the mid 1980s. The healthy newborns were infants who were born in a single delivery unit within Edinburgh. Blood was collected randomly from the placentas following delivery and all infants were full term and had a birthweight >2 kg. No infant required medical care and all left hospital when due to do so.

Table 1 Comparison of adults, healthy newborns, and perinatal deaths for apolipoprotein E genotype: distribution of ApoE alleles

Cohort	e2	e3	E4	Total
Adults (n = 400)*	66 (8%)	616 (77%)	118 (15%)	800
HN (n = 371)†	63 (8%)	538 (72%)	141 (19%)	742
PD(n = 251)	64 (13%)	365 (72%)	73 (15%)	502

*Cumming and Robertson, 1984[15]; †Becher *et al* (in press).[16] HN, healthy newborns; PD, perinatal deaths.

Statistical analysis

Comparisons of ApoE distribution between populations were made using the χ^2 test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for $p<0.05$. Similarly, χ^2 analysis was used to determine differences between perinatal deaths showing neuropathological changes and those without such change.

This study was approved by the Lothian research ethics committee (LREC 1998/6/53). Additional to standard necropsy consent, written parental consent was obtained for extensive detailed neuropathological investigation.

RESULTS

Unequivocal results with clear ApoE banding patterns were obtained for 67 liveborn infants who died at ⩽7 days of age, and 186 stillborn fetuses in this study. Both the stillborn and liveborn groups contained a pair of twins, and all four babies were found to have an ApoE 2/3 genotype. The second twin of each pair was excluded from further analysis. Spleen blocks were used in eight cases, all other results being from brain tissue.

The distribution of ApoE alleles and genotypes for the 251 perinatal deaths included in this study is shown in tables 1–3, which also includes previously published data for 400 Scottish adults[15] and for 371 healthy newborn infants.[16] Table 3 shows that departure from the Hardy-Weinberg expectation is not significant for any of the three populations. Tables 4 and 5 compare ApoE results in the present study for infants who were liveborn but who died subsequently, and those for stillbirths.

We found over-representation of ApoE e2 in perinatal deaths (13%) compared with healthy newborns (8%, OR = 1.63, 95% CI 1.13 to 2.36) and with adults (8%, OR = 1.67, 95% CI 1.16 to 2.41) (table 1). The prevalence of all genotypes possessing one or more e2 alleles (2/2, 2/3 and 2/4) was higher among perinatal deaths (24%) than among healthy liveborn infants (16%, OR = 1.56, 95% CI 1.43 to 1.68) and adults (16%, OR = 1.64, 95% CI 1.51 to 1.77) (table 2). The higher prevalence of ApoE e2 was found both in liveborn and stillborn perinatal deaths (tables 4 and 5). The prevalence of ApoE e4 was higher in healthy liveborn infants (19%) than in both normal adults (15%, OR = 1.35,

Table 2 Comparison of adults, healthy newborns, and perinatal deaths for apolipoprotein E genotype: distribution of ApoE genotypes

Cohort	2/2	3/3	4/4	2/3	2/4	3/4
Adults(n = 400)*	2 (0.5%)	233 (58.2%)	4 (1%)	51 (12.8%)	11 (2.7%)	99 (24.8%)
HN (n = 371)†	2 (0.5%)	199 (53.6%)	15 (4%)	44 (11.9%)	15 (4%)	96 (25.9%)
PD (n = 251)	5 (2%)	138 (54.9%)	3 (1.2%)	38 (15.1%)	16 (6.4%)	51 (20.3%)

*Cumming and Robertson, 1984[15]; †Becher *et al* (in press).[16] HN, healthy newborns; PD, perinatal deaths.

Table 3 Comparison of adults, healthy newborns, and perinatal deaths for apolipoprotein E genotype: distribution n of alleles with Hardy-Weinberg expectation

	e3/3	e3/−	Others	Total	p
Adults					
Observed	232	152	16	400	0.63
Expected	237	142	21	400	
HN					
Observed	199	140	32	371	0.58
Expected	195	148	28	371	
PD					
Observed	138	89	24	251	0.55
Expected	133	99	19	251	

HN, healthy newborns; PD, perinatal deaths.

Table 4 ApoE genotype in perinatal deaths, liveborn versus stillborn: distribution of ApoE alleles

Cohort	e2	e3	e4	Total
Liveborn PD (n=66)	16 (12%)	91 (69%)	25 (19%)	132
Stillbirths (n=185)	48 (13%)	274 (74%)	48 (13%)	370

PD, perinatal deaths.

95% CI 1.04 to 1.76) and stillbirths (13%, OR = 1.59, 95% CI 1.11 to 2.26). Review of tables 4 and 5 suggests that liveborn infants who die in the perinatal period have over-representation of both e2 and e4 alleles compared with normal adults, but the number of cases (n = 66) is too small to place reliance on statistical analysis.

Table 6 shows the variation in allele distribution across gestational groups. Although there appears to be an increase in e2 in extremely preterm gestations, this difference is not statistically significant.

Tables 7 and 8 show the distribution of ApoE genotype according to the presence or absence of brain damage in this cohort. No correlation was detected between ApoE status and the neuropathological findings in liveborn or stillborn babies. Similarly there was no link between ApoE and the presence of haemorrhage in the brain (table 9).

Taken together, the results of this study suggest that the absence of one or more ApoE e3 predisposes to perinatal death but not to perinatal brain injury.

DISCUSSION

This study reveals significant differences in ApoE allele distribution when comparing perinatal deaths with healthy liveborn infants and with previously published data for the adult population, all in Scotland. Cumming and Robertson[15] showed that ApoE e2 was present in 8%, e3 in 77% ,and e4 in 15% of 400 adults aged 45–60 years, chosen randomly from north Scotland. Although this is a historical cohort, the distribution of allele frequencies is similar to that of other white populations in the literature. In the review by Davignon *et al*[14] of all published data, it was found that in 5805 white subjects, the ApoE allele frequency was 8% for e2,

Table 6 Allele distributions by gestational group in all perinatal deaths

Total alleles (n=502)	e2	e3	e4
24–26 weeks (n=92)	17 (18%)	63 (68%)	12 (13%)
27–29 weeks (n=62)	7 (11%)	48 (77%)	7 (11%)
30–32 weeks (n=56)	6 (11%)	37 (66%)	13 (23%)
33–35 weeks(n=76)	12 (16%)	57 (75%)	7 (9%)
36or >wks (n=216)	22 (10%)	160 (74%)	34 (16%)
HN (n=742 alleles)	63 (8%)	538 (72%)	141 (19%)

HN, healthy newborns.

Table 7 Correlation of ApoE alleles with prenatal brain damage in liveborn babies aged ⩽3 days at death* (n=57)

	Prenatal brain damage present	No prenatal brain damage*
Infants with ApoE 3/3 (25)	12 (48%)	13 (52%)
Infants with ⩾1 ApoE 2 (12)	5 (42%)	7 (58%)
Infants with ⩾1 ApoE 4 (19)	10 (53%)	9(47%)
Infants with ApoE 2/4 (1)	0	1 (100%)

*The presence of prenatal damage cannot be determined reliably in infants aged more than three days (Becher *et al*[12]).

Table 8 Correlation of ApoE alleles with brain damage in stillborn babies (n=185)

	Established brain damage	Recent brain damage	No brain damage
Infants with ApoE 3/3 (110)	42 (38%)	34 (31%)	34 (31%)
Infants with ⩾1 ApoE 2 (30)	10 (33%)	7 (23%)	13 (43%)
Infants with ⩾1 ApoE 4 (30)	12 (40%)	11 (37%)	7 (23%)
Infants with ApoE 2/4 (15)	2 (13%)	5 (33%)	8 (53%)

Table 9 Correlation of ApoE alleles with brain haemorrhage in liveborn babies who died aged ⩽7 days (n=66), and in stillborn babies (n=185)

	Haem.	No haem.
Liveborn infants		
Infants with ApoE 3/3 (28)	14 (50%)	14 (50%)
Infants with ⩾1 ApoE 2 (13)	7 (54%)	6 (46%)
Infants with ⩾1 ApoE 4 (24)	11 (46%)	13 (54%)
Infants with ApoE 2/4 (1)	1 (100%)	0
Stillbirths		
Infants with ApoE 3/3 (110)	48 (44%)	62 (56%)
Infants with ⩾1 ApoE 2 (30)	8 (27%)	22 (73%)
Infants with ⩾1 ApoE 4 (30)	7 (23%)	23 (77%)
Infants with ApoE 2/4 (15)	4 (27%)	11 (73%)

Table 5 ApoE genotype in perinatal deaths, liveborn versus stillborn: distribution of ApoE genotypes

Cohort	2/2	3/3	4/4	2/3	2/4	3/4
Liveborn PD (n=66)	2 (3%)	28 (42.4%)	0 (0%)	11 (16.7%)	1 (1.5%)	24 (34.4%)
Stillbirths (n=185)	3 (1.6%)	110 (59.5%)	3 (1.6%)	27 (14.6%)	15 (8.1%)	27 (14.6%)

PD, perinatal deaths.

77% for e3, and 15% for e4, which is precisely the same distribution as that reported for the Scottish adult population used in this study. The exceptions in this large survey were adults in France and New Zealand, who have a raised prevalence of e2, at 13% and 12%, respectively, and the Finnish adult population, who have a reduced e2 (4%) and higher e4 (22%) prevalence.[14] These three studies comprised 223, 426, and 615 subjects, respectively, and are therefore comparable in size with our study, although the adults were younger than those in the Scottish adult study.

We have shown in 371 healthy newborn infants that the corresponding ApoE distribution was 8% for e2, 72% for e3, and 19% for e4 ($p = 0.024$ for over-representation of e4 in healthy newborns compared to healthy adults).[16] The present study of 251 perinatal deaths shows that ApoE e2 is significantly over-represented in both stillborn and liveborn perinatal deaths, compared with the lower prevalence in both healthy newborn and late middle aged adults in Scotland. Infants who are born alive but who die in the first week of life also show over-representation of e4 and resemble healthy newborns in this respect. While it is possible that these shifts in ApoE distribution have occurred by chance, the numbers in our study are not small. The shift in genotype is also borne out by examination of individual genotypes. However, the limitations in comparing newborn with adult populations should be noted, in that both ApoE e2 and e4 are associated with diseases that have their onset in middle life and carry a significant mortality risk. In addition, data from the French, Finnish, and New Zealand studies quoted above[14] indicate that the prevalence of non-e3 alleles is not entirely consistent in adults.

Support in the literature for a developmental association with different ApoE alleles comes from the small number of studies already available in human infants and pregnancy loss. Zetterberg *et al*[18] found a decreased prevalence of e4 in spontaneous abortions in Crete, 87% of which were earlier than 12 weeks gestation, and proposed that e4 may have a protective effect in pregnancy, which would be consistent with our findings in healthy newborns. The contrast between ApoE e4 prevalence in liveborn and in stillborn infants (e4 being higher in the former group), also suggests that e4 may have a protective effect. Wright *et al*[19] showed that e4 infants have a better mental developmental index at 2 years of age, suggesting that the effects of e4 at an early age may be beneficial rather than detrimental. Similar results have been demonstrated in Brazilian children.[20] Other studies suggest that the e4 allele may be associated with improved cognitive performance and higher educational achievement in early adulthood.[21 22] In contrast, possession of one or more ApoE e2 alleles appears to be detrimental to pregnancy outcome. Relatively little attention has been paid to this, the rarest ApoE allele, perhaps because it is regarded as the "good" gene with respect to Alzheimer's disease and ischaemic heart disease. ApoE e2 was reported in an early study to be associated with pre-eclampsia,[23] but this was not upheld in subsequent studies.[24 25] ApoE e2 is less often transmitted to babies who are small for gestational age[26] and perinatally growth restricted babies are more likely to develop cardiovascular disease later in life.[27] According to the antagonistic pleiotropic theory of ageing, natural selection favours genes that confer short term benefits at the expense of deterioration in later life. Such opposing effects of the e4 allele may result in beneficial effects in early development but detrimental effects in adulthood.

We found no evidence in this study for an association between ApoE genotype and vulnerability for brain damage or haemorrhage in the perinatal period. The relationship between ApoE genotype and hypoxic/ischaemic injury in the brain is complex, and conflicting results have been reported in the literature. It seems that ApoE e2 is associated with cerebral haemorrhage only in the context of amyloid angiopathy[28] while e4 is linked to poorer outcome after haemorrhagic stroke but to better survival following ischaemic damage in the adult.[3] However, no effect of ApoE allele variations was noted in neonatal mouse models transgenic for human ApoE e3 and e4 and subjected to ischaemic/hypoxic or excitotoxic insults and neuronal survival appeared to be similar in the two animal groups.[29] Our findings for human infants would be in keeping with these animal experiments, and extend the results to the e2 allele.

The role of ApoE in brain tissue is not fully understood, but appears to be neuroprotective and immunosuppressive. Protein expression of ApoE is reported to be increased in neurones following a global ischaemic insult in the human brain, but this effect is not influenced by the host genotype in respect of ApoE e3 or e4.[30] ApoE e2 was not included in this study due to the scarcity of this allele. Our findings suggest that ApoE does not display allele specific neuroprotective variations in the perinatal period.

In summary, this study demonstrates that ApoE e2 is linked to a poor gestational outcome, while e4 may confer benefits during pregnancy which make live birth more likely than stillbirth. However, we have found no evidence that ApoE status influences the likelihood of sustaining pregnancy associated brain damage. The absence of one of more ApoE e3 alleles did not predispose to brain injury or to haemorrhage. More thorough investigation of the effects of ApoE on the outcome of pregnancy may require paired parent/fetus analysis,[26] as it is possible that fetal wellbeing may vary according to the parental source of the e2 or e4 allele.

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CORRECTION

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In the Original article titled, survivin-directed RNA interference cocktail is a potent suppressor of tumour growth in vivo (*J Med Genet* 2006; **43**:119-128) figure 3B, top panel was incorrect. The original legend printed for figure 3B is unchanged. The authors apologise for this error. A full corrected figure 3B is available on the JMG website at http://www.jmedgenet.com/supplemental.