Alterations in the Cerebrospinal Fluid relating to Apolipoprotein E after Traumatic Brain Injury and Subarachnoid Haemorrhage

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Abstract

Background: The human gene coding for apolipoprotein E is polymorphic, and the *APOE* ε4 allele has been associated with less favourable outcome after acute brain injury including traumatic brain injury (TBI) and subarachnoid haemorrhage (SAH). Experimental studies identify key roles for apoE in the central nervous system such as the scavenging and recycling of lipids for cellular maintenance and repair and formation of cerebral amyloid aggregate. Human *in-vivo* evidence supporting the concept that apoE is involved in the response of the brain to acute injury is sparse. **Objectives:** This study tests the hypothesis that apoE is involved in the response of the human brain to injury, and this role is reflected by changes in cerebrospinal fluid (CSF) apoE concentration after brain injury which correlate with injury severity and outcome. In addition it was hypothesised that changes in apoE concentration would be paralleled by changes in the composition of CSF lipoprotein particles (Lps) of which apoE is a major component. Lastly, apoE is reported to chaperone amyloid-beta peptide (Aβ), therefore we hypothesised that alteration in CSF apoE after brain injury would parallel alterations in Aβ. **Methods:** Enzyme linked immunosorbant assay (ELISA) was used to determine the concentration of apoE, Aβ, S100B and Tau (as surrogate markers of brain injury) in CSF from TBI and SAH patients and a non-brain injured control group. Lipoprotein particles were isolated from CSF using size exclusion chromatography and characterised in relation to cholesterol, phospholipid, apolipoprotein E, and apolipoprotein AI composition. Injury severity was determined using the Glasgow Coma Score, and clinical outcome using the Glasgow Outcome Score. **Results:** Compared to controls there was a sustained decrease in the concentration of apoE in the CSF after TBI and SAH which was paralleled by a depletion of apoE containing lipoprotein particles. Furthermore, CSF Aβ also decreased, and the decrease correlated with injury severity and clinical outcome. In contrast the levels of S100B and Tau in brain injury CSF was substantially elevated. **Conclusion:** Despite the likely leakage of plasma apolipoprotein E into the subarachnoid space at the time of brain injury, apoE in the form of LpE is cleared from the CSF within days of injury. In addition, indirect evidence suggesting apoE-Aβ interactions *in-vivo* support the concept that apoE may form insoluble aggregates with Aβ soon after brain injury. The finding that these alterations in the CSF correlate with injury severity and outcome provides novel indirect *in-vivo* evidence that apoE is important to the response of the human brain to injury.

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Abbreviations

A	Adenine
Aβ	β-amyloid protein (1-40 or 1-42)
ACM	Astrocyte culture medium
AD	Alzheimer's Disease
A&E	Accident and emergency
ANOVA	Analysis of variance
Apo	Apolipoprotein
***APOE* ε**	Apolipoprotein E allele
APOE	Apolipoprotein E gene
***APOE*-/-**	*APOE* knockout
***APP*^{V717P}**	Amyloid precursor protein gene mutation- valine 717 to phenylalanine
Arg	Arginine
β-APP	Amyloid precursor protein
BER	Base excision repair
BSA	Bovine serum albumin
C	Cytosine
CAMP	cyclic adenosine monophosphate
CBF	Cerebral blood flow
CJD	Creuztfeldt Jakob disease
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
CI	Confidence interval
CNS	Central nervous system
CpG	Cytosine $_{\text{phosphate}}$ Guanine
CSF	Cerebrospinal fluid
CT scan	Computerised tomography scan
CV	Coefficient of variation
Cys	Cysteine
dUTP	Deoxyuridine triphosphate
EAA	Excitatory amino acid
EBIC	European Brain Injury Consortium
EDTA	Ethylene diamine tetra acetic acid
EEG	Electro-encephalogram
ELISA	Enzyme Linked immunosorbant assay
FAD	Familial Alzheimer's Disease
FC	Free (unesterified) cholesterol
FGF	Fibroblast growth factor
FPLC	Fast protein liquid chromatography
G	Guanine
GABA	γ-amino-butyric acid
GCS	Glasgow coma scale
GFAP	Glial fibrillary acidic protein
GOS	Glasgow outcome scale
HDL	High density lipoprotein
4-HNE	4-hydroxynonenal
HRP	Horse radish peroxidase
HSPG	Heparin sulphate proteoglycan
HSPG-LRP	Heparin sulphate proteoglycan-LDL receptor protein pathway
ICH	Intra-cerebral haemorrhage
ICP	Intra-cranial pressure
ICU	Intensive care unit
IDL	Intermediate density lipoprotein

IEF	Iso-electric focusing
IGFs	Insulin like growth factors
IHD	Ischaemic heart disease
IL	Interleukin
KDa	Kilo Dalton
LCAT	Lecithin: cholesterol acyltransferase
LDL	Low density lipoprotein
LDLr	LDL receptor
Lp	Lipoprotein particle
LpE	ApoE containing lipoprotein particle
LpAI	ApoAI containing lipoprotein particle
LRP	LDL receptor related protein
MAP	Microtubule-associated protein
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple Sclerosis
MVA	Motor vehicle accident
NFT	Neurofibrillary tangles
NSE	Neuron specific enolase (Protein 13-3-2)
OPD	σ- phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHF	Paired helical filaments
PLTP	Phospholipid transfer protein
PS-1	Presenilin-1
PS-2	Presenilin-2
RFLP	Restriction fragment length polymorphism
RPM	Revolutions per minute
RTA	Road traffic accident
SAH	Subarachnoid haemorrhage
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SP	Senile plaque
T	Thymine
TBI	Traumatic brain injury
TC	total cholesterol
TEMED	Tetramethylenediamine
TG	Transgenic
TG	Triglyceride
TGFs	Transforming growth factors
TMB	3`3`5`5` tetramethylbenzidine
TdT	Terminal deoxynucleotidyl transferase
TUNEL	TdT dUTP nick end labelling
TSE	Transmissible spongiform encephalopathy
USA	United States of America
VLDL	Very low density lipoprotein
WFNS	World Federation of Neurological Surgeons
WT	Wild type

1 Introduction

Traumatic Brain Injury (TBI) and spontaneous Subarachnoid Haemorrhage (SAH) are two conditions commonly encountered in neurosurgical practice. There is considerable variation in clinical outcome for patients with these conditions even after accounting for injury severity, suggesting that other unidentified factors influence recovery. The possibility that genetic factors influence the recovery after TBI and SAH recently found preliminary support from studies associating possession of the *APOE* ε4 allele with unfavourable outcome. (Niskakangas et al. 2001; Teasdale et al. 1997) Although, evidence from post mortem and experimental brain injury studies support the concept that apolipoproteinE (apoE indicates protein; *APOE*, gene) plays a key role in the response of the brain to injury, *in-vivo* evidence in humans is lacking. This thesis presents indirect *in-vivo* evidence from the analysis of cerebrospinal fluid (CSF) that apoE is involved in the response of the brain to injury. This chapter discusses TBI and SAH, the response of the brain to injury, apolipoprotein E, and the utility of CSF analysis for investigating acute brain injury.

1.1 Traumatic Brain Injury and Subarachnoid Haemorrhage

1.1.1 Definitions of TBI and SAH

1.1.1.1 TBI

TBI refers to brain injury resulting from trauma. Haemorrhage into the subarachnoid space may be a consequence of TBI, and is the commonest cause of secondary/non-spontaneous SAH. SAH secondary to TBI is often referred to as tSAH and is not to be confused with spontaneous SAH discussed below.

1.1.1.2 SAH

SAH, usually due to rupture of an intracranial aneurysm, is a spontaneous event, which results in haemorrhage into the subarachnoid space. Often the haemorrhage extends beyond the subarachnoid space into the ventricular system and brain parenchyma. Occasionally SAH is limited to the brain parenchyma with relatively little haemorrhage into the subarachnoid space.

1.1.2 Epidemiology and aetiology of TBI and SAH

1.1.2.1 TBI

In the UK, there are estimated to be at least 1 million patients presenting to hospital each year following head injury, representing 10% of all patients attending Accident and Emergency (A&E) departments. Approximately 90% of these patients have minor (Glasgow Coma Score, GCS 15-see table 1) or "mild" (GCS 13-15) head injury, and 5% have moderate (GCS 9-12), and 5% severe (GCS 3-8) head injury. (Kay and Teasdale, 2001) Approximately 20% require admission for observation, and less than 5% are transferred to neurosurgical care. It is estimated that 30-50% of trauma deaths are due to TBI accounting for 1-2% of all deaths of all causes. Importantly TBI accounts for 20% of deaths occurring between the ages of 5 and 45 years, in which group injury is the leading cause of death. Most head injuries result from a fall (40%) or an assault (20%) but most serious injuries follow a road traffic accident (RTA). These account for 58% of deaths and approximately one third of those transferred to neurosurgical care. (Teasdale, 1995) The population of patients in the regional neurosurgical unit tends to be a selected group with injuries at the more severe end of the spectrum, though differences exist due to variation in the criteria for transfer. In addition Geographical variations in aetiology exist within the United Kingdom (UK) and continental Europe, and between the European and American continents. A study of patients with severe head injury, admitted to four neurosurgical units in the UK, found that 22% of patients fell under the influence of alcohol in Glasgow compared to less than 10 % in Southampton, and that RTA accounted for less than 50% of the admissions to the Glasgow unit but nearly 70% in Southampton. (Kay and Teasdale, 2001; Murray et al. 1999) In 1999 the European Brain Injury Consortium (EBIC) published the findings from a survey of more than one thousand severely and moderately head injured patients admitted to sixty seven neurosurgical units in twelve European countries. (Murray et al. 1999) This survey found that the proportions that were injured as a vehicle occupant ranged from 11% in the UK up to 48% in the Benelux countries. In Spain only 1% of patients admitted to the neurosurgical unit had fallen under the influence of alcohol compared to 33% in Scandinavia. Gunshot wound to the head is uncommon in the UK and Europe in contrast to the United States of America (USA). These differences in aetiology and severity of injury, and the patterns of transfer to neurosurgical care, must be borne in mind when considering the relevance of findings in one unit to other units. Many of these injuries are avoidable occurring in risk taking young males with impaired judgement due to excessive consumption of alcohol.

1.1.2.2 SAH

SAH, mostly from rupture of an intracranial saccular aneurysm, accounts for only 3% of strokes and 5% of stroke deaths, but 25% of life years lost through stroke. (Johnston et al. 1998) The estimated overall incidence rate is 10.5 (95% CI: 9.9-11.2) per 100,000 life years. (Linn et al. 1996) The reported incidence of SAH is higher in Finland (22/100,000 patient years; 95% CI: 20-23) and Japan (23/100,000; 95% CI: 19-28). Women have a 1.6 (95% CI: 1.5-2.3) times higher risk than men, and black people have twice the risk of white people. (Broderick et al. 1992; Linn et al. 1996) The average age of patients with SAH is lower than for other types of stroke, peaking in the sixth decade, but higher than those with TBI. (Lanzino et al. 1996; Longstreth et al. 1993) Modifiable risk factors for SAH include smoking, hypertension, amphetamine/cocaine abuse and heavy alcohol consumption (Odds ratios of 2 - 3). (Teunissen et al. 1996) An important, but non-modifiable, risk factor is the familial predisposition to SAH such that 5-20% of patients with SAH have a positive family history. (Schievink, 1997) First degree relatives (parents, siblings, children) of patients with SAH have a three to seven fold increased risk, whilst second-degree relatives (grandparents, grandchildren, aunts, uncles, nieces, nephews) have a similar risk to the general population. (Bromberg et al. 1995; Gaist et al. 2000; Schievink, 1997) In addition, a small minority of patients with SAH have inherited connective tissue disorders. The commonest of these is Autosomal Dominant Polycystic Kidney Disease (ADPKD) and is found in 2% of all patients with SAH. (Schievink et al. 1992) Other rare genetic disorders associated with SAH include Ehlers Danlos disease IV and Neurofibromatosis type I. (Schievink et al. 1990) Saccular aneurysm rupture accounts for 85% of spontaneous SAH. It is largely unknown why some adults develop aneurysms at points of arterial bifurcation and most do not. The idea that a tunica media defect allows the inner layers to bulge through has fallen from favour recently as these defects are equally as common in vessels without aneurysms and are strengthened by densely packed collagen fibrils. Furthermore they are found in the fundus of the aneurysm, not at the neck. (Finlay et al. 1998; Fujimoto, 1996; Stehbens, 1989) The current hypothesis is that risk factors such as smoking and hypertension result in acquired local thickening of the intimal layer (intimal pads) proximal and distal to the vessel branch point producing decreased elasticity, increasing the strain on more elastic portions of the vessel. (van Gijn J. and Rinkel, 2001). SAH not due to saccular aneurysm rupture is due to perimesencephalic or prepontine haemorrhage in 10% of cases and rare conditions (e.g. transmural arterial dissection, arterial-venous malformation, dural arterio-venous fistula, septic aneurysm, and pituitary apoplexy) in the remaining 5% of cases.

1.1.3 Assessment of Injury severity

1.1.3.1 TBI and the Glasgow Coma Scale

The Glasgow Coma Scale (GCS) is the internationally recognised measure of impaired consciousness. Prior to the development of this scale in 1974, impaired consciousness was described using terms such as comatose, stupor, obtunded, drowsy and delirium. Inconsistency in the definition and interpretation of these terms made assessment of recovery and deterioration for individual patients difficult and made meaningful comparisons of different patient cohorts difficult limiting their value for research purposes. In contrast the GCS is a simple robust method for describing the level of consciousness which can have a consistency as high as 97%. The Glasgow Coma Scale enables simple description of a patient's responsiveness in terms of best motor response, verbal response and eye opening. Each of these is then stratified according to increasing impairment. The GCS grades patients according to three scales, which are summed to give a total score ranging from three to fifteen. The Glasgow Coma Scale is an artificial index obtained by adding scores for the three responses. The GCS scoring system is summarised in table 1. This score can be used as a single figure summary for purposes of head injury classification, but contains less information than separate descriptions of the three responses. Hence the Scale, not the Score, should provide the basis for monitoring and exchange of information about individual patients. The motor scale awards six points for obeying commands, five for localising a painful stimulus, four for "normal" flexion, five for "abnormal" flexion, two for "extensor posturing" and one for no motor response. In the first description of the scale the motor response had only five options with no demarcation between "normal" and "abnormal flexion". The inclusion of " abnormal flexion" to give a six point motor response, and a total score of fifteen, adds little to the monitoring of individual patients but is relevant to prognosis. The verbal scale awards five points to the patient who is orientated in time, place and person, four if speech is confused, three for inappropriate unsustained speech, two for incomprehensible sounds and one for no verbal response. Spontaneous eye opening is awarded four points, eye opening to speech three, eye opening to painful stimuli two, and no eye-opening one point. (Teasdale and Jennett, 1974; Teasdale and Jennett, 1976; Teasdale et al. 1978)

Table 1 The Glasgow Coma Score

Feature	Scale	Score
Eye Opening	Spontaneous	4
	To Speech	3
	To Pain	2
	None	1
Verbal Response	Orientated	5
	Confused	4
	Words (inappropriate)	3
	Sounds (incomprehensible)	2
	None	1
Best Motor Response	Obeys Commands	6
	Localises Pain	5
	Flexion Normal	4
	Flexion Abnormal	3
	Extends	2
	None	1
	Total Coma "Score"	3-15

Despite extensive studies supporting the repeatability, validity, and other clinimetric properties of the GCS, as few as 30% of doctors making neurosurgical referrals may be fully conversant with its use. (Morris, 1993) The motor scale alone has almost as high predictive value as the total GCS, thus the GCS is still useful if eye-opening or verbal response cannot be assessed e.g. due to facial trauma. The timing of assessment of conscious level is important as this may be influenced by the presence of sedatives, intoxication, hypoxia and hypotension. The increased use of pre-hospital sedation and intubation complicates the early assessment of injury severity using the GCS. (Marion and Carlier, 1994) The GCS does not take account of other correlates with injury severity such as the reactivity of the pupils to light, the oculocephalic response, and physiological parameters.

1.1.3.2 The World Federation of Neurological Surgeons Severity Scale for SAH

Severity of brain injury after SAH is also based upon the assessment of consciousness level graded using the GCS. In addition the grading scale of the World Federation of Neurological Surgeons (WFNS) includes an assessment of the presence or absence of focal neurological deficits as summarised in table 2. (Teasdale et al. 1988) Though poor neurological condition after SAH may be due to global ischaemic damage secondary to raised intracranial pressure from the haemorrhage, potentially reversible factors such as hydrocephalus, intracerebral or subdural haematoma also contribute to the impairment of conscious level.

1.1.3.3 Radiological assessment of severity of TBI

The computerised tomography (CT) scan is currently the means by which the nature, distribution and severity of brain injury is assessed after trauma providing satisfactory imaging for immediate management decisions. The Marshall CT classification is often used to categorise the various radiological manifestations of TBI for the purposes of clinical research. This is summarised in table 3. Even after combining the CT scan appearance with clinical information such as age and motor score from the GCS, survival is correctly predicted in less than 40% of cases. Further limitations of CT classification of TBI relate to the dynamic nature of brain injury and the time elapsed between injury and investigation, coupled to the relatively poor resolution of the technique. Thus when the initial scan shows diffuse injury, without swelling or shift, approximately one in six patients will have evidence of a mass lesion on a later scan, and will have increased risk of unfavourable outcome. (Marshall et al. 1992; Servadei et al. 2000)

Table 2 The World Federation of Neurological Surgeons Grading of SAH

WFNS grade of SAH	Glasgow Coma Scale total Score
I	15
II	13-14 no focal deficit*
III	13-14 with focal deficit
IV	7-12
V	3-6

*Cranial nerve palsies are not considered a focal deficit

WFNS: World federation of Neurological Surgeons

SAH: Subarachnoid Haemorrhage

Table 3 Radiological assessment of severity of TBI

Grade	Classification	CT findings
1	Diffuse injury	Intracranial pathology not visible on CT scan
2	Diffuse injury a) 1 only b) 2 unilateral c) Bilateral	Cisterns present with shift of 0-5 mm, lesion present, but no high or mixed density lesion >25 cc. May include bone fragments and foreign bodies.
3	Diffuse injury + swelling	Cisterns compressed or absent, shift of 0-5 mm, no high or mixed density lesion >25 cc
4	Diffuse injury + shift	Shift of >5 mm , no high or mixed density lesion >25 cc
5	Evacuated mass lesion a) Extradural b) Subdural c) Intracerebral d) >/= 2 lesions	Any lesion surgically evacuated
6	Nonevacuated mass lesion a) Extradural b) Subdural c) Intracerebral d) ≥ 2 lesions	High or mixed density lesion >25 cc, not surgically evacuated

1.1.3.4 Radiological assessment of severity of SAH

The Fisher grading system is often used to categorise radiologically the quantity of blood seen on the CT scan after SAH, for the purposes of clinical research and audit. The Fisher grading system for blood load on CT scan is summarised in table 4. The Fisher grade is reported to correlate with clinical outcome, and the development of vasospasm, but is renowned for poor inter-observer agreement. (Fisher et al. 1980)

Table 4 Fisher CT scan radiological grading system for SAH

Fisher group	Blood on CT
1	No SAH detected
2	Diffuse or vertical layers <1 mm thick
3	Localised clot and/or vertical layers>1mm thick
4	Intracerebral or intraventricular clot with diffuse or no SAH

CT: computerised tomography
SAH: Subarachnoid Haemorrhage

1.1.3.5 Classification of Clinical Outcome

Since it was described in 1975, the Glasgow Outcome Scale has become the most widely used method to summate the wide range of different sequelae of brain injury into a single overall method of classification of outcome. (Jennett and Bond, 1975) There are five grades to the scale: dead, persistent vegetative state, severe disability, moderate disability, and good recovery. The GOS is assessed six months after TBI. When assessment is performed three, six and twelve months after injury it is apparent that some patients (particularly those with severe disability at three months) improve supporting the concept of late recovery. From the European Brain Injury Consortium (EBIC) survey of 481 patients not obeying commands after TBI, less than 50% had a favourable outcome six months after injury. (Murray et al. 1999) The GOS is often assessed three months after SAH. The average case fatality rate after SAH is approximately 50% ranging from 32 -

67%. Approximately one third of survivors remain dependent and less than one third have no reduction in quality of life. (van Gijn J. and Rinkel, 2001) The GOS has proved acceptable in many parts of the world, in particular for describing the outcome in cohorts of patients, but there have been reservations about its reliability, sensitivity, validity, and relevance. These result in part from the original description having not provided explicit criteria for distinguishing between different outcome categories. As a consequence the outcome could be assigned variably, between observers. Moreover the problem could be compounded by inconsistencies in the method used to obtain information (e.g. face to face interview, telephone interview or postal questionnaire) and in the timing at which outcome is assessed. An extension of the original 5-category scale into an 8-category scale (GOSE) was described to improve discrimination but this was offset by less consistency in allocation. Recently, a structured interview based on a questionnaire has been developed to improve reliability of classification of outcome. Careful studies showed that the structured approach improved reliability and that a high level of consistency achieved by all methods of obtaining information. Furthermore, the classification produced by the structured assessment of the Glasgow Outcome Scale showed strong correlation with the results of a wide range of neuropsychological tests and with the findings of generic assessments of health and quality of life. As a consequence, the GOS remains a valuable 'overall' index, of particular use in classifying outcome in studies involving large cohorts of brain injured patients. Much experience supports the use of the GOS in studies of outcome after a severe TBI. (Pettigrew et al. 1998; Teasdale et al. 1998; Wilson et al. 1998; Wilson et al. 2000) More recently application of the GOS to survivors of so called mild TBI has challenged the assumption that most survivors are free of sequelae. A recent study of 3,000 adults with a head injury in Glasgow, confirmed that 90% had a GCS of 13-15 when they arrived at hospital. Follow-up of a representative cohort confirmed that the majority (78%) of survivors of a severe head injury were either moderately or severely disabled but produced a surprising finding that disability was also common in survivors of a moderate (54%) and mild (51%) head injury. In accord with this finding, reports of various specific problems were found to be frequent in patients with a mild injury, 79% of whom had persistent headache, 59% memory problems and 34% were unemployed. (Thornhill et al. 2000) The findings of this and similar studies questions the categorisation of patients admitted to hospital who have lost consciousness but recovered to a GCS of 13-15 as a mild injury. (Pettigrew et al. 1998; Teasdale et al. 1998) Patients with so-called mild and moderate TBI may represent a previously under-investigated subgroup that may potentially benefit from neuroprotective measures. Furthermore, the possibility that so called mild or

moderate injury may accelerate chronic neurodegeneration has not been conclusively investigated.

1.2 The response of the brain to injury

1.2.1 Classification

Evidence from experimental brain injury, and the all too frequent observation that after acute brain injury patients "talk and die", support the concept that brain injury stimulates a complex dynamic cascade of events which culminate in an accumulative burden of neuronal loss greater than that resulting from the initial injury. Thus damage occurring at the time of injury is referred to as primary injury, and that resulting from the cascades unleashed by the injury is referred to as secondary injury. More recently the concept of tertiary injury has emerged, which refers to the immunological and inflammatory changes in response to the primary and secondary injury. Neuroprotective strategies targeted at ameliorating secondary injury, though successful in experimental brain injury, have not translated to improved outcome after human brain injury. (Graham et al. 1995; Graham et al. 2000; Teasdale and Graham, 1998)

1.2.2 Primary injury

Primary injury results from the physical effects of tissue strain. Though tissue strain occurs after SAH due to raised intracranial pressure, brain shift and intraparenchymal haemorrhage, the effects have been more extensively characterised in experimental TBI. Tissue strain is the final common pathway for the forces of inertia and/or contact resulting from TBI. Contact may result in scalp laceration, skull fracture (and possibly extra-dural haematoma), cerebral contusions/laceration and intracerebral haematoma. Rapid acceleration or deceleration may result in forces of inertia causing subdural bridging veins to tear (and subdural haematoma formation) and widespread damage to white matter. Slower rates of tissue deformation tend to be tolerated better than rapid loading. Thus static loading, where the force is applied relatively slowly (more than 200 milliseconds) may only result in multiple comminuted skull fractures with little underlying brain injury. More commonly loading is dynamic (occurring in less than 200 msec and often <20 msec) due to impact (e.g. blunt object striking head) and/or impulsive (head moved or arrested rapidly, with or without contact). If a blunt object impacts the head a combination of contact and inertial forces result in tissue strain the nature of which depends on the mass, velocity and force of impact. Impulsive dynamic loading occurs when the head is rapidly accelerated or

decelerated and the brain injury results from inertial forces of translation, rotation and angulation. There is considerable variability in the types of tissue strain encountered in real life, which often occur in combination. Though primary injury may be further classified as open or closed injury, and focal or diffuse injury, such explicit categorisation may become impossible as the injury severity increases. (Graham et al. 1995; Graham et al. 2000; Teasdale and Graham, 1998)

1.2.3 Secondary injury

1.2.3.1 Hypoxic injury

Hypoxic and ischaemic injuries are major secondary events that occur after both TBI and SAH, and substantially influence clinical outcome. (Chesnut et al. 1993a; Chesnut et al. 1993b) Sustained global ischaemia occurs in the very early phase (i.e. 4-8 hours) after acute brain injury and before evacuation of mass lesions. (Marion et al. 1991) Ischaemic injury has been identified in 90% of patients dying after TBI, and episodes of transient diffuse ischaemia occur in 90% of patients in the highest quality neuro-intensive care units. (Graham et al. 1989; Jones et al. 1994) Focal ischaemia occurs in the surrounding area adjacent to cerebral contusions, intracerebral haemorrhage, beneath subdural haematomas, and tissue rendered ischaemic due to vasospasm. Hypoxic brain injury may also result from a wide range of aetiologies outside the CNS, which cause significant cardiopulmonary insufficiency, and from epileptiform seizures. Cardiopulmonary insufficiency and epileptiform seizures also occur after TBI and SAH (e.g. neurogenic pulmonary oedema) and may add to the burden of ischaemic insults in these patients.

The major component of hypoxic injury occurring after TBI and SAH is that due to reduced cerebral blood flow (CBF) due to the sudden increase in intracranial pressure at the time of haemorrhage or trauma. (Bouma et al. 1992a; Bouma et al. 1992b; Obrist et al. 1984; Schroder et al. 1996; Symon et al. 1974) The effects of the primary ischaemic injury, like the effects of tissue strain, are highly variable depending on the severity, duration, and type of hypoxic insult. Cerebral blood flow studies in the non-human primate determined the effects of reduced CBF and introduced the concepts of ischaemic thresholds, and selective neuronal vulnerability. The mean blood flow through the brain is approximately 50ml/100g/min (grey matter 80ml/100g/min: white matter 20ml/100g/min). In the healthy normally autoregulating brain cortical flow reduction (with normal arterial oxygen tension) down to 20ml/100g/min may be tolerated without functional consequences though the electro-encephalogram (EEG) may slow and the subject may develop anxiety

and drowsiness. Just below this level consciousness is lost and the brain loses the capacity to make neurotransmitter substances resulting in coma. The threshold of electrical failure has been reached resulting in flattening of EEG, and loss of evoked potentials. When flow falls below 18ml/100g/min the threshold for energy failure is reached resulting in mitochondrial dysfunction and failure of the Na^+/K^+ ATPase pump system resulting in potassium release. Between these thresholds there is a penumbra of brain that is electrically silent with normal or slightly raised potassium content, and though non-functioning is said to be capable of full recovery provided sufficient blood supply is maintained. At flows of 10ml/100g/min membrane integrity is lost, massive Ca^{2+} influx begins and the biochemical cascade of neuronal destruction begins. This cascade involves neuronal membrane deformation, cytoskeletal disintegration, ionic flux (Na^+, K^+, Ca^{2+}, H^+) disturbance and depolarisation/energy failure. Neurochemical, neurovascular, neurotransmitter and receptor changes occur due to disturbances involving catecholamine neurotransmitters, monoamine neurotransmitters, excitatory amino acids, nitric oxide, endogenous opioid peptides, platelet activating factor and various ion such as Mg^{2+} and Ca^{2+}. These mediators give rise to lipolysis, proteolysis, oxidation, phosphorylation activity, and cytoskeletal disintegration. This culminates in cell swelling, further membrane damage, free radical production with lipid peroxidation, proteolysis, axonal swelling and culminates in acute, delayed or programmed cell death (PCD). If flow is profoundly reduced e.g. to 5ml/100g/min within the distribution of one cerebral end artery for more than 60 mins, infarction is inevitable. However, when flow reduction is less marked e.g. 15ml/100g/min for 30 mins then selective neuronal loss may occur.

The most vulnerable neuronal types are:

- Hippocampal neurons (CA1).
- Cerebellar Purkinje cells.
- Cortical neurons, particularly the larger cells in the cuneate visual cortex.
- Basal Ganglia.

Classically selective neuronal loss is seen among patients who die after an episode of global ischaemia associated with cardiopulmonary arrest after which circulation has been restored. Neuronal groups may be selectively vulnerable due to differences in the microvascular anatomy that supply them (e.g. watershed changes at arterial boundary zones), though more recently evolved regions appear to be selectively vulnerable due to the physicochemical properties of the neurons themselves. A variety of phenotype differences have been postulated to account for selective vulnerability including differences in receptor expression e.g. selectively vulnerable neurons express glutamate receptors increasing

vulnerability to excitotoxicity. Other postulates include differences in cellular stores of adenosine triphosphate (ATP), or antioxidants, or apoptosis related proteins. Selective neuronal loss may be particularly important in patients with raised intracranial pressure where cerebral perfusion pressure may be marginal (e.g. 30-40 mmHg) for many hours or even days resulting in ischaemic neuronal loss especially in the hippocampus. Such bilateral hippocampal and cerebellar damage explains the high frequency of memory disorders and co-ordination difficulty seen in so many survivors of severe head injury. Even though local cerebral blood flow may not reach sub-therapeutic levels it is hypothesised that acute brain injury increases neuronal vulnerability to secondary insult. (DeGirolami et al. 1984; Pulsinelli et al. 1982; Symon, 1993; Teasdale and Graham, 1998)

1.2.3.2 Cytoskeletal injury

Cytoskeletal injury and disintegration has devastating consequences for the brain-injured patient. The original description of Diffuse Axonal Injury (DAI) defined focal lesions in the corpus callosum, focal lesions in one or both dorsolateral regions of the rostral brainstem adjacent to the cerebellar peduncles, and diffuse damage to axons that occurred after TBI. This anatomical distribution of injury accounts for the coma of brain injured patients, but has been reproduced in only one animal model of non human primates, limiting the rate of progress in understanding the underlying mechanisms. (Gennarelli et al. 1982) The term Traumatic Axonal Injury (TAI) is currently applied to animal models of axonal injury after trauma. In TAI, within two hours of injury the injured axons form focal swellings at intervals along their length which increase in size until the axons undergo disconnection four to six hours after injury. (Maxwell et al. 1997) This is called secondary axotomy and is distinct from primary axotomy where the axolemma is fragmented resulting in rapid loss of the axonal cytoskeleton. Secondary axotomy appears to result from damage to the axolemma with loss of the ionic gradient homeostatic mechanisms necessary for axonal electrical activity. Thus Ca^{2+} influx causes mitochondria to swell, microtubules to depolymerize, calpain activation and disruption of fast axonal transport. (Buki et al. 1999; Maxwell et al. 1997; McCracken et al. 1999) The definite identification of retraction bulbs (the hallmark of DAI) under the light microscope can be made approximately eighteen to twenty-four hours post injury. However, immunocytochemistry (ICC) using an antibody to Amyloid Precursor Protein (β-APP) shows evidence of axonal damage after two to three hours of survival and can be used to identify injured axons scattered amongst the population of uninjured axons. (Sherriff et al. 1994) As many axons appear to be unaffected by injury, the concept of selective neuronal

vulnerability appears to apply to cytoskeletal injury too. Possible explanations for this observation are that axons in a particular orientation relative to the axis of strain, or those changing direction and decussating, are more prone to injury. In the days following injury many irregular swellings are noted on the axons associated with oval or rounded bulbs at the end of the axon. Over the ensuing weeks these features dissipate and clusters of microglia in the white matter dominate the field as the damaged axons are phagocytosed. Months after the injury Wallerian degeneration of the white matter is noted which at the severe end of the spectrum is associated with a clinical vegetative state. (Graham et al. 1995; Graham et al. 2000) It is now recognised that axonal injury, as identified by β-APP ICC, occurs after a variety of different types of brain injury, and is not specific for TBI. (Dolinak et al. 2000a; Dolinak et al. 2000b; Geddes et al. 2000)

1.2.3.3 Vascular injury

Studies using contrast enhanced magnetic resonance imaging (MRI) and, single photon emission computed tomography (SPECT) have been used to demonstrate breakdown of the blood-brain barrier (BBB) after human acute brain injury. (Bullock et al. 1990; Lang et al. 1991; Marmarou et al. 2000) Gadolinium enhanced MRI and pertechnetate enhanced SPECT scan studies show the majority of early brain oedema, both global and focal, to be cytotoxic. Vasogenic oedema associated with opening of the BBB is seen at later time points around contusions and intracerebral haemorrhage, and is absent from patients with diffuse non-focal injury. Acute brain injury causes complex neurovascular changes resulting in altered cerebral blood flow at a number of levels. The cerebral microvasculature, which is more resistant to shear damage than axons, tends to stretch and leak rather than tear or burst. Pial vessels at the tip of the frontal poles and temporal lobes are subjected to greater focal concentrations of force and have a tendency to form contusions. Such injury to the microvasculature results in:

- Swelling of perivascular astrocytic end feet narrowing the vessel lumen.
- Increased endothelial microvacuolation and micro pseudopodial activity from increased trans endothelial flux of intravascular components.
- Perivascular haemorrhage and transvascular diapedesis of red cells.
- Increased intravascular leukocyte adherence as a result of cytokine activation due to free radical release.

In addition changes in these and larger vessels may occur due to alterations in vasomotor tone. Increased tone results in vasospasm, which may occur after both SAH and TBI, and is a major contributory factor to death and disability. Vasospasm is reported to occur in

70% of patients presenting with aneurysmal SAH, and results in symptomatic ischaemia or infarction in 36% of cases. (Biller et al. 1988) The biological mechanisms underlying vasospasm are poorly understood and treatment options are limited. The presence of blood in the subarachnoid space is the key initiator of vasospasm, and it is postulated that the oxyhaemoglobin (oxy-Hb) in subarachnoid blood induces vessel wall contraction. (Macdonald and Weir, 1991) Oxyhaemoglobin may act as a scavenger of Nitric Oxide (NO), and inactivate guanylate cyclase (GC) by oxidising a ferrous haeme group linked to the enzyme, and increases the production of radical species. NO has been identified as the major endothelial derived relaxing factor (EDRF) which upon release from endothelial cells diffuses to adjacent smooth muscle cells where it activates soluble GC resulting in production of cGMP. The cGMP activates intracellular sarcoplasmic reticulum bound calcium pumps sequestering free Ca^{2+} into intracellular stores relaxing the smooth muscle cell through reduced calmodulin mediated myosin light chain kinase activation. After SAH the released oxy-Hb may bind NO resulting in vasoconstriction and/or add to the burden of oxidative stress due to the production of globin free haemin released from Hb in the presence of H_2O_2, or oxidation of ferryl haeme, or formation of apoprotein radicals. In the absence of NO, endothelium derived constricting factors (EDCF) such as endothelin (ET), angiotensin II, prostaglandin $F_{2\alpha}$ and thromboxanes operate unopposed constricting the vessel wall. In addition, other factors such as ion channel activation, contractile protein phosphorylation, and conducted vasomotor responses may play important roles in the development of vasospasm after SAH. (Dietrich and Dacey, 2000; Laher and Zhang, 2001; Sobey, 2001) In addition to vessel spasms, acute brain injury may result in loss of autoregulation of cerebral blood flow and vasoparesis. Many patients, particularly after evacuation of intracranial haematomata, develop a phase of hyperaemic cerebral blood flow from the second through to the seventh day post injury. These cerebrovascular abnormalities following reperfusion have been attributed to the generation of oxygen-derived free radicals, and mediators of the inflammatory response.

1.2.3.4 Inflammatory response to injury

Injury results in a co-ordinated cellular response organised to clear both blood clot and necrotic brain tissue, and to reconstitute the glia limitans. The acute phase lasts until the blood clot within the subarachnoid, subdural, intraventricular or parenchymal spaces has dissolved, and serves to deliver serum, and haematogenous cells including platelets, neutrophils, monocytes, and macrophages. Cytokines are released stimulating glial reactions such that astrocytes upregulate expression of S100B and glial fibrillary acidic protein (GFAP), and microglia become active amoeboid-like cells, with shortened

processes, that actively migrate into the neuropil. The activated astrocytes play a key role in:

- Remodelling of the extracellular matrix by protease secretion
- Clearance of debris.
- Mediation of inflammation by release of transforming growth factors (TGFs) and Interleukins (ILs).
- Enhancement of neuronal survival by secretion of fibrosing growth factors (FGFs) and insulin like growth factors (IGFs).
- Metabolism of excitatory amino acids.

Demyelination of degenerating axons is initiated, but many of the processes of oligodendrocytes maintain contact with fragmenting internodal myelin segments and do not change morphologically. Clot lysis is followed by the subacute phase, which terminates with the formation of the accessory glia limitans from astrocyte processes forming a condensed layer, bound together by tight junctional complexes. Fibroblasts, which by now are more frequent than macrophages, deposit matrix and neovascularization occurs. The consolidation phase results in scar formation, and down regulation of astrocyte and macrophage activity. However, microglia appear to populate the perilesional tissue in increased numbers for a prolonged period the limit of which is yet to be defined. (Holmin et al. 1998; Logan et al. 1994)

Whilst there is little doubt that post injury immunoactivation mediates delayed neuronal damage the exact function of each mediator or activated cell type is less clear. Indeed inflammatory events after injury appear to display dual and opposing roles promoting the repair of injured tissue but also causing additional brain damage mediated by the numerous neurotoxic substances released. Most of the data supporting these hypotheses derive from experimental work based on both animal models and cultured neuronal cells. Recent evidence suggests that elimination of selected inflammatory mediators is detrimental resulting in attenuation of neurological recovery. In the peripheral nervous system, post injury inflammation is a prerequisite for regeneration and restoration of function. In the CNS the glial reaction after injury and glial scar formation inhibits axon regeneration. (Fawcett, 1997; Fawcett and Asher, 1999; Fitch and Silver, 1997) Presumably, in the CNS where regeneration is limited, the purpose of the inflammatory response is to facilitate the removal of debris in order to minimise further neuronal injury due to chronic inflammation and/or neurotoxic effects of debris degradation products. It seems likely that inflammatory mediators regulate the production or release of effectors involved in the clearance of debris after brain. (Lenzlinger et al. 2001; McIntosh et al. 1998; Morganti-Kossmann et al.

2001; Morganti-Kossmann et al. 2002; Stoll et al. 2002) We may speculate that debris removal via inflammatory processes is important for the removal of degradation products, which over time could accumulate and exert an osmotic effect resulting in raised intra-cranial pressure.

1.2.3.5 Programmed cell death

The form of cell death that occurs from primary injury is necrosis. Necrotic cell death is characterised by a loss of ionic homeostasis. The membranes of dying cells are leaky resulting in swelling and fragmentation of the cells and their organelles with spillage of their contents into the surrounding tissues damaging it, and stimulating an inflammatory response. In contrast, apoptosis (or programmed cell death, PCD) is an active orderly process by which the cell undergoes an intrinsically programmed transition from an intact, metabolically active state to cellular breakdown. Membrane bound fragments (or apoptotic bodies) contain intact cellular organelles and masses of condensed DNA that are released and quickly phagocytosed by macrophages or microglia cells. This occurs without lysis of organelles or spillage of intracellular contents and without an accompanying inflammatory response. Apoptotic cells are found singularly or in small groups and are reduced in volume. A characteristic feature of apoptotic cell death is that cellular DNA is cleaved at internucleosomal linkages generating fragments that are multiples of the internucleosomal length which produce a laddering appearance when separated by agarose gel electrophoresis. Programmed Cell death is classically associated with normal CNS development. However, there is an increasing literature that supports the concept that PCD may be triggered directly after acute brain injury by some biochemical mediators of secondary injury or indirectly as a result of secondary pathophysiological processes such as ischaemia or inflammation. Thus terminal deoxynucleotidyl transferase (TdT) mediated biotinylated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) positivity, a marker of PCD, has been identified in neurons and oligodendrocytes after both experimental and human brain injury. (Rink et al. 1995; Smith et al. 2000) In addition, anti-apoptotic cell death regulatory genes which are involved in development (e.g. the *Bcl*-2 superfamily) have been implicated in the regulation of PCD in experimental models of ischaemic, excitotoxic, and TBI. Transgenic mice over expressing human *Bcl*-2 protein have significantly less neuronal loss after experimental brain injury. (Raghupathi et al. 1998) It is postulated that Bcl-2 proteins modulate cell death and survival via mitochondrial cytochrome c, an activator of the caspase family of death related proteases. Activated caspase-1 (IL-1β converting enzyme) and caspase-3 cleave proteins that are important in maintaining cytoskeletal integrity and DNA repair, and activate

deoxyribonucleases, producing cell death with morphological features of apoptosis. Caspase-3 activation has been identified after experimental and human TBI, and central administration of a caspase inhibitor is reported to reduce post-traumatic apoptosis and neurological deficits. (Clark et al. 1999; Pike et al. 1998; Yakovlev et al. 1997) The tumour suppresser gene p53 which is typically induced and upregulated in response to DNA damage, is also activated in experimental brain injury in regions exhibiting TUNEL positive apoptotic cells. (Kaya et al. 1999; Napieralski et al. 1999) After experimental global cerebral ischaemia, base excision repair (BER) pathways are reported to decrease suggesting that neuronal apoptosis may result from a failure to repair damaged DNA. (Kawase et al. 1999) Furthermore, the nuclear protein Poly (ADP ribose) polymerase (PARP), which detects BER of double and single stranded DNA breaks is activated after experimental TBI and inhibition of PARP is reported to be neuroprotective. (LaPlaca et al. 1999; LaPlaca et al. 2001) In addition to the programmed cell death response of the brain to injury, there is evidence to support a co-ordinated intrinsic neuroprotective response, which facilitates the survival of viable neurons. Thus, proteins that modulate oxidative stress (glutathione peroxidase, and superoxide dismutase), and nitric oxide (inducible nitric oxide synthetase) production are induced after acute brain injury. Other factors produced by neurons and glia include trophic factors such as nerve growth factor, brain derived neurotrophic factor, insulin-like growth factor, etc. Examples of other genes identified as important for survival of the injured neuron include the "Immediate Early Genes" (activated within five minutes of an insult) *fos/jun*, and the so-called stress proteins such as *hsp 70*. It is postulated that these proteins activate downstream processes or act as molecular chaperones stabilising other proteins such as those making up the cytoskeleton or mobilising cell defences. However, it is estimated that there are 20,000 different proteins in the brain underlying the challenge involved in unravelling their relative contribution to intrinsic neuroprotection. (Graham et al. 2000)

1.2.4 Limitations in improving outcome after acute brain injury

Advances in understanding the chain of dynamic events that follow brain injury has prompted the development of so called "neuroprotective agents". Recognition of secondary processes and the vulnerability of the injured brain to these delayed insults suggests an opportunity to alter the "milieu" after the primary injury as a pre-treatment to the subsequent secondary insults. Recognition of the importance of ischaemia in head injury has encouraged the use of pharmacological agents developed with a view to treatment of stroke. Other approaches included hypothermia and corticosteroids. Efficacious in the controlled experimental conditions of the animal model, these agents underwent Phase II

studies with acceptable levels of safety and tolerability and in some cases preliminary evidence of improvement in outcome. However, Phase III studies have now been completed and no "neuroprotective agents" so far have reached the selected threshold for demonstrating a 10% improvement in favourable outcome. The reasons for the apparent failure of these studies are complex and require careful consideration in order to advance our understanding of the problems involved and how best to tackle them. These problems relate to heterogeneity of the target population and biological processes coupled to a dichotomous outcome whereby a relatively small proportion of patients have an intermediate outcome upon which a neuroprotective agent must exert its effect. Multicentred studies have identified prognostic factors determining outcome, however, increasingly, clinical information is less conspicuous given the earlier and increased use of ventilation. CT scanning reveals a heterogeneous array of abnormalities associated with mortalities ranging several fold. Continuous monitoring enables capture and analysis of hypoxic and hypotensive episodes that also influence outcome. Techniques such as stratification at the time of randomisation and statistical modelling are necessary to refine treatment and placebo groups for the purposes of comparison. Heterogeneity in patient management is an additional complexity, which could be minimised by adherence to guidelines. Centres where guidelines are not followed may have the greatest burden of secondary insults. Exclusion of these centres from "neuroprotection trials" may result in loss of the population of patients most likely to benefit from intervention. Not only is the brain injury population variable, so is the biological process that follows. The relevance of any particular pathophysiological or biochemical process that follows brain injury is uncertain. Hence, administration of a Glutamate antagonist, which may be of benefit in focal ischaemia predominated by excitotoxicity and intermittent depolarisation, is unlikely to benefit a population of patients with diffuse cerebral hypoxic insults. It may be that some of the failure to show clinical benefit of "neuroprotective agents " resulted from failure to meet the required criteria:

- The agent has been shown to have an effect on the relevant pathophysiological process in brain injury models
- The process is known to occur in human brain injury
- The agent can be safely administered in a regimen that is appropriate therapeutically in terms of dosage, brain penetration and time scales.

It may be that different agents are beneficial at different stages of the injury process or that multiple agents are required at a given time (a "cocktail") to minimise the multiple processes that are ongoing. A further difficulty that these trials have encountered relates to "end points" and quantification of effect. As many as 40% of patients with severe head

injury die and approximately 40% recover to independence. Hence the remainder are left in a dependent state in the "U-bend" of the outcome distribution. Given that independence is the goal, not survival in dependency, improving outcome by 10% requires "shifting" large proportions in each category up by two categories. Therefore a target of 10% improvement in favourable outcome may be unrealistic. It can be argued that the failure to show any adverse effect of the various agents supports some effect, not statistically significant at the chosen threshold that may be of benefit to the greater population of head injured patients. Furthermore, some trials were abandoned due to "futility analysis" and because of the experience of parallel trials in stroke patients. This is unfortunate given the profound differences in biological process, patient population and management of stroke and severe head injury. It is now recognised that increased focus is required upon the phase of translation from experimental brain injury to human brain injury. (Maas et al. 1999; Teasdale et al. 1999) Thus the requirement for *in-vivo* clinical research, despite the scientific challenges involved, is greater if progress in treatment of acute brain injury is to be achieved.

1.3 Apolipoprotein E

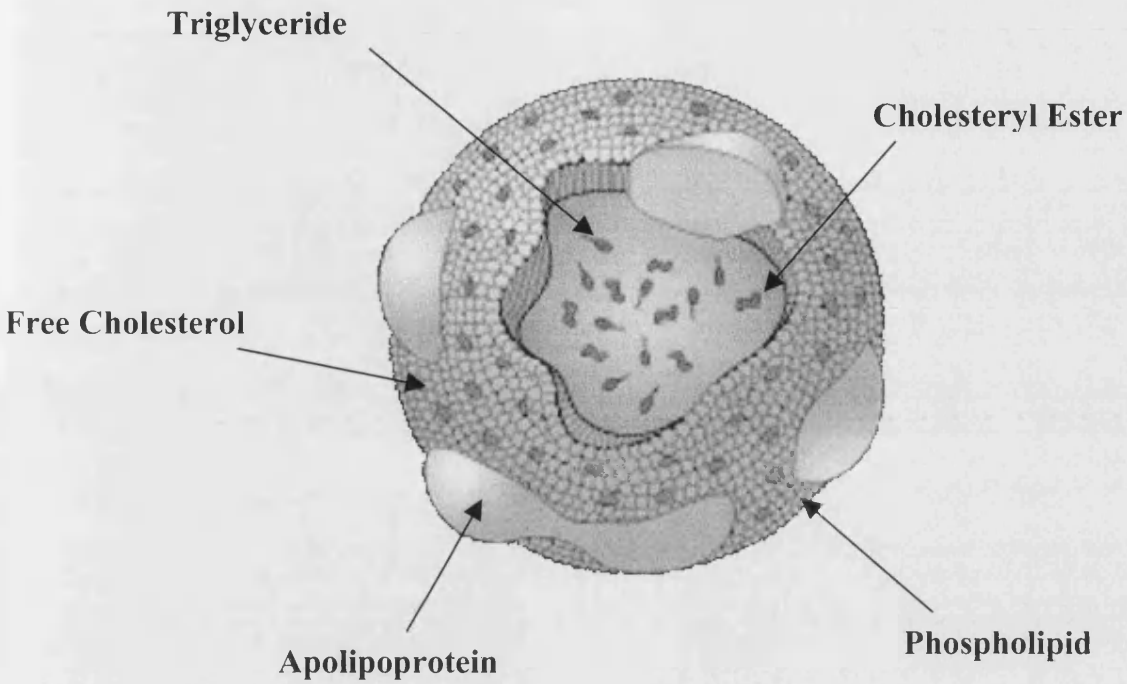
Although first described nearly thirty years ago, current understanding of the molecular and cellular biology of this 34 Kilo-Dalton (KDa) glycoprotein is far from complete. (Shore and Shore, 1973) While the relevance of apoE to clinical disorders such as atherosclerosis is now well established, a substantial body of evidence supporting a role for apoE in central nervous system (CNS) disease is accumulating. (Mahley and Huang, 1999) In relation to atherosclerosis, molecular genetic studies have established three key functions for apoE that are atheroprotectant. First, plasma apoE maintains overall plasma cholesterol homeostasis through efficient hepatic uptake of cholesterol containing lipoprotein remnants, mediated by apoE functioning as a ligand for the low density lipoprotein (LDL) family of receptors. Second, apoE (working in concert with the cholesterol esterification enzyme activator, apoAI) facilitates cellular cholesterol efflux from macrophage foam cells in the blood vessel intima. Third, apoE, by modulating both T-lymphocyte and macrophage cytokine production, influences the chronic inflammatory response integral to atherosclerosis. (Curtiss and Boisvert, 2000) However, the complexity and relevance of apoE to human disease is not limited to its range of biological functions, since in humans the gene coding for the apoE protein is polymorphic. Point mutations, at codons 112 and 158, in the *APOE* gene (on chromosome 19) result in amino acid substitutions in the protein, altering the tertiary structure. It is postulated that this natural,

genetically based, variation in the apoE protein tertiary structure underlies the risk stratification for ischaemic Heart disease (IHD), Alzheimer's Disease (AD) and recovery from TBI, and SAH. Although rare *APOE* alleles (ε) have been described in humans, three (ε2, ε3, ε4) predominate. (Kamboh et al. 1999) The *APOE* ε3 allele has the highest population frequency of approximately 77% while *APOE* ε4 and *APOE* ε2 comprise about 15% and 8% respectively with significant ethnic variations in the allele distribution around the world. (Mahley and Rall, 1999) In contrast to humans, other species, including great apes, do not display this genetic polymorphism; their gene product has sequence homology resembling apoE4 with arginine at the position corresponding to residue 112 in human apoE. (Finch and Sapolsky, 1999; Weisgraber, 1994) The two polymorphic sites in human *APOE* are C_pG sequences, which are regarded as mutation "hot spots". Thus, the methylation of C in the C_pG sequences, and their ready deamination to T, make a C to T mutation more likely than T to C, which would be necessary to obtain *APOE* ε4 from *APOE* ε3. (Hanlon and Rubinsztein, 1995) Although the *APOE* ε4 allele is less common than *APOE* ε3, it is probably the ancestral/wild type allele with *APOE* ε3 resulting from one mutational event, and *APOE* ε2 resulting from two such events. These specific point mutations enable the determination of *APOE* genotype using Restriction Fragment Length polymorphism (RFLP). Alternatively, the apoE protein phenotypes may be identified by iso-electric focussing (IEF). Functional consequences result from differences in intra-protein amino acid-amino acid interactions dictating isoform dependent lipoprotein preference and receptor binding. The amino two thirds of apoE (residues 1-191) contains both the Heparin Sulfate Proteoglycan (HSPG) binding site and the receptor binding region, of which the side chain of Arginine 150 forms a component. The carboxy terminal domain contains the major lipid-binding site. ApoE3, which has arginine at residue 158, forms a salt bridge with aspartic acid 154. This is not possible in apoE2, which has a cysteine at residue 158, instead a salt bridge forms between aspartic acid 154 and the receptor binding residue arginine 150 reducing the affinity of apoE2 for the LDL receptor. In contrast to apoE isoforms 2 and 3, which have cysteine at residue 112, apoE4 has arginine here which forms a salt bridge with glutamic acid 109 re-orientating the side chain of arginine 61 such that it interacts with glutamic acid 255 in the carboxyl terminus. This determines the preference of apoE4 for very low density lipoprotein (VLDL), while apoE3 and apoE2 prefer smaller, phospholipid rich high density lipoprotein (HDL). This might also account for differential association with the high capacity, low affinity cell surface HSPGs in the HSPG-LRP pathway. (Mahley, 1988; Mahley and Huang, 1999; Mahley et al. 1996)

1.3.1 Apolipoprotein E and the central nervous system

In contrast to the periphery where a variety of apolipoproteins are found, there are relatively few apolipoproteins in the CNS. Though mRNA has not been detected for apoAI, apoAII, apoAIV, apoB, apoCII, apoCIII, apoF and apoH in the brain, apoE mRNA expression in the brain is second only to the liver. (Elshourbagy et al. 1985) CNS apoE is synthesised entirely intrathecally since CSF apoE isoform type is unaltered by liver transplantation from a donor of different genotype. (Linton et al. 1991) ApoE, which does not cross the intact "blood brain barrier", is synthesised by astrocytes and microglia; neurons probably do not synthesise apoE under normal circumstances. (Boyles et al. 1985; Diedrich et al. 1991; Elshourbagy et al. 1985; Poirier et al. 1991; Stone et al. 1997; Zlokovic, 1996) However, neurons do have the capacity to internalise apoE-containing lipoproteins and apoE has been localised within neurons of apparently normal brains, and in response to acute and chronic injury. (Beffert et al. 1998; Han et al. 1994; Horsburgh et al. 1997; Horsburgh et al. 2000b; Horsburgh and Nicoll, 1996; Metzger et al. 1996; Schmechel et al. 1993) Using immuno-electron microscopy on human brain sections from young epileptic patients, apoE is reported to localise to the cytoplasm of cell bodies and proximal dendrites in association with the external membrane surface of some organelles supporting the concept of intracellular apoE trafficking. (Han et al. 1994; Nicoll et al. 2001) Thus apoE appears to play a principal role in the CNS in the transport of cholesterol and lipid from glial cells to the neuronal cytoplasm. The vehicle in which the lipid and cholesterol are transported is the apoE lipoprotein particle (LpE).

Figure 1 Lipoprotein Particles



Five classes of lipoprotein particle are described in plasma. Chylomicrons have the lowest density (d): < 0.95 g/mL and a mean particle diameter of 500 nm. They are rich (85%) in triglyceride, but have the lowest (2%) apoprotein (CI, CII, CIII, B-48, E, AI, and AII) content. Very low density lipoproteins (VLDL) have smaller diameter (43 nm) and d < 1.006 g/mL. They have less triglyceride (50%), more cholesterol (7%), cholesteryl ester (13%) and phospholipid (20%) and the same apoproteins as chylomicrons. Intermediate density lipoproteins (IDL) arise from the catabolism of chylomicrons and VLDL. IDL density = 1.006 - 1.019 g/mL, and mean diameter = 27 nm. They contain a higher proportion (18%) of apoprotein (apoB-100 and apoE), and lipid, but less triglyceride (26%). Low density lipoproteins (LDL) arise from the catabolism of VLDL and comprise three subfractions (LDL I, II, III). Apoprotein (apoB-100 and apoE) represents 25%, triglyceride 10% and cholesteryl ester 37% of the composition. LDL I has a diameter of 27 nm (d = 1.02 - 1.03 g/mL), LDL II = 26.6 nm (d = 1.03 - 1.04 g/mL) and LDL III = 26 nm (d = 1.04 - 1.06 g/mL). High density lipoproteins (HDL) occur as three main subfractions (HDL_1, HDL_2, and HDL_3); HDL_1 is rarely seen in human plasma. HDL_2 has a mean particle diameter of 9.5 nm and density range of 1.063 - 1.125 g/mL. HDL_3 has a mean particle diameter of 6.5 nm and density range of 1.125 - 1.210 g/mL. HDL has the highest (55%) apoprotein (AI, AII, D, CII, CIII, and E) and phospholipid (24%) content and the lowest (4%) triglyceride. See also figures 21 and 22.

1.3.1.1 CSF lipoprotein particles

As in plasma, apoE forms a key component of CSF lipid transport vesicles known as lipoprotein particles (Lps), which facilitate the solubilisation of lipids within an aqueous environment, and their transport to appropriate metabolic pathways. In plasma, these vesicles have a nonpolar core containing triglyceride and cholesteryl esters, surrounded by a monolayer of phospholipid (see figure 1). The polar head groups of the phospholipids are orientated toward the aqueous environment stabilising the lipoprotein particle allowing them to remain in solution. The surface monolayer contains unesterified cholesterol and the apoproteins, which function as ligands for cell surface receptors and regulators of enzymes involved in particle remodelling. (Mahley et al. 1984) Phospholipid is the most abundant lipid in CSF lipoproteins followed by cholesterol and cholesteryl esters. The CNS accounts for 2% of body mass and 25% of total body free cholesterol. The cholesterol content of brain tissue is high making up 1% of grey matter (wet weight) and 4% of white matter. The CNS cholesterol pool is non-exchangeable with the plasma and its interchangeable pools. Evidence from experimental cholesterol turnover studies suggests that brain cholesterol demand for development and day to day maintenance is met entirely by intrathecal cholesterol synthesis. (Dietschy and Turley, 2001) Though the CNS apoE lipoprotein particle most likely plays a key role in regulating cholesterol homeostasis, as it does in plasma, differences between the plasma and CSF particles have been identified and have been an area of focus for those researching neuronal degeneration. (Montine et al. 1997) In contrast to plasma where five different lipoprotein classes exist, Lps in the CSF are more homogeneous having a size similar to plasma HDL. At least two subclasses of HDL- like CSF Lps have been identified which appear to be either relatively rich apoAI or apoE. CSF HDL contrasts plasma HDL in that the larger apoE rich HDL_1- like particle found in CSF are a very minor component of plasma HDL where smaller HDL_2 and HDL_3 particles predominate. There is very little HDL_1 in human plasma as cholesteryl ester is transferred via cholesteryl ester transferase protein (CETP) to the larger apoB containing lipoprotein, LDL. Normal CSF does not contain apoB so apoE lipoproteins play a special role in cholesterol transport by virtue of their ability to expand to HDL_1. Other smaller Lps have been identified in the CSF but are yet to be fully characterised. In contrast to plasma, the concentration of apoE in CSF is reported not to vary according to genotype. (Rebeck et al. 1998; Schiele et al. 2000a; Vincent-Viry et al. 1998) ApoE phenotype has been reported to affect the distribution profile of apoE containing lipoproteins in the CSF suggesting that metabolism of apoE containing lipoproteins depends on the apoE isoform present. ApoE2 and apoE3 (but not apoE4) can form heterodimers with apoAII, which have greatly diminished LDL receptor binding compared to apoE. Therefore apoE4 Lps,

which do not form apo (E-AII) and apo (AII-E-AII) complexes, may be metabolised more efficiently than apoE3 and apoE2 containing lipoproteins. CSF associated with apoE4 is reported to have a lower cholesterol concentration (contrasting the situation in plasma) than CSF associated with apoE3 and apoE2. (Borghini et al. 1995; Borghini et al. 1993; Guyton et al. 1998; Montine et al. 1998; Montine et al. 1997; Pitas et al. 1987) However, others have found no significant differences between CSF Lps with respect to the different apoE isoforms. (Fagan et al. 2000; Koch et al. 2001) CSF Lps may not be representative of the population of Lps present in the neuropil. Lps isolated from serum free rat astrocyte culture media (ACM) contain only apoE and apoJ in contrast to human CSF Lps, which contain apoE and apoA. Rat astrocyte Lps are discs that contain less cholesteryl and have the potential to accumulate cholesterol ester via lecithin, cholesterol acyltransferase (LCAT) and CETP and remodel to cholesterol ester laden CSF spheres. Thus these particles may represent a population of particles participating in the recycling of cholesterol within the brain parenchyma. (Albers et al. 1992; Smith et al. 1990) ApoE appears to be important to the regulation of cholesterol cycling as *APOE* -/- (knockout) mice serum free ACM contains little or no free cholesterol or phospholipid and lipoprotein particles were not detectable under the electron microscope. (Fagan et al. 1999)

1.3.2 Apolipoprotein E and neuronal plasticity

In contrast to the CNS, peripheral nerves have the capacity to regenerate. Following rat sciatic nerve crush injury, macrophage apoE secretion increases one hundred fold, reaching a peak one week after injury and normalises within a few months when regeneration is largely complete. The secreted apoE facilitates the recycling of cholesterol from the degenerating neuron, which may then be used by the growth cones of neurites via upregulated LDL receptors at their tips. (Goodrum, 1990; Goodrum, 1991; Goodrum et al. 2000a; Goodrum et al. 2000b; Ignatius et al. 1987a; Ignatius et al. 1987b) However, when *APOE* -/- (knockout) mice undergo peripheral nerve injury, there is no apparent reduction in regeneration suggesting that in the mouse peripheral nervous system, lipoproteins other than apoE (e.g. apoJ) may be utilised for the same purpose. (Goodrum et al. 1995; Popko et al. 1993) The CNS of APOE knockout mice are reported to have synapto-dendritic structural abnormalities, measured using Microtubule-Associated Protein (MAP-2) and synaptophysin immunoreactivity, in hippocampal and cortical areas, which increase with age. Amelioration of these changes by intraventricular infusion of exogenous apoE or endogenous expression of human apoE3 (but not human apoE4) suggests there is a degree of synaptic plasticity that is apoE dependent. (Masliah et al. 1996; Masliah et al. 1997) If these apoE knockout mice experience hippocampal injury, then clearance of lipid-

laden products of neurodegeneration is impaired supporting the concept that apoE plays a role in lipid and cholesterol recycling. (Fagan et al. 1998; White et al. 2001a) A number of *in- vitro* studies utilising peripheral and central nervous system cell cultures, and more recently organotypic hippocampal cultures from human *APOE* transgenic mice, have shown that apoE3 promotes neurite extension to a greater extent than apoE4. The effect requires the apoE to be associated with a lipoprotein transport vehicle directing it to the HSPG-LRP pathway. The mechanism by which apoE promotes neurite extension and the reason for greater effect with apoE3 has not been conclusively elucidated. (Bellosta et al. 1995; DeMattos et al. 1998; Fagan et al. 1996; Holtzman et al. 1995; Nathan et al. 1994; Nathan et al. 1995; Sun et al. 1998) In vitro studies support a role for apoE in the stabilisation and remodelling of the neuronal cytoskeleton. Binding of apoE3 to the microtubule associated protein Tau stabilises the microtubules and prohibits hyperphosphorylation. It has been postulated that deficient apoE4- Tau interactions result in Tau self association and hyperphosphorylation, laying down the foundations for Paired Helical Filament (PHF) formation and thereafter neurofibrillary tangles (NFT). If cultured Neuro-2a cells are treated with neurite outgrowth inhibiting lipidated apoE4 there are fewer microtubules, and a lower ratio of polymerised to monomeric tubulin, compared to cultures treated with neurite outgrowth promoting lipidated apoE3 treated cells. (Nathan et al. 1995) There is additional evidence to support a role for apoE in cytoskeletal remodelling from the *APOE* transgenic and knockout mice. In addition to cytoskeletal abnormalities, *APOE* knockout mice have increased phosphorylated Tau immunoreactivity compared to wild-type mice. (Genis et al. 1995; Masliah et al. 1995) The transgenic mice expressing human *APOE*ε4 have more cytoskeletal abnormalities than those expressing *APOE*ε3. (Buttini et al. 1999) However, the relevance of these cytoskeletal-remodelling observations to human pathology such as Alzheimer's Disease (AD) is uncertain given the failure to demonstrate apoE in the neuronal cytosol, and the lack of consistent evidence supporting an association between *APOE* genotype and NFT burden in AD brain. (Beffert et al. 1998; DeMattos et al. 1999)

1.3.3 Apolipoprotein E and amyloid β protein

The function of the transmembrane protein beta-amyloid precursor protein (β-APP) is not known. However, as a part of normal cellular processing, secretase enzymes cleave specific regions of the protein generating peptides, known as amyloid beta-protein (Aβ), of varying length, e.g. $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$. For unknown reasons these hydrophobic peptides form a major component of AD plaques. Although the 42 amino-acid peptide ($A\beta_{1\text{-}42}$)

constitutes the minority (10%) of Aβ, it is the most hydrophobic species, and forms the *seed* of the amyloid plaque. The Aβ peptides are known to be chaperoned by apoE (and apoJ) containing HDL-like lipoprotein particles in the CNS, and the proportion of $A\beta_{1-40}$ appears to be influenced by *APOE* genotype. Thus CSF Lps isolated from *APOE* ε4 subjects have significant elevation of the $A\beta_{1-40}$ ratio than CSF Lps from non-*APOE* ε4 subjects. (Biere et al. 1996; Fagan et al. 2000; Koudinov et al. 1996; LaDu et al. 1995; Tokuda et al. 2000; Wisniewski et al. 1993) There is therefore evidence to support direct interactions between apoE and Aβ, which results in the formation of insoluble aggregates. However, human *in-vivo* evidence for such an interaction is sparse. Microglia scavenge Aβ-apoE aggregates via scavenger and lipoprotein receptors and deliver it to the lysosomal pathway for degradation. Microglia have the capacity to secrete apoE which may then capture Aβ for internalisation by these receptors (secretion capture), possibly in conjunction with HSPGs. (Cole and Ard, 2000) Interest in Aβ degradation has increased following reports that Aβ immunization not only reduces Aβ deposit formation, but also established Aβ deposits. (Schenk et al. 1999) Although it is not clear whether apoE directly regulates Aβ degradation or indirectly regulates degradation by promoting aggregation and deposition, evidence from human post-mortem studies and work with transgenic mice support a role for apoE controlling the ultimate fate of Aβ. (Bales et al. 1999; Bales et al. 1997) *In vitro* studies provide supporting evidence for both the chaperone role and an effect of apoE directly promoting assembly of Aβ into amyloid fibrils, though the effects appear to be dependent on the lipidation and oxidation status of the apoE. (Sanan et al. 1994; Wisniewski et al. 1994) *In vitro* apoE4 is more amyloidogenic than apoE3 and apoE2, which is consistent with reports that *APP* TG mice expressing human *APOE4* exhibit accelerated Aβ deposition. (Holtzman et al. 2000a; Holtzman et al. 2000b; Ma et al. 1994) In addition, apoE may indirectly influence APP processing via the regulation of cellular cholesterol, which is reported to regulate the activity of the intra-membranous secretase APP cleavage enzyme. (Dodart et al. 2002b; Simons et al. 1998) The concept that cholesterol reduction may decrease Aβ production has received recent attention due to the observation that cholesterol-lowering agents may reduce the risk of Alzheimer's disease. (Jick et al. 2000; Wolozin et al. 2000)

1.3.4 Apolipoprotein E and oxidative stress

Given that apoE2 possesses two cysteine residues, at positions 112 and 158, apoE3 has one at position 112 while both these positions are arginine in apoE4, it has been suggested that differences in free radical scavenger ability exist between isoforms. Differences in

oxidative stress have been identified in TG mice but the relevance to human brain injury is unknown. The *APOE* -/- transgenic (TG) mice are reported to be in a state of chronic oxidative stress resulting with increased protein nitration in the cortex, hippocampus, cerebellum and brainstem, indicating formation of the neurotoxic free radical, peroxynitrite. (Lomnitski et al. 1999; Matthews and Beal, 1996) *APOE* knockout and *APOE4* transgenic mice are reported to be more susceptible to closed head injury and some of this effect is reported to be attributable to reduced anti-oxidant activity. (Chen et al. 1997; Lomnitski et al. 1997; Sabo et al. 2000) Furthermore, *APOE* -/- knockout and wild-type (WT) mice, when subjected to global cerebral ischaemia, show reduced neuronal damage and lipid peroxidation if apoE is infused into the ventricle supporting a role for apoE as an antioxidant. (Horsburgh et al. 2000a; Kitagawa et al. 2002)

1.3.5 Apolipoprotein E and Alzheimer's Disease

Alzheimer's disease (AD) accounts for 70% of late onset dementia and 100,000 deaths per year in the USA. Clinical features include personality change, language and visuo-spatial problems, memory loss and global cognitive decline. The neurological deficit results from selective neuronal dropout and synaptic loss. (DeKosky and Scheff, 1990; DeKosky et al. 1996) Currently, the clinical diagnosis of probable AD can only be confirmed post-mortem, by the presence of abundant neurofibrillary tangles (NFTs) and β-Amyloid (Aβ) plaques distributed in characteristic and specific brain regions, cells types and laminae. Though the pathology of AD is characterised by the accumulation of these insoluble aggregates, and despite compelling evidence supporting their role in the pathogenesis of AD, neither has been universally accepted as the primary aetiological factor. NFTs are the insoluble intracellular structures consisting of abnormal cytoskeletal elements, called paired helical filaments (PHFs), the major component of which consists of abnormally phosphorylated microtubule associated Tau protein. (Goedert, 1993) Hyper-phosphorylation of Tau results in reduced flexibility disrupting axonal transport and microtubule binding, resulting in intra-neuronal accumulation. NFTs are not diagnostic of AD since they are seen in other neurodegenerative disorders (Tauopathies) such as progressive supranuclear palsy, cortico-basal degeneration, Pick`s disease, Parkinson-dementia complex of Guam, post-encephalitic Parkinsonism and the chronic TBI disorder, dementia pugilistica. (Goedert, 1999) Tangle formation appears to represent a stereotypical cellular reaction to a range of pathological initiators. (Wisniewski et al. 1979) In addition to NFTs, two other neurofibrillary structures are recognised which, despite morphological differences, share Tau epitopes and occur in the same neuronal pool. These are neuropil threads, which are the distal dendrites of tangle containing neurons, and dystrophic

neurites, surrounding senile plaques and neuropil threads. (Braak and Braak, 1988) The second major manifestation of AD is the senile or neuritic plaques comprising extracellular lesions containing a dense central core of β-amyloid protein, surrounded by dystrophic neurites, reactive astrocytes and microglia. The β-amyloid protein is a hydrophobic fragment of the transmembrane glycoprotein Amyloid Precursor Protein (APP), which although its` function is uncertain, appears to be up regulated by a wide range of pathologies causing axonal injury. (Graham et al. 1995)

1.3.5.1 Apolipoprotein E and AD-epidemiological evidence

Although several genetic loci, including APP, Presenilin-1 (*PS*-1) and *PS*-2 contribute to Familial Alzheimer's disease (FAD), the commonest known genetic susceptibility factor for the population at large is *APOE*. (Adroer et al. 1995; Basun et al. 1995; Corder et al. 1993; Frisoni et al. 1995; Goate et al. 1991; Levy-Lahad et al. 1995; Lucotte et al. 1993; Mayeux et al. 1993b; Okuizumi et al. 1994; Payami et al. 1994; Saunders et al. 1993a; Sherrington et al. 1995; St et al. 1995; Strittmatter et al. 1993). Estimates of attributable risk as high as 50% have been reported for the development of AD based on *APOE* genotype. (Corder et al. 1993) In both sporadic and familial late-onset AD, the prevalence of the ε4 allele is increased from approximately 15% to 40%. (Poirier et al. 1993; Saunders et al. 1993b) Approximately 80% of familial and 64% of sporadic late-onset AD cases carry at least one copy of the *APOE* ε4 allele compared to 30% of controls. Possession of *APOE* ε4 reduces the age of onset of AD and thus the frequency of this allele is low in the very elderly. Thus *APOE* ε4 homozygotes represent 40% of AD patients at 55 years of age but less than 5% over 91 years of age. (Corder et al. 1993; Rebeck et al. 1994) In contrast *APOE* ε2 is protective in that it delays the onset and reduces the risk of AD. (Corder et al. 1994; Talbot et al. 1994; West et al. 1994) For patients with FAD, age of onset appears to be influenced by *APOE* ε4 in patients with *APP* mutations but not *PS*-1 mutations. (St George-Hyslop et al. 1994; Van et al. 1994) However, the *APOE* ε4 allele does not appear to influence the rate of disease progression once started. (Beffert et al. 1998) A meta-analysis of 5930 AD patients for *APOE* genotype AD associations, found *APOE* ε4 to be a risk factor for AD across a wide range of ethnically different groups, but that though women were more likely to develop AD, this was not attributable to differences in *APOE* genotype frequency. (Farrer et al. 1997)

1.3.6 Apolipoprotein E and Alzheimer's Disease pathology

After Strittmatter reported the overrepresentation of the *APOE* ε4 allele among AD patients, several studies examined the relationship between possession of the *APOE* ε4 allele and markers of AD pathology such as amyloid containing senile plaques (SP) and intra-neuronal neurofibrillary tangles (NFT). (Beffert et al. 1999; Beffert and Poirier, 1996; Berg et al. 1998; Chen et al. 1999; Gearing et al. 1996; Gomez-Isla et al. 1996; Marz et al. 1996; McNamara et al. 1998; Nagy et al. 1995; Ohm et al. 1995; Polvikoski et al. 1995; Schmechel et al. 1993; Strittmatter et al. 1993) Senile plaque density is reported to correlate with *APOE* ε4 in a dose dependent manner while *APOE* ε2 is reported to be associated with less amyloid deposition. (Lippa et al. 1997) The association between *APOE* ε4 and amyloid deposition has not been reported unanimously though some of the inconsistency may be attributable to different methods for labelling amyloid deposits as immunostaining with Aβ identifies the correlation between *APOE* ε4 and Aβ better than silver stains. (Berg et al. 1998; Chen et al. 1999) Furthermore, few studies discriminate between $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ plaque, the former of which increase with *APOE* ε4. (Gearing et al. 1996) Although the presence of abundant amyloid plaques is a prerequisite for the pathological diagnosis of AD, they may be present in cognitively normal individuals and it is the presence of intracellular neurofibrillary lesions that correlate best with the presence of dementia. (Arriagada et al. 1992a; Arriagada et al. 1992b). However, there is even less correlation between NFTs, which are generally regarded as correlating with AD severity better than SPs, and possession of *APOE* ε4 and NFTs. (Beffert et al. 1998) Thus, there appears to be some evidence in humans that *APOE* ε4 is relatively more amyloidogenic and may influence formation of tangles, yet some continue to question this and the relevance to the aetiology of AD.

1.3.6.1 Apolipoprotein E and Aβ deposition in transgenic mouse models of AD

Prior to the creation of transgenic (TG) mice, recreating AD pathology in animal models of AD has been unsuccessful. These TG mice overexpress a mutant form of the human amyloid precursor protein (APP) gene known to cause FAD (APP_{V717P}) and develop age related and region specific Aβ immunoreactivity, fibrillar amyloid deposition, glial activation and neuritic dystrophy similar to that observed in AD. (Games et al. 1995) Crossing these *APP* mutant mice with *APOE* -/- knockout mice results in reduced Aβ immunoreactivity, compared to age matched littermates expressing wild-type mouse apoE,

providing in vivo evidence for mouse apoE promoting amyloid deposition. (Bales et al. 1999) When APP_{V717F} transgenic mice are crossed with *APOE* transgenic mice expressing either human *APOE*3 or *APOE4*, there is almost no detectable Aβ immunoreactivity unless 15 month old mice are used, which develop fibrillar Aβ deposits associated with greater neuritic plaque density in *APOE* ε4 crosses compared to *APOE* ε3. (Holtzman et al. 2000a; Holtzman et al. 2000b)

1.3.7 Apolipoprotein E and Traumatic Brain Injury

1.3.7.1 Pathological studies

The brains of approximately 30% of patients who die after TBI have deposits of Aβ in the cerebral cortex; patients younger than 60 years of age only have Aβ if they die after TBI. The *APOE* ε4 allele frequency in Aβ positive cases is significantly higher than in the Aβ negative cases. There is also an allele dosage effect in that 10% of patients without *APOE* ε4 had Aβ compared to 35% with one copy and 100% with two copies of the ε4 allele. One interpretation of this association is that TBI is an acute trigger for deposition of Aβ and this occurs predominantly in patients with the *APOE* ε4 allele. (Nicoll et al. 1995) Alternatively, age related Aβ deposition may occur in individuals with the *APOE* ε4 allele, and patients with the *APOE* ε4 allele are more likely to die after TBI. (Roses and Saunders, 1995) Some support for the argument of increased mortality in TBI patients possessing the *APOE* ε4 allele comes from the higher frequency of this allele in the ninety patients with fatal TBI than in the control population. (Nicoll et al. 1995) Studies of patients who die after hypoglycaemia, status epilepticus, herpes encephalitis and global ischaemia identify selective neuronal injury with increased neuronal apoE immunoreactivity within a few days of injury. (Horsburgh et al. 1999a; Nicoll et al. 2001) It should be noted that these intriguing findings have not been extensively replicated by others, and the possibility exists that findings from the West of Scotland population may not reflect the brain injury population at large. The influence of environmental factors (e.g. diet, ischaemic heart disease, cigarette smoking etc) upon brain apoE and Aβ are not known.

1.3.7.2 Human *APOE* transgenic mice and TBI

The majority of studies using human *APOE* TG or knockout mice models of experimental brain injury focus upon AD pathology and ischaemia. (Chen et al. 1997; Genis et al. 2000; Horsburgh et al. 1999b; Horsburgh et al. 2000a; Horsburgh et al. 2000b;

Laskowitz et al. 2000; Laskowitz et al. 1997; Lomnitski et al. 1999; Lomnitski et al. 1997; Lomnitski et al. 2000; Sheng et al. 1998; Sheng et al. 1999) The absence of association between possession of *APOE* ε4 and poor outcome after human ischaemic stroke questions the relevance of findings from experimental models of ischaemia to human TBI, even though both focal and global ischaemia are major components of TBI. (Graham et al. 1995; McCarron et al. 2000) Nevertheless studies of global ischaemia in *APOE4* TG mice suggest they have greater neuronal vulnerability than *APOE*3 TG mice, suggesting that *APOE4* is less protective than *APOE*3. (Horsburgh et al. 2000a; Horsburgh et al. 2000b)

1.3.7.3 Clinical studies

Following on from the human *APOE* transgenic mice, and human neuropathology studies, prospective observational studies in TBI patients have identified an association between possession of the *APOE* ε4 allele and an unfavourable outcome (dead or severely disabled) six months after TBI. In the Glasgow study, after correcting for age and injury severity, the likelihood of an unfavourable outcome was doubled by possession of the *APOE* ε4 allele. (Friedman et al. 1999; Teasdale et al. 1997) A separate study of TBI patients completing a rehabilitation program also associated possession of the *APOE* ε4 allele with reduced functional recovery. (Lichtman et al. 2000) Possession of the *APOE* ε4 allele has also been associated with more severe chronic TBI in boxers. High exposure boxers (i.e. those with 12 or more professional bouts) have significantly higher Chronic Brain Injury (CBI) scores compared to low exposure boxers, and boxers with *APOE* ε4 have significantly greater CBI scores than high exposure boxers without *APOE* ε4. Furthermore a gene dosage affect is apparent as boxers with severe impairment have at least one copy of the *APOE* ε4 allele. (Jordan, 2000) Possession of *APOE* ε4 has also been associated with reduced cognitive performance in older football players, and patients who undergo cardiopulmonary bypass for cardiac surgery. (Kutner et al. 2000; Newman et al. 1995; Tardiff et al. 1997) Other studies have identified associations between possession of the *APOE* ε3 allele and more favourable outcome following non-TBI such as SAH, spontaneous intracerebral haemorrhage, cardiopulmonary bypass and resuscitation. (Alberts et al. 1995; Niskakangas et al. 2001; Schiefermeier et al. 2000; Tardiff et al. 1997; Schiefermeier et al. 2000; Schiefermeier et al. 2000)

1.3.8 Traumatic Brain Injury and Alzheimer's Disease

1.3.8.1 Epidemiological evidence supporting TBI as a risk factor for AD

Compared to other risk factors for AD such as age, sex, level of education and family history epidemiological evidence consistently supporting an association with TBI is lacking. This may be partly due to the failure to conduct rigorous long-term prospective studies exploring this specific hypothesis. Conducting these studies presents several challenges to the epidemiologist. First, the diagnosis must be defined using the criteria of the American Psychiatric Association (DSM-III-R) to diagnose dementia, and those of the National Institute of Neurological and Communicative disorders and Stroke/Alzheimer's Disease and Related Disorders Association, to diagnosis probable AD. (Letenneur et al. 2000; McKhann et al. 1984) Second, pathological confirmation of the diagnosis of AD has often not been established. (Mendez et al. 1992). Third, TBI among probable AD cases is often identified on the basis of patient, spouse or sibling reported measures introducing recall bias, or from medical register data which may also be unreliable. Unfortunately there is relatively little high quality information concerning long term outcome years or decades after TBI and though some studies have stratified injury severity according to the presence or absence of loss of consciousness, the degree and duration of unconsciousness is often not described. Thus many case control studies have shown no significant risk for AD after TBI. (Broe et al. 1990; Chandra et al. 1989; Chandra et al. 1987; Ferini-Strambi et al. 1990; Fratiglioni et al. 1993; Li et al. 1992; Mendez et al. 1992; Ryan, 1994) Some studies do report an association between TBI and the development of AD but several of these are of borderline significance (Breteler et al. 1995; French et al. 1985; Graves et al. 1990; Guo et al. 2000; Mayeux et al. 1993a; Mortimer et al. 1985; Mortimer et al. 1991; None, 1994; O'Meara et al. 1997; Rasmusson et al. 1995; Salib and Hillier, 1997; Shalat et al. 1987; van et al. 1992). Few longitudinal cohort studies report increased risk of AD in patients with a history of TBI, though some of these studies have short follow-up intervals. (Katzman et al. 1989; Launer et al. 1999; Mehta et al. 1999; Schofield et al. 1997; Williams et al. 1991) Nemetz reports that TBI reduces the age of onset of AD by 8 years in individuals susceptible to the disease. (Nemetz et al. 1999)

1.3.8.2 Pathological evidence linking TBI to AD

The brains of people dying with AD share some pathological features with those dying after TBI. Plaque-like deposits of Aβ are found in the cerebral cortex of 30% of fatal cases of TBI. Amyloid is derived from the cleavage of APP, a transmembrane protein, by α,β,γ

secretases to form mostly Aβ $_{1-40}$ and Aβ $_{1-42}$ the latter of which is insoluble and deposited as β amyloid. Immunostaining for Aβ $_{1-42}$, the earliest cleavage product species deposited in AD brain, is also the predominant species after fatal head injury. (Gentleman et al. 1993; Gentleman et al. 1997; Gentleman et al. 1993; Gentleman et al. 1995; Graham et al. 1995; Graham et al. 1996; Horsburgh et al. 2000; McKenzie et al. 1994; McKenzie et al. 1996; Roberts et al. 1991; Roberts et al. 1994) However, a study of twenty one long-term survivors of TBI, who died from other causes up to twenty years later, failed to identify more Aβ deposits compared to controls. (Macfarlane et al. 1999) Thus it is possible that deposits of Aβ present after TBI are at a later stage cleared via inflammatory pathways including activated microglia, which are upregulated in the acute phase after TBI. (Griffin et al. 1994) Chronic Traumatic Brain Injury (CTBI) e.g. due to boxing, where in its` most extreme form it is referred to as dementia pugilistica, also shares pathological features with AD. Extensive plaques and NFTs with the same PHF formation as in AD have been observed in the brains of boxers with dementia pugilistica. Repetitive head injury in young adults appears to be associated with neocortical NFT formation around blood vessels particularly penetrating vessels in the depths of the sulci contrasting AD where cytoskeletal pathology does not cluster around blood vessels. Furthermore, repetitive head injury pathology in young adults does not appear to be associated with Aβ deposition, which along with the early involvement of the neocortex would be unusual in AD. The absence of amyloid in repetitive head injury patients contrast dementia pugilistica where amyloid is often but not always noted. (Corsellis, 1989; Dale et al. 1991; Jordan, 2000; Roberts, 1988; Roberts et al. 1990)

1.3.8.3 Animal model evidence linking AD to TBI

Attempts to reproduce these human pathological observations in rodent models of TBI have largely failed though a diffuse increased expression in β-APP is observed. Using a nonimpact head rotation acceleration model to generate DAI in pigs, Aβ, Tau and NF are reported to co-localise to damaged axons throughout the white matter 3-10 days after trauma. A proportion of the pigs with DAI had diffuse Aβ-containing plaque-like profiles in both the grey and white matter, and accumulations of Tau and NF rich inclusions in the neuronal perikarya. (Smith et al. 1999) TG mice expressing excessive levels of heavy chain neurofilament protein (*NFH-LacZ* mice) are more histologically and behaviourally vulnerable to TBI than wild type mice supporting the concept that neurofilament rich intraneuronal inclusions have deleterious effects on neuronal function. (Galvin et al. 2000) When the APP_{717} (PDAPP) TG mice, which overexpress APP ten fold, are subjected to

Controlled Cortical Impact (CCI) both young (4 months of age) and older (2 years of age) APP_{717} TG mice undergo prominent loss of neurons, gliosis and atrophy near the site of CCI. When the cortex of young mice eight months after injury is compared to the contralateral unlesioned cortex, Aβ deposition is reduced in the ipsilateral hippocampus and cingulate cortex and there is hippocampal atrophy. In contrast, hippocampal atrophy and reduced Aβ deposits are not seen in hippocampus or cingulate cortex of sham-injured (PDAPP) APP_{717} TG mice or in any WT mice. The older mice too, four months after injury demonstrate marked regression in ipsilateral hippocampal Aβ plaque burden compared to the contralateral hippocampus. This work supports the possibility that plaque resulting from progressive amyloidosis in AD brain may be reversible. (Nakagawa et al. 1999; Nakagawa et al. 2000) Furthermore, non injured PDAPP mice have impaired memory function compared to non injured wild-type littermates and brain-injured PDAPP mice had more profound memory dysfunction than brain-injured wild-type littermates. (Smith et al. 1998) When young PDAPP transgenic mice are immunized with $A\beta_{1-42}$, amyloid deposition is prevented; astrocytosis is dramatically reduced as is the Aβ-induced inflammatory response. In addition, $A\beta_{1-42}$ immunization appears to arrest the progression of amyloidosis in older PDAPP mice. Thus $A\beta_{1-42}$ immunization (e.g. administered via a nasal spray) appears to increase clearance of amyloid plaques, and is a potential therapeutic or preventative strategy for AD. (Bard et al. 2000; Games et al. 2000; Janus et al. 2000; Schenk et al. 1999; Weiner et al. 2000) To date the clinical trial assessing the safety and efficacy of $A\beta_{1-42}$ immunization in patients with AD has halted due to adverse events underlining the requirement for increased understanding of Aβ biology. If such a treatment strategy proves beneficial in AD then consideration could be given to extending the application to patients with TBI.

1.4 CSF proteins and the response of the brain to injury

All of the currently available research tools used to investigate the *in-vivo* response of the human brain to injury have limitations, and these must be taken into account when interpreting findings from studies in acute brain injury. Imaging type investigations, such as XeCT, PET, MRI, SPECT and MRS, allow *in vivo* studies of metabolic processes and alterations in cerebral blood flow, but require substantial financial investment and are limited to single or a few time points after injury. These techniques have not yet been applied to large-scale studies of human brain injury. Techniques for continuous monitoring of physiological and biochemical parameters are available, but these are restricted to patients with severe brain injury, and for limited time intervals. Although multimodal

monitoring and capture of physiological variables such as intracranial pressure (cerebral perfusion pressure), cerebral oxygen content and pH, and jugular bulb oxygen saturation ($SjV0_2$) enables trend analysis, it is often challenging to correlate these observations with clinical end points such as clinical outcome six months after injury. Microdialysis can be used to continuously monitor biochemical changes after brain injury such as glutamate, lactate, and aspartate but substantial variations are observed depending on the location of the microdialysis membrane in relation to the area of injured brain monitored. Uncertainty regarding the true location of the dialysis membrane in relation to the area targeted for monitoring compound these problems. Analysis of CSF is limited to patients who require CSF access for the purpose of treating acute hydrocephalus, or the monitoring of intraventricular pressure after acute brain injury. Analysis of the constituents of ventricular CSF at best facilitates a global reflection of upstream (parenchymal) events. In contrast to microdialysis CSF analysis allows larger molecules such as structural proteins to be assayed, and is a relatively cheap and simple technique. Through the analysis of CSF proteins in the first instance, CNS proteins have been identified and in some cases their release has correlated with injury severity and outcome. The identification of proteins in the CSF that act as surrogate markers of brain injury may in the future be extrapolated to studies in plasma and serum, which would be applicable to a wider spectrum of brain injured patients, including those with "mild" injury.

1.4.1 Cerebrospinal Fluid

Cerebrospinal Fluid (CSF) is essentially an ultrafiltrate of plasma. In normal CSF eighty percent of proteins are transudated from plasma and the remainder are synthesised by the brain. All known proteins pass from plasma into CSF, but do so in inverse proportion to their molecular size. In addition lipophilicity, hydration, and charge influence transfer to the CSF. The term "barrier" should not be taken literally to mean "impervious" as this is not in fact the case. There are six main "barriers":

- Blood-CSF barrier-choroid plexus accounts for sixty percent of CSF production.
- Blood-brain barrier-the remainder of the brain vasculature accounts for one third of CSF production.
- Blood-dorsal root barrier-representing the vasculature within the dorsal root ganglion, which is more permeable than the rest of the brain vasculature.
- Meninges-(do not normally produce CSF)
- Wandering cells-normal CSF contains a cell ratio of two thirds lymphocytes (blood derived) and one third monocytes (blood derived and from microglia) (blood contains mainly granulocytes which are essentially absent from normal CSF).

- Structural cells-neurons, astrocytes, oligodendrocytes, and microglia.

The adult CSF space is a volume of approximately 140 ml whilst CSF is produced at a rate of approximately 500ml per day, which equates to a four times a day turnover rate. Thus a sample of CSF will reflect brain protein concentration at that particular point in time. Serial sampling shows whether the protein concentration is changing, and over what time interval. Although proteins have been identified in the CSF, which have been described as "brain specific", none are exclusively brain specific. A small number however, have emerged which almost fit this definition and are under evaluation as research tools for the investigation of a variety of neurological disorders. Though none appear to be of sufficient specificity or sensitivity for the purposes of disease diagnosis, some have been correlated with injury severity and outcome, and may reflect cell-specific response to injury. Neuronal markers include "neuron specific enolase" (NSE or Protein 14-3-2), Protein 14-3-3 (γ isoform the most brain specific), Tau (discussed below) and many others. Measurement of these proteins in CSF is limited by the availability of antibodies specific for the "brain specific" isoforms, availability of purified proteins, quality control, reagent cost, and physicochemical properties of the analyte (e.g. NSE is unstable). CSF protein markers for astrocytic cells include Glial Fibrillary Acidic Protein (GFAP), and S100B (discussed below), both of which are amenable to assay. CSF Ferritin measurement reflects microglial activity in response to brain injury. A CSF marker protein for Oligodendrocytes is myelin basic protein. (Thompson and Keir, 1990)

1.4.2 S100 protein

S100 belongs to a family of low molecular weight acidic calcium binding proteins, which are found in much higher concentrations in the CNS then in any other tissues. (Donato, 1991; Hidaka et al. 1983; Kindblom et al. 1984) S100 proteins contain no carbohydrate, lipid, nucleic acid or phosphate and exist as homodimers or heterodimers of two subunits α and β, which have molecular weights of 10.5 and 10.4 KDa respectively. (Fano et al. 1995) These subunits contain hydrophobic regions in both the C and N termini and a Ca^{2+} binding site which when occupied by Ca^{2+} causes conformational changes in the protein with the exposure of some aromatic amino acids, hydrophobic residues and two sulphydryl groups. (Baudier and Cole, 1988b; Heizmann and Hunziker, 1991) The subunits share 45% sequence homology, but have been shown by complement fixation and cross-immunofixation to be antigenically different. The subunits are the product of two separate genes, the locus of the α subunit being1q21 and that for the β subunit being 21q22.2-21q22.3. The classical S100 proteins are S100B ($\beta\beta$), S-100ao ($\alpha\alpha$) and S-100a ($\alpha\beta$).

S100B is found in Schwann cells and within the astrocytes (90% cytosolic, 10% membrane bound) where it reaches the concentration of 10μM. Approximately 90% of the total S100B pool is found within the brain, with the remainder being located in non-neuronal tissues such as the testes, melanocytes and T-lymphocytes. (Fano et al. 1995; Hidaka et al. 1983; Kindblom et al. 1984) S100ao is found in neuronal cells, particularly the hippocampal neurones, of the central nervous system in much lower concentrations than S100B. Outside the CNS, S-100ao is found in high concentrations in cardiac and skeletal muscle, and within the kidneys. S-100a is found within astrocytes but not Schwann cells. (Kato and Kimura, 1985; Takashi et al. 1988) Thus it is the S-100β subunit which is regarded as a protein marker for the astrocytic cells of the CNS.

In-vitro studies suggest both intracellular and extracellular functions for S100. Intracellular S100B appears to modulate the activity of target proteins through calcium-dependent interactions effecting cell-signalling systems, cell metabolism and cell structure. For example, in the presence of GTP, S100B can stimulate the activity of the cerebral cortex membrane adenylate cyclase system, and inhibits membrane bound phospholipase-C thus influencing membrane-induced transduction. (Fano et al. 1995; Zimmer et al. 1995) In-vitro, S100B binds fructose-1, 6-bisphosphate aldolase suggesting a role in the regulation of astrocytic energy production via the glycolytic pathway. (Zimmer and van, 1986) S100B may influence cell morphology through interactions with protein like Tau preventing phosphorylation, and GFAP preventing polymerisation. (Baudier and Cole, 1988a; Bianchi et al. 1993) Both interleukin-1 (IL-1) and cAMP are reported to stimulate astrocytic S100B expression. (Kahn et al. 1991) The extracellular effects of S100B are to stimulate astrocyte proliferation and hypertrophy, promote neuritic growth, increase neuronal survival, and elevate neurone and glial intracellular calcium concentrations. (Selinfreund et al. 1991; Whitaker-Azmitia et al. 1990) S100B has also been reported to cause apoptosis. (Fano et al. 1993) The effect of S100B on cell proliferation appears to be dose dependent with cells increasing in number at lower doses of S100B but when the concentration is increased the effect is reversed, resulting in cell death. S100B has been shown to stimulate inducible nitric oxide synthase (iNOS) in rat cortical astrocytes, and to cause the death of neurones co-cultured with astrocytes by the production of nitric oxide. (Hu et al. 1996; Hu et al. 1997)

1.4.2.1 Utility of CSF measurement

Raised concentrations of S100B have been reported to occur in the CSF of patients with a wide variety of neurological disorders including strokes, encephalitis, meningitis, Creutzfeld-Jakob disease, brain tumours, Guillain-Barré syndrome, epilepsy, Parkinson's disease and dementia. (Jimi et al. 1992; Mokuno et al. 1983; Noppe et al. 1986; Otto et al. 1997; Persson et al. 1987; Sindic et al. 1982) Elevated concentrations of CSF S100B have been found in the acute phase of Multiple Sclerosis (MS) but only marginally increased concentrations in the non-acute phase of the disease. (Massaro et al. 1985; Michetti et al. 1980; Mokuno et al. 1983; Noppe et al. 1986; Sindic et al. 1982) The raised concentration of S100B in the CSF may result from cell damage, or be due to an active response from the brain to the injury. (Jongen et al. 1997; Mokuno et al. 1983). Raised concentrations of S100B have been reported in the CSF of patients with large cerebral infarcts, but not in smaller infarcts or transient ischaemic attacks. Thus, S100B has emerged as a surrogate marker for injury severity. In addition, the concentrations of S100B in the CSF within twenty-four hours of SAH has been correlated with clinical outcome assessed using the GOS. (Persson et al. 1987) The utility of measuring S100B in CSF is limited by the invasive means required to obtain samples. A number of studies report elevated S100B concentrations in plasma or serum after stroke, cardiac arrest, cardio-pulmonary bypass, TBI and SAH, corroborating the findings in CSF. (Abraha et al. 1997; Blomquist et al. 1997; Buttner et al. 1997; Ingebrigtsen and Romner, 1996; Missler et al. 1997; Rosen et al. 1998; Taggart et al. 1997; Waterloo et al. 1997; Westaby et al. 1996; Wiesmann et al. 1997) Thus plasma S100B measurement as a surrogate marker for acute brain injury is emerging as a useful research tool to be used as an adjunct to injury severity classification.

1.4.3 Tau protein

Tau protein is a microtubule-associated phosphoprotein found predominantly within the axons of CNS neurons, although a form of Tau, known as big Tau, has been found within the peripheral nervous system. Tau promotes the assembly and stabilisation of neuronal microtubules. Six isoforms are found in the CNS of healthy adults, which range from 352-441 amino acids in length, and differ from each other by the size of the N-terminal inserts, and the presence of three or four tandem repeat regions of 31-32 amino acids in the carboxyl terminal end. Studies with recombinant Tau have shown that it is in these repeat regions in the carboxyl terminal regions which are the microtubule binding domains and

each contain a characteristic Pro-Gly-Gly-Gly motif. The Tau gene is located on chromosome 17q21 and contains 15 exons, 11 of which are used to encode the major Tau protein isoforms. (Goedert et al. 1991; Goedert et al. 1989; Hardy et al. 1998) The one or two phosphorylation sites on each isoform are serine or threonine residues followed by proline, suggesting that protein kinases with a specificity for seryl-proline and threonyl-proline residues are responsible for phosphorylation. Protein kinases such as mitogen-activated protein (MAP) kinase, glycogen synthase kinase-3 and proline-directed protein kinase have been shown to phosphorylate recombinant Tau on at least some of the residues seen in adult or foetal Tau. The phosphorylation status of a protein depends on a balance of phosphorylation and dephosphorylation and Tau phosphorylation by MAP kinase is only dephosphorylated by phosphatase 2A. (Goedert et al. 1992) The phosphorylation status of Tau affects its ability to bind to microtubules, such that, Tau phosphorylated by MAP kinase has one-tenth the ability of non-phosphorylated Tau, to bind to microtubules. (Drechsel et al. 1992) The relevance of these observations to neurodegeneration and cytoskeletal disintegration after acute injury is not currently known.

There are a number of degenerative diseases of the CNS, which are associated with the intracellular or extracellular inclusion bodies consisting of a hyperphosphorylated form of Tau protein. These include Alzheimer's disease (AD), Pick's disease (PiD), and progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). Although methods are available for the determination of CSF Tau concentration, it is not possible to reliably differentiate between these conditions. As with S100B, CSF Tau is elevated by a wide range of neurological conditions. (Jensen et al. 1995; Vandermeeren et al. 1993; Vigo-Pelfrey et al. 1995) Thus the utility of measuring CSF Tau for the purpose of diagnosing dementia is limited. To date only one systematic study reports CSF Tau concentration after TBI, utilising an in-house ELISA to quantify axonal injury. (Zemlan et al. 1999)

1.5 Study hypotheses

- Alterations in the concentration of apoE occur in the CSF reflecting the role of apoE in the response to CNS injury.
- Altered CSF apoE concentration after CNS injury is related to injury severity and clinical outcome.
- After CNS injury altered CSF apoE concentration is related to changes in CSF amyloid-beta concentration.
- Alterations in CSF lipoprotein particles occur reflecting the role of apoE in cholesterol recycling in response to CNS injury.

2 Materials and Methods

This chapter describes the subjects investigated, and the methods used for CSF analysis. The subjects investigated comprise a control group of patients without acute brain injury, and an acute brain injury group. The acute brain injury group comprises patients with either TBI or SAH. The CSF of these patient groups was investigated in two ways. First, quantification of apoE, S100B, Tau, Aβ, albumin, and total protein was undertaken on CSF. In addition, the time course of these alterations was investigated in a subgroup of patients for whom serial CSF samples were available. The relationship between the concentration of the proteins assayed and clinical measures of injury severity, and outcome were determined. Second, CSF lipoprotein particles were isolated and analysed from a subgroup of patients from the control and brain injury groups to determine whether differences observed in the concentration of apoE in nascent CSF were paralleled by changes in CSF lipoprotein particle composition.

2.1 Patients investigated

CSF from three categories of patients were investigated:

- Patients with no acute brain injury-the control group.
- Patients with TBI.
- Patients with SAH.

The control CSF samples were obtained from patients admitted to the Institute of Neurological Science (INS) in Glasgow (discussed later). SAH CSF for the analysis of lipoprotein particles by size exclusion chromatography (discussed later) was obtained from patients admitted to the INS in Glasgow. The remainder of the brain injury CSF analysis was performed on CSF from the Neurosurgical Unit at the University Hospital, Pittsburgh, Pennsylvania, USA.

An absolute requirement for participation in the study was the granting of informed consent by patients (or assent from next of kin of patients incapable of giving informed consent) participating in the study. The study had approval from the local hospital ethics committee.

2.1.1 Ethics and Consent

This study commenced immediately after the Medical Research Council (MRC) issued *Interim Operational and Ethical guidelines for use of human tissue and biological samples in research.* (Medical Research Council, 1999) A summary of the issues to address when obtaining consent, as outlined in the MRC guidelines, is given in the appendix. The consent form was based upon that recommended by the MRC (see appendix for consent form and patient information sheet).

Consent was sought for:

- Collection, storage and analysis of residual CSF for apoE determination.
- Determination of *APOE* genotype using DNA isolated from CSF, or a buccal swab from the oral cavity.
- Collection, storage and analysis of residual CSF for other protein relevant to brain injury.
- Analysis of other genes of relevance to brain injury.
- Retrieval, analysis and storage of relevant personal (e.g. age, sex) and clinical (e.g. diagnosis) information.
- Publication, and presentation of anonymised data resulting from the study.

Patients were given assurances regarding confidentiality and restriction of access to data collected to members of the research team. Patients were given a written information sheet stating the goals of the research, the types of tests to be done, the diseases to be investigated, and how the results might affect their interests. In addition patients were informed that the study had local ethics committee approval. The patient information sheet was approved by the local ethics committee, and was adapted from that used in previous studies undertaken at the Southern General Hospital, University Department of Neurosurgery, investigating the association between *APOE* genotype and recovery after acute brain injury. In addition the study was approved by the consultant neurosurgeons and neurologists at the Institute of Neurological Sciences.

Informed consent was undertaken by face to face interview in the majority of patients. Where patients were incapable of giving informed consent, assent from the next of kin was obtained. Many patients were discharged from hospital before it was possible to obtain informed consent by face to face interview and these patients were contacted by post, and of those who were contactable the majority returned the signed consent form. These patients (and those consented by face to face interview) were encouraged to telephone or write if they had any questions or required more information. Consent to perform *APOE*

genotype determination, and retain and analyse CSF in Pittsburgh was undertaken by the Pittsburgh research team. The study protocol had approval of their local ethics committee.

2.1.2 Selection and characteristics of controls

Sampling CSF requires the use of invasive techniques such as lumbar puncture, cisterna magna puncture, or ventricular puncture. The risks associated with these invasive techniques limit the sampling of CSF to patients for whom the potential clinical benefits of the procedure outweigh the small, but definite risks. The risks of CSF sampling preclude the study of healthy, asymptomatic "normal" subjects. Control group CSF was selected according to the results of CSF analysis and clinical exclusion criteria. Thus a substantial number of consecutive CSF samples sent to the department of neuropathology for analysis were used to create a population from which a smaller control population was selected on the basis of "normal" CSF analysis (see below). In addition, from knowledge of the patients' clinical diagnosis, it was possible to select patients according to clinical indication for CSF analysis, and estimate the effect of confirmed neurological disease upon the study parameter. This approach enabled identification of an age and sex matched control group of similar size to the acute brain injury groups.

2.1.2.1 CSF exclusion criteria for control group

The control group was selected by excluding patients with CSF indices as follows:

- Presence of xanthochromia.
- Nucleated cell count greater than five cells per cubic millilitre.
- Albumin concentration outside the 95th centile of the population (CSF samples with no xanthochromia, and cell count less than 5 nucleated cells per mm^3) mean (i.e.> 273 mg/l).
- Total protein concentration outside the 95th centile of the population mean (i.e.> 576 mg/l).
- S100B greater than the 95th centile of the population mean (>0.39 ng/ml).
- Presence of Oligoclonal bands.

2.1.2.2 Control group clinical exclusion criteria

These included patients with clinical features consistent with SAH, TBI and, for "health and safety reasons", microbiological infection and transmissible spongiform encephalopathy (TSE). Patients investigated for demyelination or dementia formed a separate subgroup for comparison with the control group to estimate the impact of these

disorders upon CSF parameters. Clinical information was obtained prospectively from the patients' clinical records, and from discharge summaries.

2.1.3 Patients with acute brain injury

The availability of CSF is limited due to the tendency to restrict ventriculostomy to patients with severe brain injury. It is argued that in this category of patients, the risk of ventriculostomy is outweighed by the benefits of CSF drainage for the treatment of raised intracranial pressure/or hydrocephalus. In the USA, intraventricular pressure monitoring is considered the gold standard and is recommended for patients with severe brain injury. (Brain Trauma Foundation, 1996) In the UK intracranial pressure monitoring is usually undertaken using intraparenchymal pressure sensors because ventricular cannulation is generally regarded as having an unacceptably high risk of brain injury secondary to cannulation and infection. The quantification of CSF proteins in CSF after SAH and TBI was therefore limited to CSF obtained by the neurosurgical unit in Pittsburgh, USA. CSF was available from all patients within three days of injury, and in a subgroup serial samples were available for as long as two weeks after injury. CSF lipoprotein particles were isolated and analysed from SAH and control CSF from patients at the INS, Southern General Hospital, in Glasgow. The studies of TBI CSF lipoprotein particles utilised CSF from the Neurosurgical unit in Pittsburgh, USA.

2.1.3.1 Characteristics of TBI patients

CSF samples were available from 27 TBI patients within three days of injury, and serial samples from 13 TBI patients for up to one week after injury. The characteristics of these patients are summarised in table 5. In addition to the demographic and clinical features of the TBI patients, radiological features were classified according to the CT findings at admission. The radiological features of the TBI patients are summarised in table 6.

2.1.3.2 Characteristics of SAH patients

Serial CSF was available from 19 SAH patients for up to two weeks after haemorrhage. The clinical and radiological characteristics are summarised in table 7. Ruptured intracranial aneurysms were identified in 18 SAH patients (16 anterior circulation, 2 posterior circulation), and one patient was unfit for investigation.

Table 5 Characteristics of TBI patients

Characteristic	Number of patients (%)	
	CSF within three days of TBI	Serial TBI CSF
Age (years)		
Average	32	33
Median	25	37
range	16-65	16-65
Gender		
Male	22 (81)	10 (77)
Female	5 (19)	3 (23)
Total	27	13
***APOE* genotype**		
ε33	20 (74)	10 (77)
ε34	5 (18)	1 (8)
ε32	2 (7)	2 (15)
Best GCS		
Average	5	6
Median	5	6
range	3-8	3-8
Mechanism of TBI*		
RTA**	18 (67)	8 (62)
Fall	6 (22)	3 (22)
Other	2 (7)	1 (8)
Assault	1 (4)	1 (8)

*TBI: Traumatic Brain Injury

**RTA: Road Traffic Accident

GCS: Glasgow Coma Score

Table 6 Radiological features of TBI patients

Marshall Grade	Classification	Number of patients	
		within 3 days	serial
1	Diffuse injury	1	1
2	Diffuse injury	0	0
	a) 1 only		
	b) ≥ 2 unilateral		
	c) Bilateral		
3	Diffuse injury + swelling	1	1
4	Diffuse injury + shift	1	1
5	Evacuated mass lesion		
	a) Extradural	3	2
	b) Subdural	0	0
	c) Intracerebral	2	0
	d) ≥ 2 lesions	5	3
6	Nonevacuated mass lesion		
	a) Extradural	1	0
	b) Subdural	3	1
	c) Intracerebral	3	2
	d) ≥ 2 lesions	7	2

CT: computerized tomography

Table 7 Characteristics of SAH patients including radiological features

Characteristic	N (%)
Age (years)	
Average	56
Median	54
range	35-74
Gender	
Male	6 (32)
Female	13 (68)
Total	19
***APOE* genotype**	
ε33	11 (58)
ε34	7 (37)
unknown	1 (5)
Best GCS	
Average	10
Median	10
range	3-15
Fisher CT Grade	
1	0
2	1
3	9
4	9

2.1.4 Assessment of clinical outcome after acute brain injury

Clinical outcome was assessed using the Glasgow Outcome Scale (GOS). Outcome was assessed six months after TBI injury and three months after SAH. Outcome assessments were determined by Dr Marion (blinded to the results of CSF analysis) at the outpatient clinic review at the Department of Neurosurgery in Pittsburgh.

2.2 Analysis of CSF proteins

2.2.1 CSF collection, storage and analysis

CSF was collected from the lateral ventricle in all acute brain injury patients, and the lumbar subarachnoid space for the majority of the controls. Ventricular CSF was obtained from ventricular catheters sited for the purposes of intraventricular pressure monitoring in patients with acute brain injury. CSF samples from patients with suspected hydrocephalus or shunt dysfunction, but no acute brain injury, were used as ventricular CSF controls for comparison with control CSF obtained by lumbar puncture, and as the control group for comparison with serial brain injury CSF samples. CSF obtained from the lumbar subarachnoid space was obtained by lumbar puncture for the purpose of assessing intrathecal pressure, treatment of communicating hydrocephalus or benign intracranial hypertension, or for analysis of CSF for investigation of suspected neurological disease.

CSF was collected in Polypropylene tubes and centrifuged at approximately 450g at 4° C for 10 minutes to eliminate cells and debris and was aliquoted prior to freezing and storage at -80° C until subsequent analysis. CSF from brain injury patients and controls was processed and stored identically for simultaneous analysis at a later date. Analysis of CSF from controls and acute brain injury patients was performed simultaneously with cases and controls on each analysis plate. It was not possible to blind the observer to sample type due to the difference in macroscopic appearance of CSF from controls and patients with acute brain injury. CSF samples underwent one freeze-thaw cycle prior to analysis, and 5µl of protease inhibitor cocktail (Product number P 2714, SIGMA, Missouri, USA) was added at a final concentration of 1mM.

2.2.2 Determination of CSF apoE concentration

A method for assaying apoE of sufficient sensitivity for the concentration range present in CSF was not readily available and was therefore developed for the purpose of this study. The technique used was Enzyme Linked Immunosorbant assay (ELISA) based upon the method described by Stark and Siest. (Starck et al. 2000) The results of the assay evaluation for the purposes of this study are described in detail in the chapter 3. The principal of the assay involves binding a capture antibody to the surface of a microtitre plate, which specifically captures apoE from the CSF. This antibody-apoE complex is then bound by a second (detection) antibody, to which a third antibody is targeted. The third antibody is conjugated to horseradish peroxidase (HRP) which catalyses the peroxidation of a substrate to a coloured product detectable spectrophotometrically.

2.2.2.1 CSF ELISA method

In brief, the capture antibody for the apoE assay was rabbit polyclonal anti-human apoE antibody (Dako, Ely, UK), diluted in 0.02 M Citrate buffer. The coated plate was washed with Phosphate Buffered Saline (PBS) and non-specific binding sites were blocked with 2% bovine serum albumin (BSA). The blocked plate was washed with PBS/Tween, prior to incubation at 37°C with diluted CSF samples and the apoE standard curve (1.5-100 ng/ml) in duplicate. After further washing with PBS/Tween, goat anti-human apoE antibody (Chemicon, Harrow, UK) was used as detection antibody, followed by HRP conjugated rabbit anti-goat IgG as the secondary antibody for the colour reaction. A detailed description of the apoE ELISA is given in the appendix. There was parallelism between the apoE calibration curve and serial dilutions of control CSF, brain injury CSF, brain homogenate and mixtures of CSF and plasma or lysed red blood cells. The range of assay recovery was 92-98%. The intra-assay and inter-assay coefficients of variation were 7.4% and 8.6 % respectively. These data are presented in chapter 3.

2.2.3 S100B ELISA

The S100B ELISA used was a previously established, and extensively evaluated, *in house* assay for the determination of S100B in CSF. (Green et al. 1997) In brief, 96 well micro-titre plates were coated with 200µl of 0.05M carbonate buffer containing monoclonal anti-S100B (Affinity Research Products, Exeter, UK). The plates were washed with 0.67 M Barbitone buffer containing 5mM Calcium Lactate, 0.1% BSA, and 0.05% Tween, and then blocked with 2% BSA and washed again. CSF samples diluted in 0.67 M Barbitone

buffer containing 5 mM Calcium Lactate were added in duplicate. After incubation and washing, HRP conjugated polyclonal anti S100B (Dako, Copenhagen, Denmark) was used as detecting antibody. The σ-phenylenediamine (OPD) colour reaction was stopped with 1M hydrochloric acid and the absorbance read at 492 nm and 405 nm. The antigen concentration was calculated from an internal standard curve ranging from 0.01 to 2.5 ng/ml. The intra and inter-assay coefficient of variation for the S100B assay was 9.3% and 8.1% respectively. The recovery of S100B added to CSF was 94%.

2.2.4 Tau ELISA

CSF Tau measurements were performed using the Innotest hTau Antigen, and enzyme-linked immunoassay supplied by Innogenetics, Belgium (UK suppliers: Autogen Bioclear, Wiltshire, UK). This assay uses a monoclonal capture antibody (AT120) which reacts with both the normal and the hyperphosphorylated forms of Tau. (Vandermeeren et al. 1993) The microtitre plates are supplied pre-coated with this monoclonal antibody, and after blocking of the unbound protein sites on the microtitre plate wells, standards and CSF samples were added in duplicate. Tau calibrants were prepared from recombinant Tau protein and a series of calibrants 0, 75, 150, 300, 600 and 1200 pg/ml were used for the standard curve. A pair of biotinylated monoclonal antibodies (HT7 and BT2) were added to each well of the microtitre plate and the plate incubated over night at room temperature. The HT7 monoclonal antibody reacts with both normal and phosphorylated Tau protein, whilst the BT2 monoclonal antibody reacts preferentially with the hyperphosphorylated Tau protein. (Goedert et al. 1994) After overnight incubation the microtitre plate was washed and peroxidase conjugated streptavidin added. The streptavidin binds to the biotin on the monoclonal antibodies to form a complex, the quantity of which is proportional to the amount of Tau protein in the standard or CSF sample. The microtitre plate was washed and the substrate containing 3`, 3`, 5`, 5` tetramethylbenzidine (TMB) and hydrogen peroxide added. The peroxidase activity converts the colourless TMB into an insoluble blue precipitate. The peroxidase activity was stopped by the addition of sulphuric acid, converting the insoluble blue precipitate to a soluble yellow product. The absorbance of the yellow product was measured at 450 nm being proportional to the amount of Tau protein originally present in the sample. The manufacturer claims the lower limit of detection to be 59 μg/l. The intra and inter assay coefficient of variation are both reported to be below 6%, and the recovery of Tau added to plasma is reported to be 92%. Detailed evaluation of this commercial ELISA was beyond the scope of this study.

2.2.5 Amyloid ELISA

$A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were also assayed using commercially available ELISA kits (BioSource International, Inc. California, USA) according to manufacturers instructions. The kits were identical for each peptide other than the antibody specificity. In brief, 100 µl of Aβ standard (0-1000 pg/ml) and CSF samples were aliquotted in duplicate into the wells of the 96 well microtitre plate. Control and brain injury CSF samples were distributed within the same plate. The wells, which were pre-coated with capture antibody specific for $A\beta_{1-40}$ (or $A\beta_{1-42}$), were incubated for two hours, at room temperature, on a plate shaker. After washing, rabbit anti human Aβ detection antibody was added to each well and allowed to incubate for a further two hours. After this incubation and further washing, HRP conjugated anti-rabbit antibody was added and incubated for a further 2 hours. After further washing steps, chromogen (OPD) was added, and after 30 mins (in the dark) the reaction was stopped with acid, and the absorbance read at 450 nm. The coefficient of variation for intra-assay precision is claimed by the manufacturer to be 3.6%, and inter-assay precision 3.7%. The recovery of Aβ added to serum is reported to be approximately 100%. Again detailed evaluation of these commercial ELISAs was beyond the scope of this study.

2.2.6 CSF total protein concentration

The concentration of protein in the CSF was determined using a turbidimetric assay in which protein precipitation was achieved using benzethonium chloride, EDTA, and NaOH. (Luxton et al. 1989) To a volume of 10 µl of CSF was added 190 µl of precipitating reagent (5.0 g of benzethonium chloride, 20 g of EDTA, and 10 g of NaOH per litre). The plate was left at room temperature for 15 minutes before the absorbance of each well was measured at 410 nm (Dynatech MR700 microtitre plate reader) after blanking the instrument with precipitating reagent. The amount of precipitated protein (e.g. albumin and globulin) is proportional to the concentration of protein present in the CSF, and results in proportionate absorbance to light. The method is rapid, requires only 10 µl of CSF, and can be performed in a microtitre plate with between batch coefficient of < 5%. The protein calibration standards used were prepared by dissolving 150mg of human albumin (Sigma, Poole, UK) and 50 mg of human gamma globulin (Sigma, Poole, UK) in 100ml of isotonic saline (NaCl 0.85 g/l) containing 10 g of sodium azide per litre to give a stock 2000 mg/l standard for total protein. From this solution serial dilution was performed to create a set of working standards in the 50 to 1500 mg/l concentration range.

2.2.7 CSF Albumin rocket electrophoresis

CSF albumin concentrations were determined using rocket electrophoresis as detailed in the appendix. In brief, CSF and an albumin standard curve are loaded onto an agarose gel containing polyclonal anti-human albumin antibody and electrophoresed overnight, washed and stained for protein with brilliant Coomassie blue. The distance (measured in mm) migrated from the well is proportional to the albumin concentration and values derived from the albumin standard curve electrophoresed in parallel to the CSF samples.

2.3 Detection of CSF apoE by SDS-PAGE and Western blotting

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on control and acute brain injury CSF samples in order to:

- Identify the presence of small molecular weight apoE epitopes/fragments, which might conceivably arise from proteolysis.
- Estimate the influence of apoE denaturation (SDS and thermal energy) upon immunoreactivity (i.e. evidence for epitope masking).
- Obtain an approximate corroboration of the CSF concentration obtained by the ELISA method, and the semi-quantitative SDS-PAGE method.

2.3.1 SDS-Polyacrylamide gel electrophoresis

SDS-PAGE separates proteins according to their molecular weight, independent of intrinsic electrical charge. Prior to electrophoresis the CSF sample is diluted with a buffer containing sodium dodecyl sulphate (SDS), and a reducing agent, and the mixture is heated to 100°C. The reducing agent breaks inter and intra subunit disulphide bonds, and the SDS (an anionic detergent) denatures the protein by wrapping around the polypeptide backbone. As a result the proteins in the sample become rods of negative charge with equal charge per unit length. Acrylamide is used for preparing electrophoretic gels for separating proteins by size. A mixture of acrylamide and bis-acrylamide polymerises to form a cross-linked network with a pore size that is dependent on the concentration of acrylamide used. The higher the concentration of acrylamide, the smaller the pore size. The migration of the protein in the gel is thus a function of its size and the intensity of the band when visualised gives a semi-quantitative estimate of the amount of protein present in the CSF sample. The gel used for this investigation was a 10% running gel, and 4% stacking gel assembled according to manufacturers instructions (Amersham Pharmacia Biotech, Herts, UK).

2.3.1.1 Sample preparation

Seven CSF samples with known apoE concentration (determined by ELISA) were run on the gel. Three were control CSF samples: one with high apoE concentration (ventricular CSF), one with intermediate apoE concentration, and one with low apoE concentration. Four TBI samples were run: two with low apoE concentration and two with intermediate apoE concentration. For each CSF sample 60μL was diluted with an equal volume of sample diluent (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8) and placed in a boiling water bath for 4 minutes.

2.3.1.2 Running the SDS-PAGE gel

The comb was carefully removed from the stacking gel and the wells were rinsed with electrophoresis buffer (0.025 M Tris, 0.192 M glycine, 0.1 % SDS, pH 8.3). After fitting the gaskets to the upper tank chamber it was placed onto the gel and secured using the cams supplied. The lower tank chamber was filled with 3 L of electrophoresis buffer and the gel plus upper chamber were lowered into the lower tank chamber, being careful to avoid trapping any bubbles at the bottom of the gel. The upper chamber was filled with electrophoresis buffer, making sure that there was no leakage of buffer into the lower chamber. To each well 100 μL of diluted CSF was added and molecular weight markers were included on each run. The gel was run at 250 V, 50mA for 3 hours using an EPS 200 power supply (Amersham Pharmacia Biotech, Herts, UK).

2.3.2 Western blotting

"Western blotting" or electroblotting refers to the process of transferring the separated proteins from the gel onto a nitrocellulose matrix to which they bind and become immobilised. It is necessary to transfer the proteins to the nitrocellulose matrix from the gel matrix because the gel matrix restricts access to the detecting antibody. In addition to the accessibility of protein immobilised on the nitrocellulose matrix to the detecting antibody, nitrocellulose is less fragile.

2.3.3 Gel to nitrocellulose protein transfer step

At the end of the electrophoresis, the gel was removed from the electrophoresis system and the lane containing the molecular weight markers were removed and placed in Coomassie Brilliant Blue stain for 1 hour and destained using deionised water. The remaining gel was placed into a pre-chilled transfer buffer for 30 minutes. Two pieces of filter paper and a piece of nitrocellulose were cut to the same dimensions of the gel and placed into the transfer buffer, along with two fibre pads supplied with the Trans-blot® Electrophoretic Transfer cell (Bio-Rad, Herts, UK). After 30 minutes one fibre pad was placed onto the plastic gel holder and onto this was placed a piece of wet filter paper ensuring that there were no trapped air bubbles. The gel was removed and placed on top of the filter paper. A piece of pre-wetted nitrocellulose was placed on top of the gel, being careful not to trap any bubbles. The remaining filter paper and fibre pad were placed on top of the gel, all bubbles removed and the gel holder closed. The transfer cell was filled with pre-chilled transfer buffer (0.025 M Tris, 0.192 M glycine, 0.1 % SDS, pH 8.3, containing 20% methanol) and the cooling coil was placed inside the cell and attached to a free flowing water system. The gel holder was placed into the Trans-blot® Electrophoretic Transfer cell with the gel facing the cathode. The transfer of proteins was carried out using a constant currant of 400 mA and a starting voltage of 50 V for 16 hours.

2.3.4 ApoE immunoblotting technique

After transferring the apoE onto nitrocellulose, empty protein binding sites were blocked by incubating the membrane in 2% non-fat milk in PBS for 30 minutes at room temperature. The nitrocellulose membrane was rinsed with running water and then washed with PBS containing 0.05% Tween 20 (wash solution), and placed in 50 mL PBS/milk containing rabbit anti-human apoE. After incubating the nitrocellulose membrane overnight at 4°C, the membrane was rinsed with running water and washed and then placed into 50 mL PBS/milk containing swine anti-rabbit immunoglobulin HRP conjugate. After incubating the nitrocellulose membrane for 1 hour at room temperature, the membrane was rinsed with running water and washed with several changes of wash solution over 1 hour. The membrane was developed with aminocarbazole colour regent for 20-30 minutes at room temperature, then rinsed under running water and then washed for 30 minutes before being dried.

2.4 Isolation and analysis of CSF lipoprotein particles

In addition to the quantification of apoE of controls and patients with SAH or TBI, CSF lipoprotein particles were isolated from the CSF, and their composition analysed. Because the concentration of these particles in the CSF is low, a substantial volume (excess of 20 ml) of CSF is required. The volume of residual CSF obtained from control and TBI patients is relatively low (e.g.1 ml), therefore it was necessary to pool CSF from at least twenty patients. The approach of pooling CSF to generate the volume required for the isolation of CSF lipoprotein particles has been used by others for the same reasons. (Demeester et al. 2000; Koch et al. 2001) Obtaining sufficient volumes of CSF from patients with SAH is less problematic as a daily drainage volume of approximately 200 ml of CSF is not unusual.

2.4.1 CSF size exclusion chromatography

Twenty millilitres of CSF was concentrated 50 times by filtration through Centricon YM-10 concentration filters (Amicon, Beverly, MA, USA). These filters have a pore size 10 KDa (Molecular weight of apoE protein is 34.2 KDa) and a recovery of 99.9%. The concentrated CSF was fractionated by size exclusion chromatography as follows. The concentrated CSF (500 µl) was loaded onto a Superdex 200 size exclusion chromatography column (sepharose 6HR 10/30) and eluted with elution buffer (10mM Tris, 150mM NaCl, 0.01% EDTA, 1mM NaN_3) at a flow rate of 0.25ml/min, and flow pressure of 0.5 Mpa. Fractions of 250 µl were collected and assayed for a free cholesterol (FC), phospholipid (PL), triglyceride (TG), apoE, and apoAI as follows. Lipid and lipoprotein concentrations were determined from the elution profile by calculating the area under the curve (AUC). Molar concentrations and ratios were calculated from the molecular weight (KDa) of apoE (34.2), apoAI (30.7), apoB (549), FC (38.6), and PL (77.4). The control CSF pools had no detectable apoB confirming the absence of plasma contamination.

2.4.2 Free cholesterol assay

Free cholesterol was determined colorimetrically using a commercially available kit (Wako Chemicals, Japan) according to manufacturer's. instructions. In this assay free cholesterol is oxidised by cholesterol oxidase to 4-cholestenone generating hydrogen peroxide. The hydrogen peroxide formed, in the presence of peroxidase, oxidises phenol and 4-aminoantipyrine resulting in a red colour product. The amount of free cholesterol in the test sample is determined by measuring the absorbance of the red colour at the maximum

absorption wavelength of 505 nm on a Hitachi 704-auto analyser. The coefficient of variation for the assay was 1.9%.

2.4.3 Phospholipid assay

Phospholipid was determined colorimetrically using a commercially available kit (Roche Diagnostics GmbH, Germany) again using the hydrogen peroxidase reaction, measuring the absorbance of the coloured product at 505 nm on Hitachi 704-auto analyser. The coefficient of variation for the assay was 1.5%.

2.4.4 Triglyceride assay

Triglyceride was also assayed using the Hitachi 704 auto-analyser using a commercial Triglyceride kit (Roche Diagnostics GmbH, Germany). In this case Lipoprotein lipase hydrolyses triglyceride to glycerol, which is oxidised to dihydroxyacetone phosphate and hydrogen peroxide which is measured as above. The coefficient of variation for the assay was 2.1%.

2.4.5 Apolipoprotein turbidimetric immunoassay

Commercially available turbidimetric immunoassay kits are commonly used in clinical laboratories for the determination of plasma apoE, apoB, apoAI, apoAII, apoCI, apoCII and apoCIII. The concentration of these proteins in native CSF is generally below the limit of detection of this method. However, the method can be used to assay the concentration of these proteins in concentrated CSF fractionated by size exclusion chromatography. The principle of the assay is identical for all the proteins analysed. The concentrated CSF fraction is mixed with the buffer and a solution containing antibody to the lipoproteins. The apolipoprotein in the sample then combines with anti-human antibody specific for that apolipoprotein in the reagent to yield an insoluble aggregate, which causes an increase in the turbidity of the sample. The degree of turbidity is measured optically and is proportional to the amount of analyte in the sample. These kits are compatible with the Hitachi 704 auto-analyser. The linear range of detection for apoE, apoAI and apoB were 0-12 mg/dl, 0-70 mg/dl and 25-200 mg/dl respectively.

2.5 *APOE* genotyping

APOE genotyping was performed, blind to the CSF concentration, using a hot start polymerase chain reaction (PCR) method. DNA was extracted from the *buffy coat* layer of haemolysed blood, or CSF, by ethanol extraction. Residual ethanol wash was removed by vacuum desiccation for 15 minutes, or overnight evaporation. To each sample was added 50µl of proteinase K (800 µg/ml), 20 µl of 10X PCR buffer, and 130 µl of Analar water. The solutions were overlaid with 100 µl of mineral oil and incubated at 37°C overnight. The samples were then heated to 95 °C for 10 minutes to inactivate the proteinase K, and 2.5 µl of the resulting solution was used as a target for PCR with the *APOE* primers. (Wenham et al. 1991) The reactions took place in a total volume of 50µl comprising 35µl of master mix A (see below), which was separated initially by a wax barrier to allow a "hot start", from 12.5µl of master mix B (see below) and 2.5µl of target. Master mix A contained the following reagents, in volumes multiplied by the number of samples to be amplified: 19.5 µl Analar water; 3.5 µl 10X PCR buffer; 5 µl dNTP solution containing 2 mM dTTP; 5 µl dimethyl sulfoxide; and 1µl of each primer to give final concentrations in the reaction of 0.2 µM. Thirty-five microlitres of Master mix A were aliquoted to each 500-µl reaction tube. An Ampliwax gem (Perkin Elmer Cetus) was added to each tube, heated to 75-80°C for 5-10 minutes to melt the gem, and cooled to allow it to resolidify. Master mix B contained the following reagents, in volumes multiplied by the number of samples to be amplified: 10.75 µl of Analar water, 1.5µl 10X PCR buffer, and 0.25 µl *Taq* polymerase; 12.5µl of Master mix B was added to each reaction tube overlying the Ampliwax gem followed by 2.5 µl of target, prepared as described above. The samples were heated to 95°C for 7 minutes, followed by 40 cycles of 95°C for 1 minute, 65°C for 1 minute and 70°C for 2 minutes. Ten units *Hha*I was added directly to the PCR product and incubated overnight at 37°C. The products of the digestion were separated on a 10% polyacrylamide gel, stained with ethidium bromide and visualised by ultraviolet-induced fluorescence. (Nicoll et al. 1997)

2.6 Data collection, storage and protection

2.6.1 Clinical data

Clinical (and demographic) data was collected prospectively from clinical case notes and entered onto a paper proforma. Patients were assigned an anonymous study identity

number, and this was entered, with the clinical data of relevance to the study, to an ACCESS database minus the patients name, address, and hospital number, which were stored separately. The study identity number was used to link the clinical data to the relevant CSF sample. This data was stored on a network-protected system, and access to the database was password protected.

2.6.2 CSF analysis data

Data from analysis of CSF was recorded in paper copy form as raw data, which was imported manually to an EXCEL database for analysis of mathematically derived values from standard curves etc. The CSF samples were labelled with the identity number only. This data was also stored on a network, and password, protected system.

2.7 Statistical Analysis

2.7.1 Statistical software

Statistical analysis was performed using Microsoft Excel, GraphPad Prism and InStat software.

2.7.2 Types of data analysed

2.7.2.1 Categorical data

- Male/female
- TBI, SAH, control
- Glasgow Coma Score (ordered categorical)
- WFNS grade (ordered categorical)
- Glasgow Outcome Score (ordered categorical)
- Marshall/Fisher CT grade (ordered categorical)
- *APOE* genotype (nominal)

2.7.2.2 Numerical

- Number of patients (discrete)
- Age (continuous)
- Cell count, albumin, total protein, apoE, S100B, Tau, Aβ etc.

2.7.2.3 Other

Ratios and percentages are used to describe relative frequency. Examples include:

- ApoE to albumin ratio, apoE to total protein ratio
- Molar ratios of lipoprotein particle constituents

2.7.3 Data description

2.7.3.1 Measures of central tendency

- Arithmetic mean (average) e.g. CSF apoE concentration (μg/l)
- Median e.g. CSF concentration (μg/l)

2.7.3.2 Description of variability

Variability was described by plotting the continuous data using relative frequency histograms. The approximation of the data distribution to a Gaussian distributed was estimated using Dallal and Wilkinson`s approximation to Lilliefors` method (mindful of the limitations of this test when the sample size is small). Log transformation of positively skewed data was performed to approximate normality where necessary.

2.7.3.3 Quantification of variability

- Range e.g. GCS, GOS, and age.
- Centiles-95th centile.
- Standard deviation (SD).
- Coefficient of variation (standard deviation divided by the mean and expressed as a percentage). This was used to describe the precision of the assays.
- 95% confidence intervals.

2.7.4 Comparing groups of data

The analysis performed depended upon the data type, the distribution, and the variance.

2.7.4.1 Comparing group parameters to parameters of one sample

The one sample t-test was used to compare concentrations derived from the area under the curve of a variable from one pool of TBI patients (comprising 27 patients) with that of several (6) pools of control patients (comprising 20 patients in each control pool).

2.7.4.2 Comparing two groups (unpaired) of continuous data

If the data was normally distributed or could be normalised, t-tests (with Welch`s correction if the variance of the two groups was significantly different) were used to compare group means. If the data did not pass the normality test and/or log transformation failed to normalise the data, or the sample size was small and appeared not to have a Gaussian shaped frequency distribution histogram, or the group variances were significantly different, the Mann-Whitney test was used. For example, comparing the median ventricular CSF apoE with lumbar CSF apoE.

2.7.4.3 Comparing three (or more) groups of continuous data

Normally distributed data was analysed using parametric ANOVA with Tukeys multi-comparison test. However, non-parametric analysis was performed for all the data for consistency of approach, as some data sets did not satisfy the criterion for parametric analysis. Data groups, which did not pass the normality test or where the difference between group variances was significant, were compared using the Kruskall-Wallis test, with Dunn`s multi-comparison test to compare group medians.

2.7.4.4 Comparing groups of categorical data

Fisher's exact test was used to compare the proportions within columns and rows of frequency tables. For example, *APOE* genotype and GOS (dichotomised into favourable and unfavourable) after SAH. In addition, continuous data was assigned to a category according to an arbitrarily selected threshold and analysed as categorical data according to the proportion of values above or below the threshold. For example, the proportion of TBI patients with CSF apoE concentration below the lowest value observed in controls. Where all brain injury CSF values were above the highest control value, the daily mean CSF Tau concentration after TBI (or SAH) was determined, and patients were categorised according to whether the value recorded on that day was above or below the mean value for all TBI patients that day. The proportion of patients above or below the daily mean was then compared with the proportion of patients with favourable or unfavourable outcome (or injury severity) using Fisher's exact test.

2.7.4.5 Relation between two variables

The association between two variables was analysed using Spearmans` rank correlation (Spearman rank correlation coefficient). The results are summarised in the form of a correlation matrix, and the data plotted using scatter diagrams.

3 Results

This chapter comprises four sections. The first section presents the results from the evaluation of the ELISA used to assay apoE in the CSF. The second section presents the results of the analysis of CSF from different categories of control patients. The third section presents the results of the analysis of apoE and other proteins in CSF from TBI, SAH patients, and selected controls. The final section presents the results from the isolation and analysis of CSF lipoprotein particles in control, SAH and TBI CSF.

3.1 Apolipoprotein E ELISA evaluation

3.1.1 Calibration of the apoE ELISA

Recombinant human apoE3 (Panvera, U.S.A.) was serially diluted in the dilution buffer to give concentrations of 100, 50, 25, 12.5, 6.2, and 3.1 μg/l. Aliquots of these were stored at -20° C. Each batch of standards was calibrated by analysing the new batch in parallel with the old batch on at least 20 separate occasions. One way analysis of variance demonstrated that there was no statistically significant difference between consecutive calibration curves. See figure 2, panel A. There was no significant difference between calibration curves using recombinant apoE3, apoE4 or apoE2.

3.1.2 The lower limit of detection of the apoE ELISA

The lower limit of detection was calculated from the mean plus three standard deviations of 40 separate consecutive replicate analysis of apoE free sample (incubation buffer). The lower limit of detection was 2.9 μg/l. See figure 2, panel B.

3.1.3 The linearity of the apoE ELISA and the presence of parallelism

CSF was serially diluted with dilution buffer and the apoE concentration of the diluted CSF was determined confirming the presence of linearity. Parallelism was investigated by assaying doubling dilutions of control, TBI, SAH CSF and the apoE3 calibration standard. The resulting optical densities were normalised by expressing the absorbance obtained for each dilution as a percentage of that given by the highest value for that series. Analysis of covariance confirms that the slopes are not different and are therefore parallel. In addition serial dilution of CSF mixed with plasma, or lysed red cells, demonstrated parallelism with

the calibration curve. Thus there are no systematic differences in the ability of the assay to detect apoE in a variety of matrices over a range of concentrations.

3.1.4 Within and between batch precision of the apoE ELISA

One hundred control CSF samples were pooled and diluted (1 in 250) such that the absorbance from the apoE assay was in the upper region of the linear portion of the standard curve. This diluted pool was then aliquotted and frozen simultaneously with the calibration curve aliquots, under identical storage conditions. These aliquots represent the high CSF quality control (high CSF QC). One hundred patient plasma samples were pooled and diluted (1 in 2500) such that the absorbance from the apoE assay was in the upper region of the linear portion of the standard curve. This diluted pool was then aliquotted and frozen simultaneously with the calibration curve, and CSF QC aliquots, under identical storage conditions. These aliquots represent the high plasma quality control (high plasma QC). The within batch precision (intra-assay coefficient of variation = CV%) was calculated from the mean and standard deviation of 40 replicate analysis of the CSF and plasma QC performed on one micro-titre plate. The intra-assay variation for the CSF and plasma QCs were 8.5% and 7.3% respectively. The scatter plot of the apoE concentration of the QCs versus position of the QC on the ELISA plate illustrates the low variability within the plate. See figure 3. Statistical analysis of the regression line confirmed that the slope of the line was not significantly different from zero. In addition, the variation in apoE concentration attributable to the position of the sample on the ELISA plate is estimated to be less than 10% (CSF and plasma QC: $R^2 = 0.08$).

The between batch precision was calculated from 20 replicate analyses of the plasma and CSF QCs performed on consecutive microtitre plates. See figure 3. The between batch precision (inter-assay variation) for the CSF and plasma QC were 9.6% and 9.4% respectively. The regression line for serial apoE measurements is not significantly different from zero, and estimates that less than 5 % of the variation in apoE concentration is attributable to plate order (CSF and plasma QC: $R^2 = 0.004$). Furthermore, there was no statistically significant reduction in the apoE signal of the QCs with time, indicating that the apoE was stable in CSF and plasma under the conditions of storage (-20°C) used for the period of storage (6 months).

Figure 2 ApoE ELISA precision and linearity

Figure 2A: Precision of the apoE ELISA from the mean (SD) normalised absorbance ratio relative to the highest standard of 20 consecutive calibration curves. There was no significant difference between the slope of each calibration curve (ANOVA).

Figure 2B: Linearity of the assay for the measurement of CSF apoE over a range of dilutions.

Figure 2C: There is linearity of the control CSF dilution curve, which is parallel with that of the calibration curve, and that of brain injury CSF.

Figure 2A : ApoE ELISA callibration curve

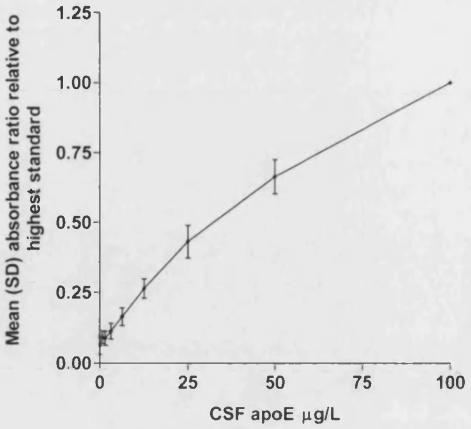


Figure 2B : Linearity of the apoE ELISA over a range of CSF dilutions

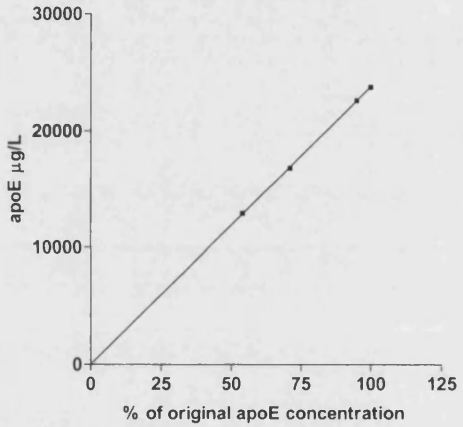


Figure 2C : Paralell serial dilution lines for CSF and apoE callibrant

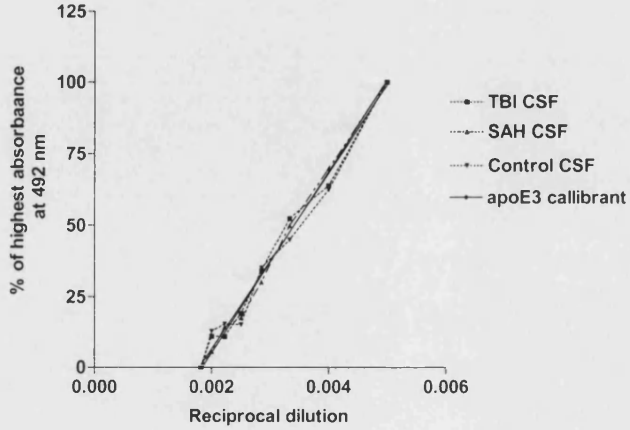


Figure 3 ApoE ELISA intra and inter-assay variation

Figure 3A: Scatter diagram of CSF quality control (QC) determinations within one ELISA plate.

Figure 3B: Scatter diagram of plasma quality control (QC) determinations within one ELISA plate The intra-assay coefficient of variation (CV% = SD/mean * 100) of the CSF and plasma QC were 8.5 % and 7.3 % respectively.

Figure 3C: Scatter diagram of CSF QC determinations from 20 consecutive ELISA plates.

Figure 3D: Scatter diagram of plasma QC determinations from 20 consecutive ELISA plates. The inter-assay coefficient of variation (CV% = SD/mean * 100) of the CSF and plasma QC were 9.6 % and 9.4 % respectively. From linear regression analysis there was no significant variation between each microtitre plate.

Figure 3A : Intra-assay variation of CSF QC

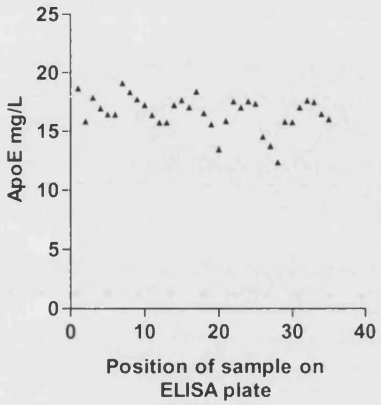


Figure 3B : Intra-assay variation of plasma QC

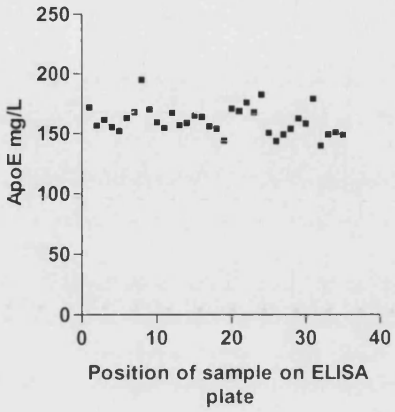


Figure 3C : Inter-assay variation of CSF QC

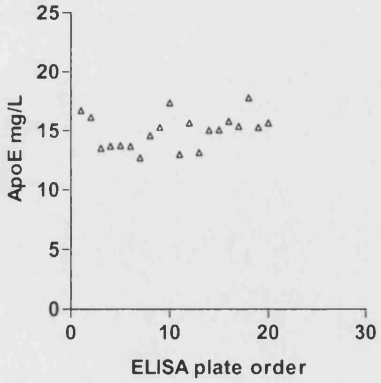


Figure 3D : Inter-assay variation of plasma QC

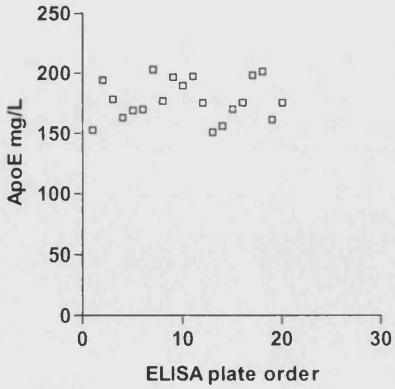


Figure 4 Recovery of apoE added to CSF and absence of albumin interference

Figure 4A: The column bar graph demonstrates that apoE added to CSF and brain homogenate can be efficiently detected, and is not significantly different between the mixtures to which the apoE spike is added.

Figure 4B: There was no significant decrease in the apoE signal in the presence of increasing albumin concentration.

Figure 4A : Recovery of apoE added to CSF and brain homogenate

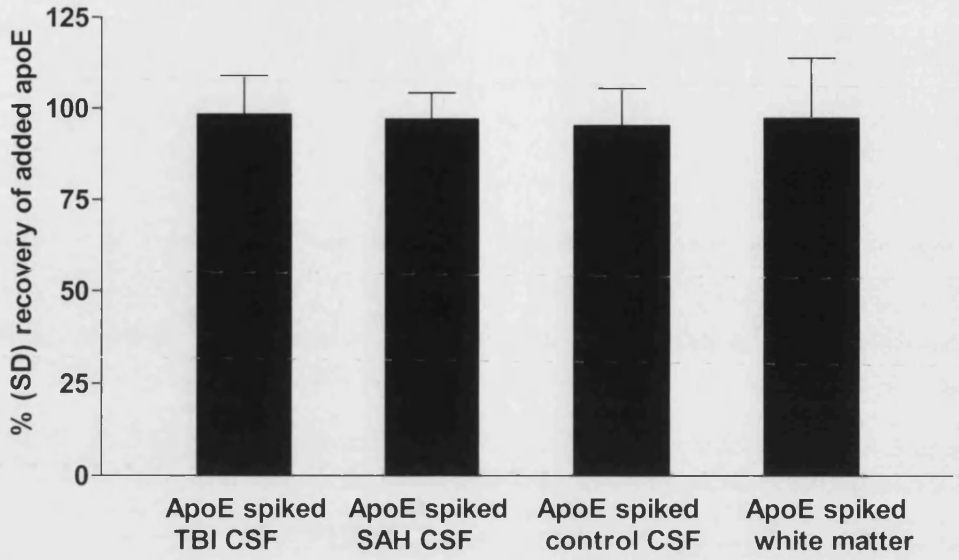
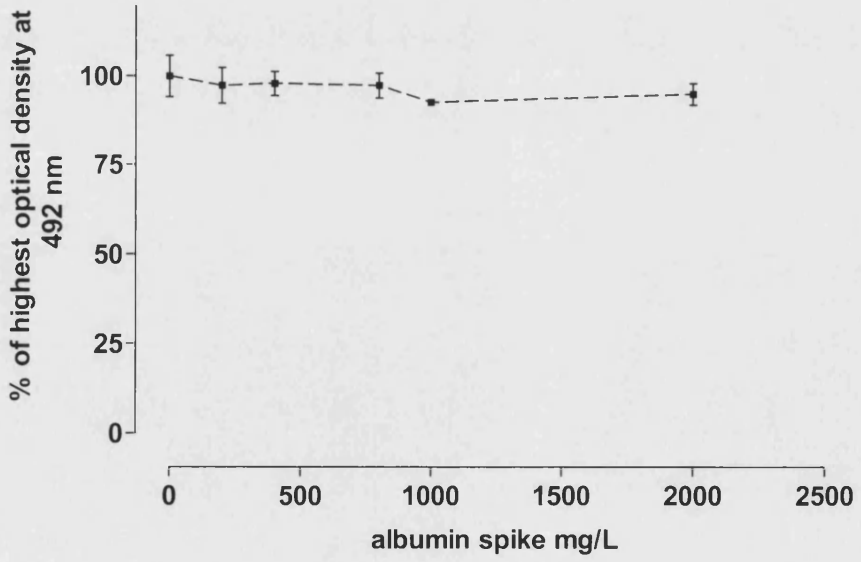


Figure 4B : Recovery of apoE in the presence of increasing concentrations of albumin



3.1.5 The recovery of apoE added to CSF and white matter homogenate

The ability of the apoE ELISA to measure apoE added to TBI, SAH, and control CSF was tested by analysing CSF to which a known amount of apoE had been added. In addition the recovery of apoE added to human cerebral white matter homogenate, obtained post mortem, was determined (see appendix). Both the spiked and unspiked samples were analysed and the difference in apoE concentration calculated as a percentage of that added to the spiked sample. An equal volume of the apoE top standard (100μg/l) was added to equal volumes of TBI (n=3), SAH (n=3), control (n=6) CSF, and white matter homogenate (n=3). The mean recovery (SD) in TBI CSF was 98% (10%), in SAH CSF was 97% (7%), in control CSF was 95% (10%), and white matter 97% (16%). The difference between mean values is not statistically significant. See figure 4.

3.1.6 The specificity of the apoE ELISA for apoE

The specificity of the apoE ELISA for apoE has been extensively investigated. (Starck et al. 2000) No interference in blank values, or apoE spiked samples, was detected when increasing concentrations of apoAI, apoAII, apoB, apoCI, apoCII or apoCIII were added indicating that the assay is specific for apoE. In the present study there was no significant difference between the calibration curves obtained using recombinant apoE3, apoE4, or apoE2, justifying the use of apoE3 as the calibrant for the quantification of apoE in CSF from patients of different genotype. In addition, there was no significant interference from human albumin or protein (as used for albumin and total protein standard curves) added at increasing concentrations (to levels encountered in brain injury CSF) to the apoE standards. See figure 4.

3.1.6.1 Summary of evaluation of apoE ELISA

In summary, the apoE ELISA developed for the quantification of apoE in CSF from control, TBI, and SAH patients was robust, sensitive, precise, and specific. Importantly, there was no significant interference from substances present in brain injury CSF that are not present in control CSF.

3.2 Determination of CSF apoE concentration in controls

This section presents the results from the analysis of control CSF

3.2.1 Control selection according to CSF analysis

It is not ethical to sample CSF in humans unless there are substantial clinical indication for CSF analysis. The control group for this study was selected from a population of 229 consecutive patients admitted to the neuroscience unit for investigation of suspected neurological disease, and this included analysis of CSF. None of these patients had a recent history of SAH or TBI. CSF analysis using spectrophotometry confirmed the absence of xanthochromia. Microscopic examination of the CSF enabled exclusion of CSF containing more than five nucleated cells per cubic millilitre. Analysis of the total protein and albumin concentration of the CSF enabled identification and exclusion of CSF with values beyond the 95% confidence interval of the sample population. The results of the selection of controls according to this analysis are summarised in table 8.

Displaying these data as a column scatter graph illustrates the wide concentration range for apoE in control CSF. See figure 5. Of the 39 CSF samples with normal cell count, and total protein and albumin concentration within the 95% confidence interval of the population mean, the oligoclonal band status was determined in 28, and found to be negative. The mean apoE for this group was 12.4 mg/L (median 12.1, SD: 4.8 mg/L). Inspection of the frequency distribution histogram for the control subgroups shows that the distribution of the mean concentrations of apoE in control CSF is positively skewed. Analyte concentrations are often positively skewed, and appear more Gaussian after log transformation. The advantage of the log transformation is that parametric tests can be used, which are more sensitive than non-parametric tests, and provide a confidence interval (geometric mean ratio). However, the number of patients in the brain injury groups is small, and normality tests become even less reliable with small sample sizes. Data, which passed the normality test, sometimes appeared to be skewed on the frequency distribution histogram, even after log transformation. In addition, there was data, which appeared to be parametrically distributed, but had more than twice the variance of the control group. Therefore, one way analysis of variance was performed using non-parametric tests. One-way analysis of variance using the Kruskall-Wallace test with Dunn`s post- test found no statistically significant difference in the median CSF apoE concentration of these control CSF subgroups.

Table 8 CSF apoE according to biochemical analysis

Control CSF category	Number	Mean ± SD apoE mg/L	Median apoE mg/L	95 % CI
[1]All CSF	229	11.8 ± 7.4	11.3	10.9-12.8
[2]Cell count <5/mm^3	138	13 ± 6.9	12.0	11.9-14.2
[3]Total protein <576 mg/L	117	13.5 ± 6.0	12.8	12.2-14.8
[4]Albumin <273 mg/L	82	12.5 ± 5.5	11.9	10.7-14.3

SD: Standard Deviation.

95 % CI: ninety-five percent confidence interval.

[1] No xanthochromia present but cell count not known in 91 patients

[2] No xanthochromia present, and cell count less than or equal to 5 / mm^3

[3] CSF with mean total protein within 95% CI of the <5 cells/mm^3 group (21 not known)

[4] CSF with mean albumin within 95% CI of the group with <5 cells/mm^3, and total protein within 95%CI (35 not known)

The concentration of apoE in the CSF according to the various indications for lumbar puncture are summarised in table 9. Displaying the raw data as a column scatter plot again shows a wide range for apoE concentration across diagnostic groups and a lower median apoE concentration in CSF from patients with definite MS. As discussed above the frequency distribution histograms of the clinical subgroups also demonstrate that CSF apoE concentration has a positively skewed distribution, and this appears more Gaussian with log transformation. As justified above, for consistency of approach, non-parametric tests were used for analysis of variance. Using the Kruskal-Wallace test, the difference between the median apoE in CSF from patients with definite MS and other control groups is statistically significant ($p<0.001$). There is no significant difference between the median CSF apoE concentration in the other control subgroups.

3.2.2 The relationship between site of CSF sampling and apoE concentration.

The median concentration of apoE in ventricular CSF is higher than that of lumbar CSF, and using the U-Mann-Whitney test, this difference is statistically significant ($p=0.036$). The albumin and total protein concentration was higher in the lumbar CSF, but the difference was not statistically significant. See figure 6, panel A.

3.2.3 The relationship between gender and CSF apoE concentration

The mean (SD) apoE concentration in CSF from female controls (n=57) was 12.5 (6.4) mg/L, and 13.8 (8.1) mg/L in the CSF of (n= 33) males. The difference between the median concentration of apoE in CSF from male versus female controls was not statistically significant. See figure 6, panel B.

3.2.4 The concentration of apoE during continuous CSF drainage

ANOVA found there was no statistically significant change in the concentration of apoE in CSF when sampled daily for seven consecutive days from six patients undergoing continuous CSF drainage for management of CSF rhinorrhoea. There was no significant decrease in CSF apoE concentration *in-vitro* between CSF before and after passage through a ventricular drainage catheter. In order to investigate the possibility that CSF apoE is lost from the supernatant during centrifugation, CSF was assayed before and after centrifugation and was found not to be significantly different. See figure 7.

Table 9 CSF apoE in controls according to indication for CSF analysis

Indication for CSF examination	Number	Mean ± SD apoE mg/L	Median apoE mg/L	95 % CI
[5]No disease identified	14	15.7 ± 9.6	13.4	10.2-21.3
[6]Other	23	14.3 ± 7.5	13.7	11.1-17.6
[7]BIH	7	11.3 ± 7.6	10.4	4.2-18.3
[8]Dementia	82	13.5 ± 6.9	11.5	12.0-15.0
[9]Possible MS	18	12.4 ± 3.9	12.4	10.5-14.3
[10]Probable MS	10	15.2 ± 7.1	11.9	10.1-20.3
[11]Definite MS***	44	6.5 ± 4.5	5.8	5.1-7.9

***P<0.001

SD: Standard Deviation.

95 % CI: ninety-five percent confidence interval.

[5] All Investigations negative

[6] CSF leak, Guillan Barre Syndrome, ?malignancy, ?Vasculitis,

[7] BIH="Benign" Intracranial Hypertension

[8] Dementia=all types (un-specified)

[9] Possible MS

[10] Probable MS

[11] Definate MS

Figure 5 Concentration of apoE in the CSF of non-acutely brain injured subjects

Figure 5A displays the column scatter plots for CSF apoE concentration from potential control subjects categorised according to:

- Nucleated cell count of<5/mm^3.
- Nucleated cell count of<5/mm^3 and CSF total protein within 95th centile of population.
- Nucleated cell count of<5/mm^3, CSF total protein within 95th centile of population, and CSF albumin within 95th centile of population.
- Nucleated cell count of<5/mm^3, CSF total protein within 95th centile of population, CSF albumin within 95th centile of population, and oligoclonal bands absent.

Figure 5B displays column scatter plots for CSF apoE concentration from potential control subjects categorised according to indication for CSF analysis as follows:

- No objective evidence of neurological disease identified.
- *Benign* Intracranial Hypertension (BIH).
- Dementia (type unspecified)
- Possible Multiple Sclerosis (MS).
- Probable MS.
- Definite MS. The concentration of apoE in definite MS CSF is significantly ($p<0.001$) lower than in the other groups.
- Other (e.g. CSF leak).

Horizontal bar represents the median concentration (mg/L)

Figure 5A : CSF apoE concentration according to analysis indices

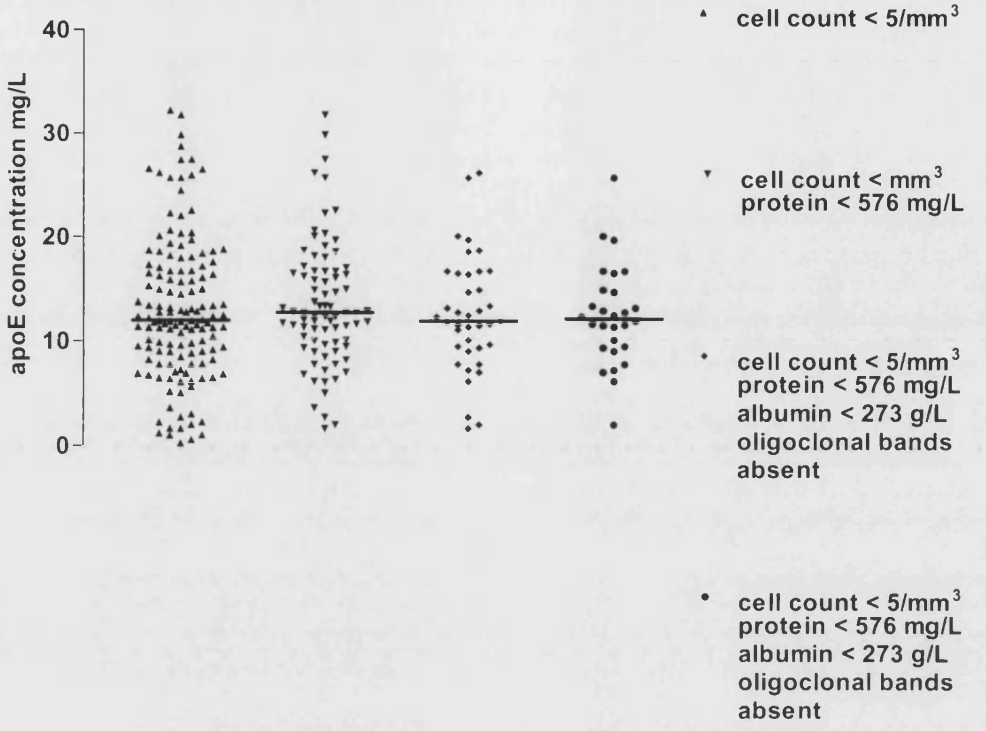


Figure 5B : CSF apoE concentration according indication for lumbar puncture

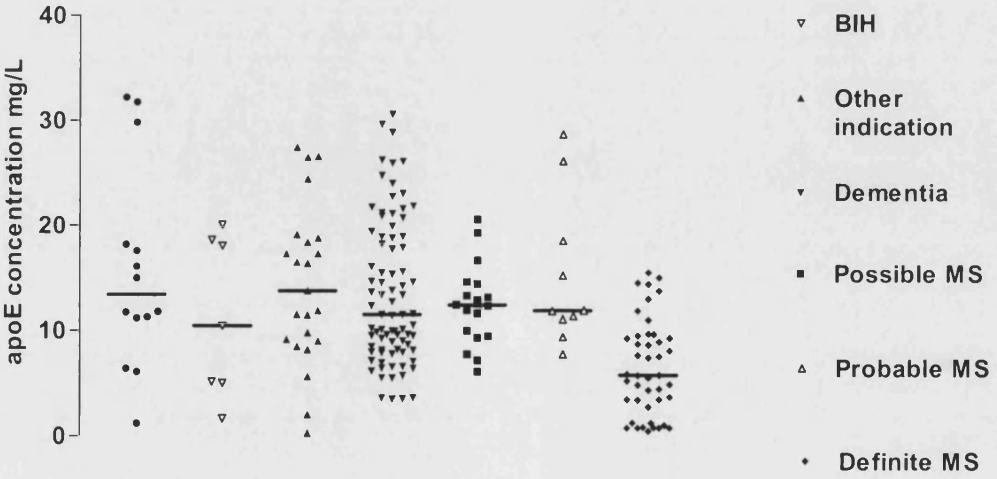


Figure 6 Column scatter plot of CSF apoE concentration according to anatomical sampling site and gender

Figure 6A: Column scatter plot for CSF apoE concentration (mg/L) in lumbar and ventricular CSF from control subjects without evidence for acute brain injury or raised intracranial pressure. The median apoE concentration (represented by horizontal bar) was significantly (Mann-Whitney, p=0.036) higher in ventricular CSF than CSF sampled via the lumbar subarachnoid space. The apoE concentration gradient is the result of intrathecal synthesis of CSF apoE. The nucleated cell count of the ventricular and lumbar CSF samples was $<5/mm^3$. The ventricular CSF albumin and total protein concentrations were within the 95^{th} centile of the control group mean.

Figure 6B: Column scatter plot for CSF apoE concentration (mg/L) in male and female subjects. There is no significant difference in control CSF apoE concentration according to gender.

Figure 6A : CSF apoE concentration according to site of sampling

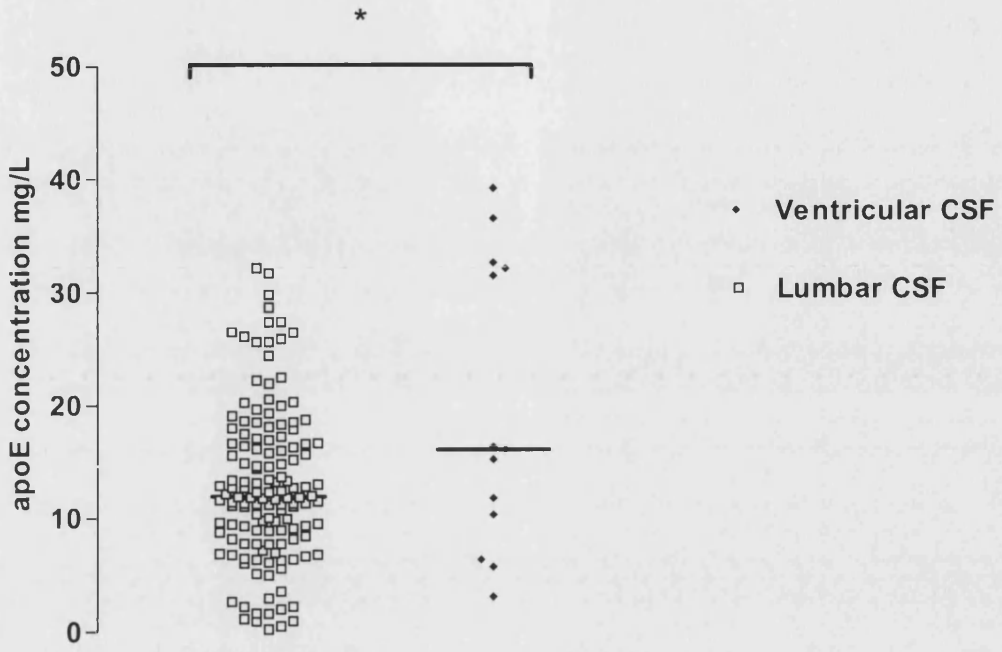


Figure 6B : CSF apoE concentration of male and female controls

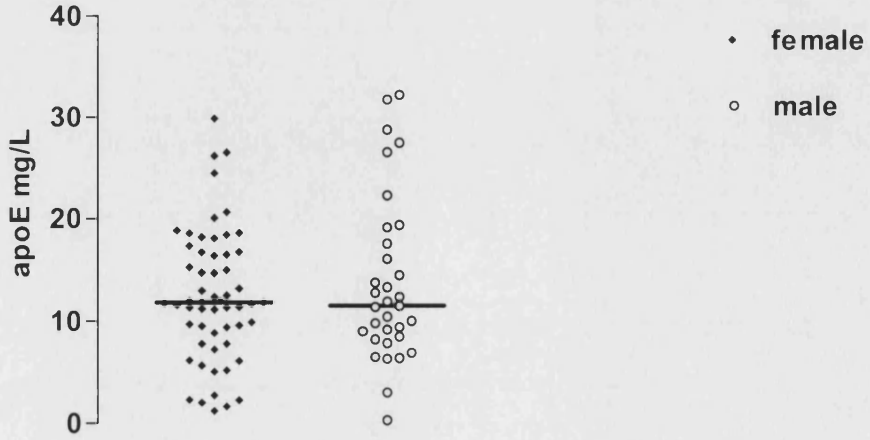


Figure 7 The concentration of CSF apoE during continuous drainage

There was no significant change in CSF apoE concentration relative to the initial value obtained on the first day after initiation of continuous external CSF drainage. The CSF was obtained from patients undergoing continuous CSF drainage as treatment for or prevention of CSF leakage. The patients (n=6) had no neurological deficits, no acute brain injury, and the CSF concentration of albumin and protein was within normal limits.

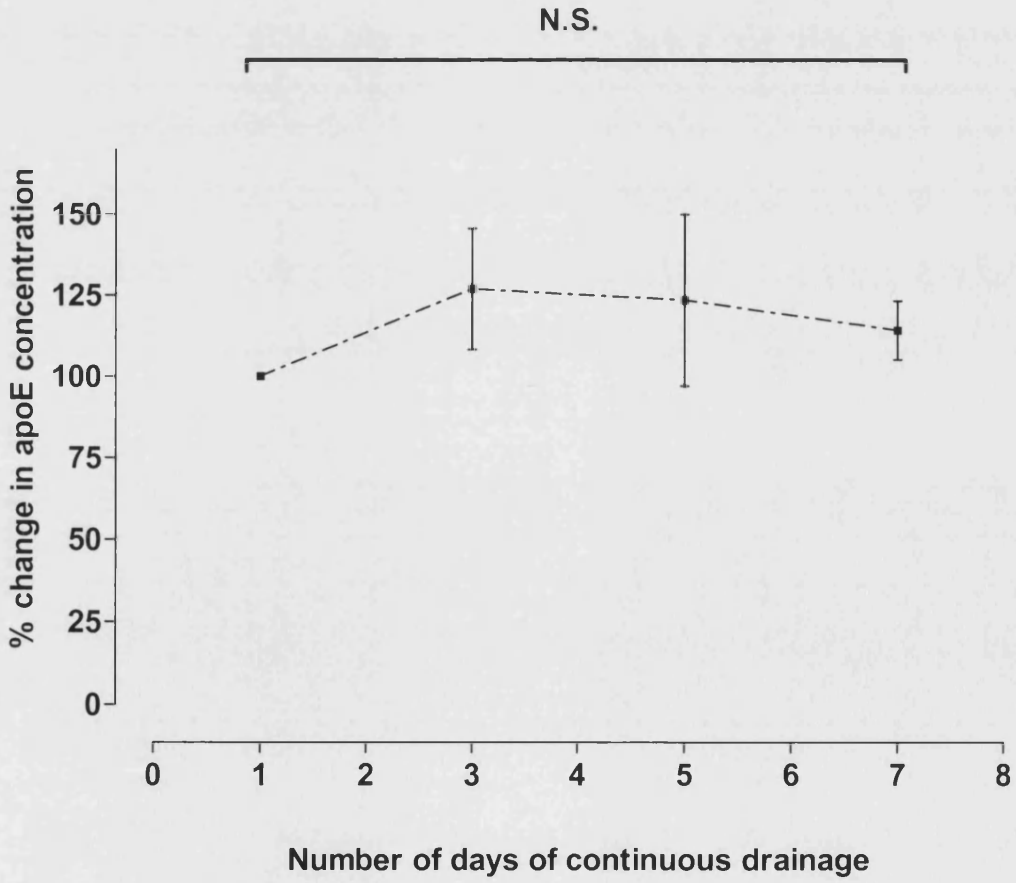


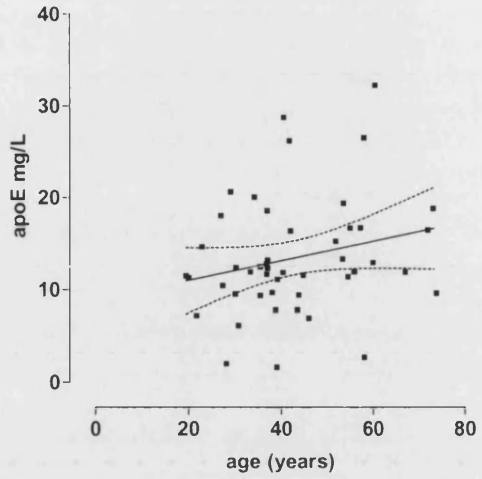
Figure 8 Relationship between CSF apoE, albumin, and apoE to albumin ratio and subject age

Figure 8A: Scatter plot of CSF apoE concentration in controls of different ages, with the linear regression line and the 95% confidence interval for the slope of the regression line (dotted line). Spearman rank correlation failed to reach statistical significance at the $p<0.05$ level.

Figure 8B: Scatter plot of CSF albumin concentration in controls of different ages, with the linear regression line and 95% confidence interval for the slope of the regression line. There was significant ($p< 0.0001$) correlation between CSF albumin concentration and age.

Figure 8C: Scatter plot of CSF apoE to albumin concentration ratio in controls of different ages, with the linear regression line and the 95% confidence interval for the slope of the regression line. Spearman rank correlation failed to reach statistical significance at the $p<0.05$ level.

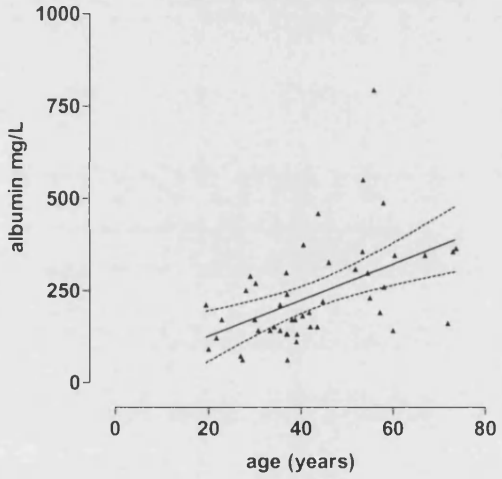
Figure 8A : Relationship between CSF apoE concentration and age



Spearman r = 0.23

p = 0.12

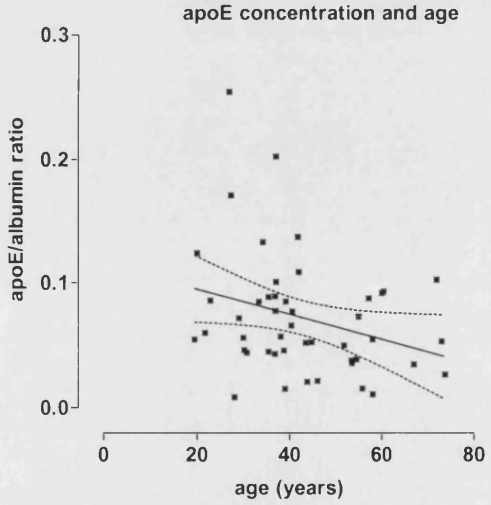
Figure 8B : Relationship between CSF albumin concentration and age



Spearman r = 0.55

p < 0.0001

Figure 8C : Relationship between CSF apoE concentration and age



Spearman r = - 0.27

p = 0.067

3.2.5 The relationship between *APOE* genotype and CSF apoE concentration

There were no ε44 or ε22 controls, and only one ε24 control. There was no significant difference between ε33 (average ± SD apoE = 10.1 ± 4.7 mg/L), ε34 (6.6 ± 4.5 mg/L) and ε23 (7.3 ± 2.4 mg/L) CSF apoE concentration.

3.2.6 The relationship between CSF apoE and albumin concentration

There is no significant rank correlation (Spearman r = 0.267, P = 0.0176, 95% CI: 0.042 to 0.466) between CSF apoE and albumin concentration in 79 controls. Regression analysis suggests that only 2% of the variation in apoE is due to variation in albumin in controls (R^2 = 0.02).

3.2.7 The relationship between age and CSF apoE concentration

The association between age and CSF concentration was determined using rank correlation analysis of a group of 90 control CSF samples with known age, apoE and albumin concentration. There was no significant correlation between CSF apoE and age (r = 0.23, P=0.116). See figure 8, panel A. There was statistically significant correlation between CSF albumin and age (r = 0.55, P<0.0001, 95% CI: 0.30-0.72). See figure 8, panel B The correlation between the ratio of CSF apoE to albumin and age (r = -0.27, P = 0.067, 95% CI: -0.52 to 0.03) just failed to reach statistical significance. See figure 8, panel C. Regression analysis suggests that approximately 5% of the variation in CSF apoE concentration is attributable to age (R^2 = 0.055). In contrast, approximately 25% of the variation in CSF albumin is attributable to age (R^2 = 0.245).

Using the Mann-Whitney test, there is no statistically significant difference between the median apoE/albumin ratio of non-demented controls compared to patients investigated for dementia.

3.2.8 Summary of quantification of control CSF apoE

The concentration range for apoE in the CSF of controls is wide, but the difference between diagnostic groups is not significant except for patients with definite MS. The

concentration of apoE in controls is not significantly affected by age, CSF albumin concentration, *APOE* genotype, or continuous CSF drainage. The concentration of apoE in ventricular CSF is higher than that from the lumbar subarachnoid space.

3.3 The concentration of apoE and brain specific proteins in the CSF after TBI and SAH

This section presents the results of quantification of apoE, and other proteins, in the CSF of TBI, SAH, and control patients. Due to the limited availability of CSF from patients with acute brain injury, particularly TBI, the investigation comprises two data sets. The first is the analysis of twenty-seven TBI, and nineteen SAH patients for whom CSF was available within three days of brain injury. The second data set investigates a subgroup of these patients for whom serial CSF samples were available for up to two weeks after brain injury. Due to the expense of the commercial assay kits used for Aβ and Tau fewer time points were assayed using these kits compared to assays done using *in house* techniques (i.e. apoE, S100B, albumin, and total protein).

3.3.1 CSF protein concentrations within three days of TBI and SAH

3.3.1.1 The control group

The control group used for this analysis was matched for age (mean age 40, median 37, and range 19-73 years) with the acute brain injury group. It was selected from the larger control population of non-xanthochromic CSF samples, with less than five nucleated cells per cubic millilitre, on the basis that oligoclonal bands were known to be absent, and the albumin and total protein concentration were within the 95^{th} centile of the control population mean. This resulted in a control group of similar size (n=28) to the brain injury group. The median concentration of apoE in this control group was 12.1 mg/L.

3.3.1.2 CSF apoE within three days of TBI and SAH

The characteristics of the patients in this TBI and SAH group are summarised in tables 5, 6 and 7. The mean concentration of apoE in the CSF (one CSF sample per patient) sampled from patients within three days of TBI or SAH is approximately one third that of the control group. The median concentration of apoE in the CSF from TBI patients was 1.9 mg/L, and 3.9 mg/L in SAH patients. These data are summarised in table 10. ANOVA using the nonparametric Kruskall-Wallis test, with Dunn`s post test, determined that the

median concentration of apoE in control CSF was significantly higher than in TBI ($P<0.001$), and SAH ($P<0.001$) CSF. In addition Dunn's post test determined that the median concentration of apoE in TBI and SAH CSF was not significantly different. See figure 9.

3.3.1.2.1 Estimation of CSF apoE concentration in control, TBI, and SAH CSF using PAGE

In addition to apoE quantification using ELISA, denaturing PAGE was used to determine the relative amount of apoE in control (n=3), TBI (n=3), and SAH (n=3) CSF as described in methods. Denaturing PAGE is at best semi-quantitative, but is capable of detecting apoE epitopes present due to proteolysis. In addition, because denaturing conditions are used the possibility that signal loss due to epitope masking may be evaluated. We found the signal intensity from the denaturing PAGE approximately correlated with the apoE ELISA determined concentration. No immunoreactive proteolytic fragments were identified. See appendix.

3.3.1.3 The ratio of CSF apoE to albumin within three days of TBI or SAH.

The mean (SD) concentration of albumin in control CSF was 177 (40) mg/L, in SAH CSF was 957 (1610) mg/L, and TBI CSF was 716 (1096) mg/L. Thus, the concentration of albumin in the acute brain injury CSF is approximately three times higher than in the control group. From the Kruskall-Wallis ANOVA the median CSF albumin concentration is significantly increased in TBI ($P<0.001$) and SAH ($P<0.001$) compared to control CSF. Although the CSF albumin concentration after SAH is higher than after TBI, the difference is not statistically significant. The mean (SD) apoE to albumin ratio of control CSF was 0.07 (0.03) and was seven times higher than that in TBI and SAH CSF (mean ratio 0.01, SD: 0.02). Thus, after acute brain injury, compared to controls, the seven-fold decrease in the apoE to albumin ratio is disproportionate to the three-fold increase in albumin concentration. From the ANOVA for the apoE to albumin ratio, the ratio is significantly decreased after TBI ($P<0.001$ and SAH $P<0.001$) but the difference between TBI and SAH is not significant. See figure 10.

Table 10 ApoE, S100B, albumin and total protein in controls, SAH and TBI CSF

CSF Concentration	Patient group		
	Control N=28	SAH N=19	TBI N=27
ApoE (mg/L)	12.4 (12.1) ± 4.7 [0.34]	4.6 (3.9) ± 3.8 [0.13]	3.7 (1.9) ± 4.2 [0.10]
S100B (μg/L)	0.39 (0.29) ± 0.37 [0.018]	19.9 (5.8) ± 30.1 [1.5]	23.3 (14.7) ± 21.7 [1.76]
Albumin (mg/L)	177 (171) ± 40 [2.7]	957 (359) ± 161 [14.5]	716 (330) ±1096 [10.8]
Total protein (g/L)	0.32 (0.27) ± 0.12.	1.97 (1.81) ± 1.34.	3.34 (1.57) ± 5.37

Mean (median) CSF protein concentration ± standard deviation is given. Micromolar (μM) concentration is given in square brackets.

Figure 9 CSF apoE and S100B concentration in controls and patients within three days of TBI and SAH

Figure 9A: Column scatter plot of apoE concentration in control CSF and CSF from patients within three days of TBI or SAH. Horizontal bar represents the group median.

Figure 9B: Column scatter plot of S100B (Log concentration) in control CSF and CSF from patients within three days of TBI or SAH. From ANOVA using the Kruskall-Wallis test the median concentration of apoE and S100B in TBI ($p<0.001$) and SAH ($p<0.001$) CSF is significantly different from controls. CSF apoE is less than controls after acute brain injury contrasting S100B, which is increased. The controls were age matched lumbar CSF samples from patients without acute brain injury with no objective clinical or biochemical evidence of CNS disease.

Figure 9A : ApoE concentration in SAH, TBI and control CSF

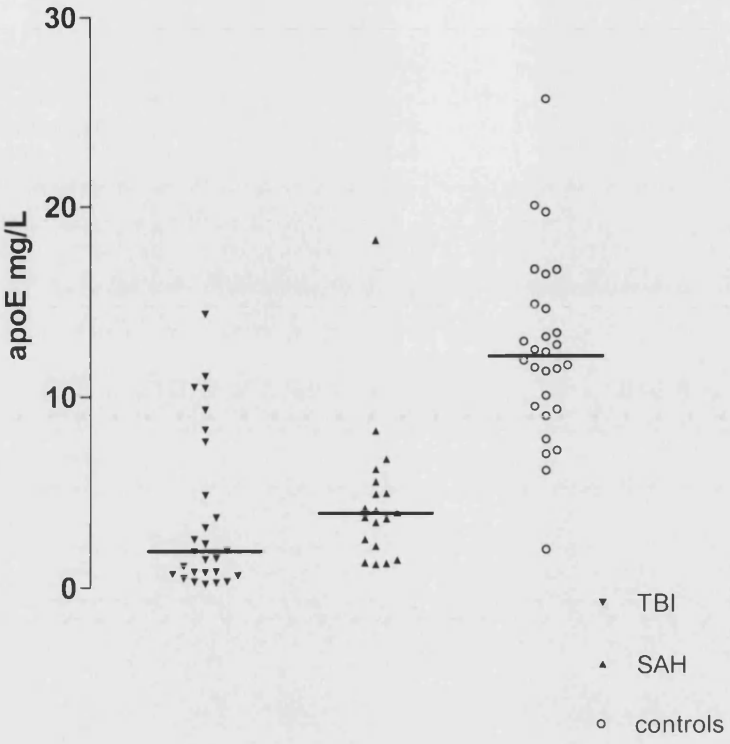


Figure 9B : S100B concentration in SAH, TBI and control CSF

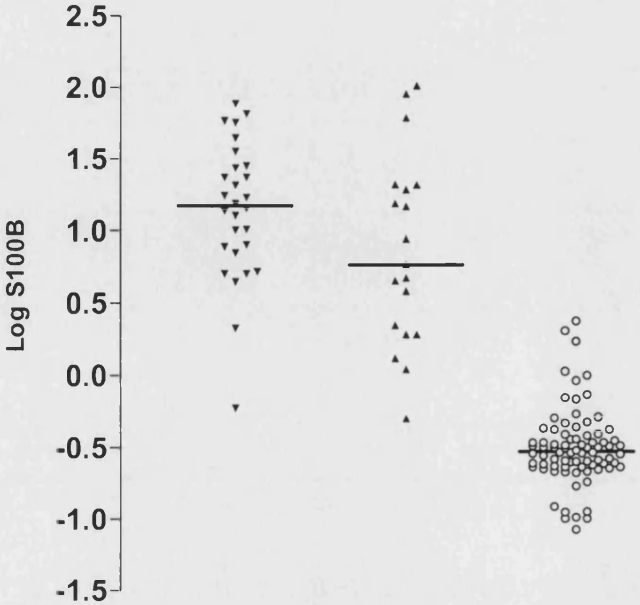


Figure 10 Ratio of apoE/albumin and apoE/ total protein in CSF fom controls and patients within three days of TBI and SAH

Figure 10A: Column scatter plot of apoE/ albumin concentration ratio in CSF from controls and patients within three days of TBI and SAH.

Figure 10B: Column scatter plot of apoE/ total protein concentration ratio in CSF from controls and patients within three days of TBI and SAH.

From ANOVA using the Kruskall-Wallis test the ratio for TBI and SAH CSF is significantly different ($p<0.001$) from controls for each ratio. After TBI or SAH CSF albumin and total protein concentration increase compared to control CSF. Despite the release of plasma apoE into CSF after brain injury, CSF apoE concentration is decreased. The controls were age matched lumbar CSF samples from patients without acute brain injury with no objective clinical or biochemical evidence of CNS disease.

Figure 10A : CSF apoE/albumin ratio in patients with SAH, TBI and controls

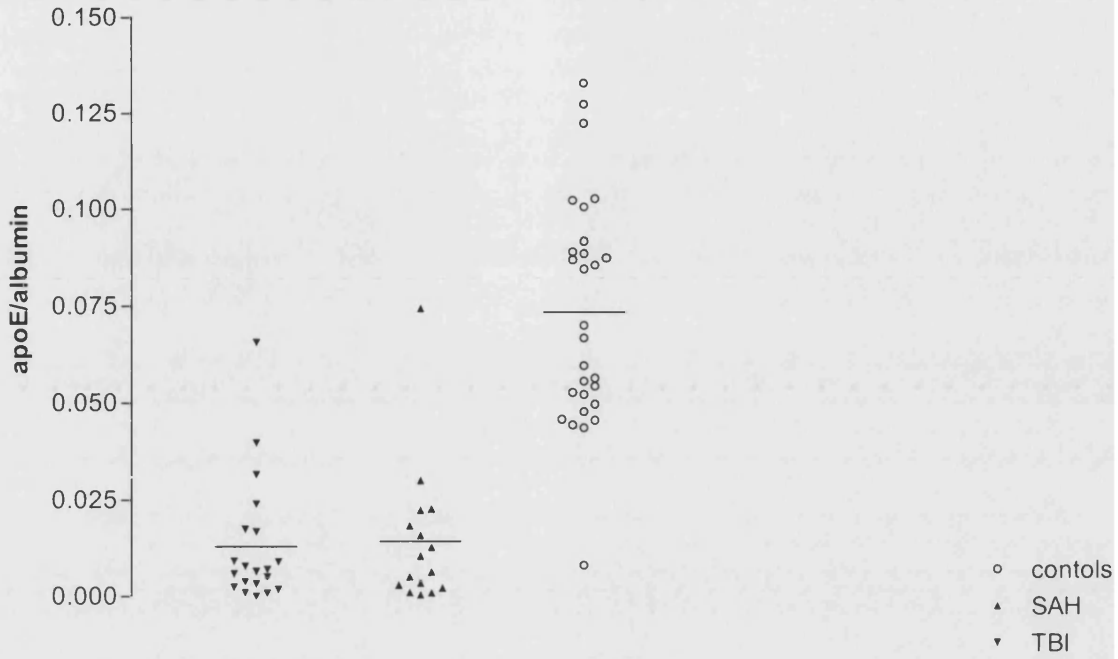


Figure 10B : CSF apoE/total protein ratio in patients with SAH, TBI and controls

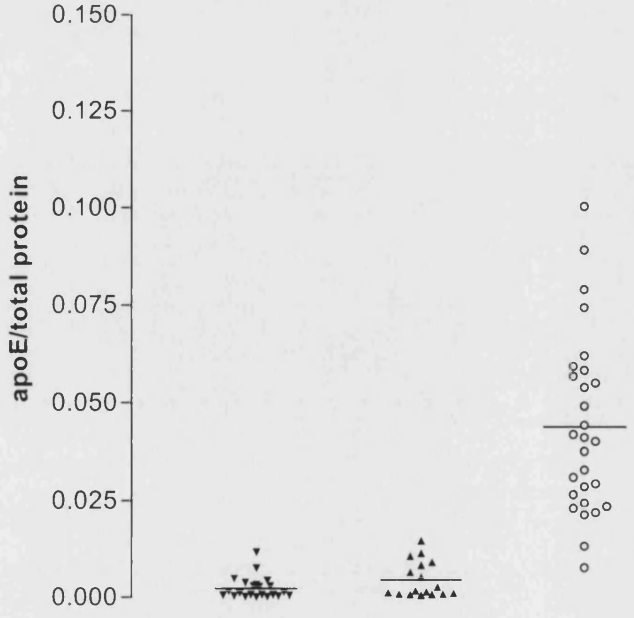


Figure 11 Relationship between CSF apoE, injury severity and clinical outcome

Figure 11A: Relationship between CSF apoE concentration and best recorded level of consciousness (GCS) within 24 hours of SAH.

Figure 11B: Relationship between CSF apoE concentration and clinical outcome (GOS) three months after SAH.

Figure 11C: ApoE concentration in the CSF of patients with favourable and unfavourable outcome after SAH. Patients with unfavourable outcome scores have significantly lower CSF apoE within three days of SAH (Mann-Whitney, $p=0.03$).

Figure 11A : Relationship between CSF apoE and GCS after SAH

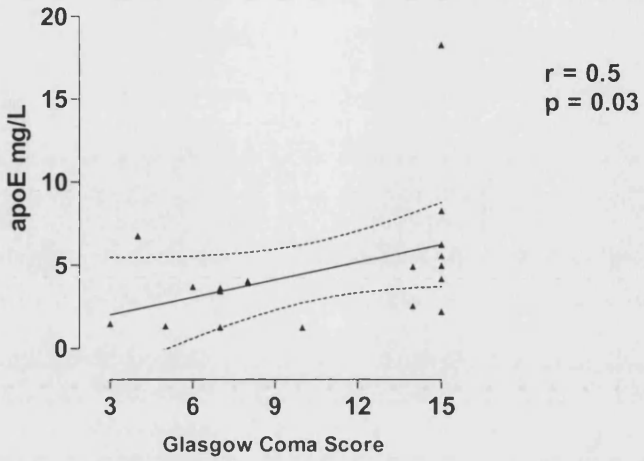


Figure 11B : Relationship between CSF apoE and GOS after SAH

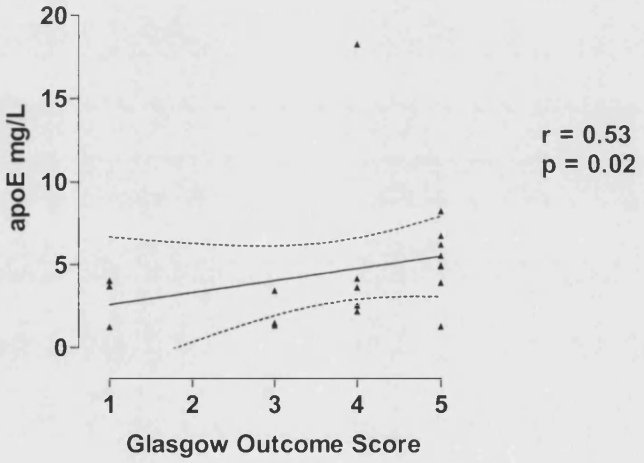
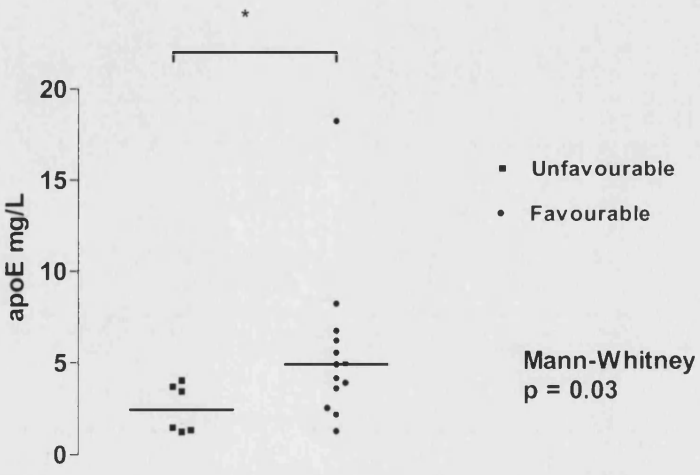


Figure 11C : CSF apoE concentration after SAH and clinical outcome



3.3.1.4 The ratio of CSF apoE to total protein within three days of TBI or SAH

Within three days of injury, the mean concentration of total protein in the CSF is substantially higher than that of the control group. The mean (SD) concentration of total protein in control CSF was 324 (273) mg/L, in SAH CSF was 1974 (1343) mg/L, and TBI CSF was 3399 (5367) mg/L. From the Kruskall-Wallis ANOVA the median CSF protein concentration is significantly increased after TBI ($P<0.001$) and SAH ($P<0.001$) compared to control CSF. Although the total protein concentration is substantially higher in TBI CSF compared to SAH CSF, the difference is not statistically significant. The mean (SD) apoE to total protein ratio in control CSF was 0.044 (0.023), in SAH CSF was 0.0045 (0.0046), and in TBI CSF was 0.0023 (0.0028). Thus after SAH, there is approximately six fold greater total protein concentration and ten fold decrease in the apoE to total protein ratio. After TBI, the total protein concentration increases by order of magnitude but there is nearly a twenty fold decrease in the apoE to total protein ratio. From the ANOVA for the apoE to total protein ratio, the ratio is significantly decreased after TBI ($P<0.001$), and SAH ($P<0.001$), but the difference between TBI and SAH is not significant. Thus the reduction in the CSF apoE after brain injury is substantially greater than the increase in total protein. See figure 10.

3.3.1.5 The concentration of S100B in the CSF within three days of TBI and SAH

CSF S100B increased substantially after acute brain injury. The mean (SD) concentration of S100B in control CSF was 0.39 (0.37) μg/l, in SAH CSF was 19 (30) μg/l, in TBI CSF was 23 (22) μg/l. ANOVA using the Kruskall-Wallis test determined that there was a statistically significant difference between group medians ($P<0.0001$). Dunn's multiple comparison test determined this difference to be significant for TBI ($P<0.001$) and SAH ($P<0.001$) compared to controls, but not between TBI and SAH. See figure 9.

3.3.1.6 The relationship between CSF apoE and S100B within three days of injury and GCS and GOS

The proportion of patients in coma (GCS<8) after TBI was significantly higher than the proportion after SAH (Fishers' exact test, $p = < 0.0001$). The injury severity (GCS) after TBI did not correlate significantly with clinical outcome (GOS). The correlation between GCS and GOS after SAH failed to reach statistical significance (Spearman $r = 0.4$, 95%

CI: -0.08 to 0.73, p = 0.09). There was no significant correlation between CSF S100B and apoE, GCS, or GOS after TBI or SAH. There was no significant correlation between CSF apoE and GCS, or GOS, and there was no significant difference between the concentration of apoE (or S100B) in the CSF of patients with favourable versus unfavourable outcome after TBI. After SAH, there was significant correlation between CSF apoE concentration and injury severity (Spearman r = 0.5, p<0.03, 95% CI: 0.04-0.78). See figure 11, panel A. However, there was no significant difference between the concentration of apoE in the CSF of SAH patients in coma, and those with GCS of 9-15. There was significant correlation between the concentration of apoE in the CSF of SAH patients and clinical outcome (Spearman r = 0.53, p = 0.018, 95% CI: 0.09-0.80). See figure 11, panel B. The concentration of apoE in the CSF of SAH patients with unfavourable outcome was significantly lower than the concentration of apoE in the CSF of patients with favourable outcome (Mann-Whitney: p = 0.03). The proportion of patients with unfavourable outcome and CSF apoE concentration below the lowest control value, was significantly greater than those with favourable outcome (Fishers` exact test: p = 0.02). See figure 11, panel C. The sample size was too small to determine any possible influence of *APOE* genotype upon any of these data.

3.3.1.7 Summary of findings

The concentration of apoE in the CSF within three days of acute brain injury is substantially less than that of controls. The concentration of albumin, total protein, and S100B increase substantially. The decrease in apoE is far greater than the increase in albumin and total protein. Decreased CSF apoE concentration is associated with more severe injury and outcome after SAH. The results from the investigation of the time course of these changes in apoE are presented in the next section, and are related to changes in other proteins.

3.3.2 Temporal alterations in CSF proteins after TBI and SAH

In addition to the proteins assayed at one time point within three days of injury, Tau, $A\beta_{1-40}$, and $A\beta_{1-42}$ were assayed in serial CSF samples from the nineteen SAH patients and a subgroup of thirteen TBI patients for whom serial samples were available. The characteristics of the patient subgroups are summarised in tables 5,6 and 7. The proportion of patients in coma (GCS<8) after TBI was significantly (Fishers exact test p<0.0001) greater than after SAH. The median concentration of protein at each time point was compared to the control group median using non-parametric ANOVA, assuming that the

concentration of the various proteins in the CSF of non-brain injured individuals does not vary significantly with time. From analysis of the change in concentration with time after injury, the maximum or minimum value for each patient protein time series was identified and used to determine the time from brain injury to the minimum/maximum value. In addition the maximum or minimum values were used to investigate the relationship between injury severity and outcome with the magnitude of the change in protein concentration. The control group comprised 13 patients (mean age 36, median 32, range 16-61 years) with suspected shunt dysfunction, or chronic hydrocephalus, with no history of acute brain injury or impaired conscious level, requiring drainage/examination of ventricular CSF. CSF found to have a cell-count greater than five cells per millilitre, xanthochromia, or albumin and total protein concentration above the 95th centile of the population, were excluded to produce a group of controls with "normal" ventricular CSF parameters as far as was feasible.

3.3.2.1 The change in CSF concentration of apoE with time after TBI and SAH

After TBI, the decrease in CSF apoE concentration compared to controls was not statistically significant one day after injury, but was significant on day two ($p<0.01$), day three ($p<0.001$), day four ($p<0.0001$) and day five ($p<0.05$). After SAH, the decrease in CSF apoE concentration compared to controls was not significant on day two and days five to twelve. However, the decrease was statistically significant ($p<0.05$) on days three and four after SAH. See figures 12 and 13, panel A.

3.3.2.2 The change in CSF concentration of albumin and total protein with time after TBI and SAH

One day after TBI, CSF albumin concentration increased significantly ($p<0.05$) compared to control values. The increase in albumin after TBI is not statistically significant from day two to five. After SAH, the CSF albumin concentration is significantly ($p<0.0001$) increased, compared to controls, on days one to three, and on day five, but not on day four or days six to eleven. The apoE to albumin ratio is significantly decreased compared to the control group on days two to five after TBI ($p<0.01$), and after SAH on days two and three ($p<0.001$), and day nine ($p<0.05$). The SAH apoE to albumin ratio on day ten is not significantly different from the controls. See figures 12 and 13, panel B.

Figure 12 Time course for alterations in the CSF apoE concentration and the ratio of apoE to albumin and total protein after TBI

Figure 12A: Serial column scatter plot of apoE concentration (mg/L of CSF) after TBI.

Figure 12B: Serial column scatter plot for ratio of CSF apoE to albumin concentration. Mean (± SD) CSF albumin concentration of control group was 135 (37) mg/L. Mean (± SD) albumin concentration in TBI CSF was: day 1 = 454 (457), day 2 = 221 (164), day 3 = 193 (138), day 4 = 181 (154) and day 5 = 170 (144) mg/L.

Figure 12C: Serial column scatter plot for ratio of CSF apoE to total protein concentration after TBI. Mean (± SD) CSF total protein concentration of control group was 313 (112) mg/L. Mean (± SD) total protein concentration in TBI CSF was: day 1 = 2773 (1532), day 2 = 995 (671), day 3 = 1170 (1621), day 4 = 683 (577) and day 5 = 761 (667) mg/L.

Non-parametric ANOVA was used to compare the median concentration (represented by horizontal bar) of apoE in the CSF of the TBI patients at each time point with the control group median. No comparison was made between serial samples from the TBI group. Differences that are not statistically significant are summarised as ns (i.e. $p>0.05$), * represents $p<0.05$, ** represents $p = 0.01\text{-}0.001$, *** represents $p<0.001$. The CSF albumin, and total protein, concentration increased significantly after TBI and then decreased towards normal thereafter. The control CSF is from the ventricles of age matched subjects without acute brain injury and CSF profile within normal limits (i.e. no xanthochromasia, < 5 nucleated cells per ml of CSF, and normal biochemical profile including S100B etc).

Figure 12A : CSF apoE after TBI

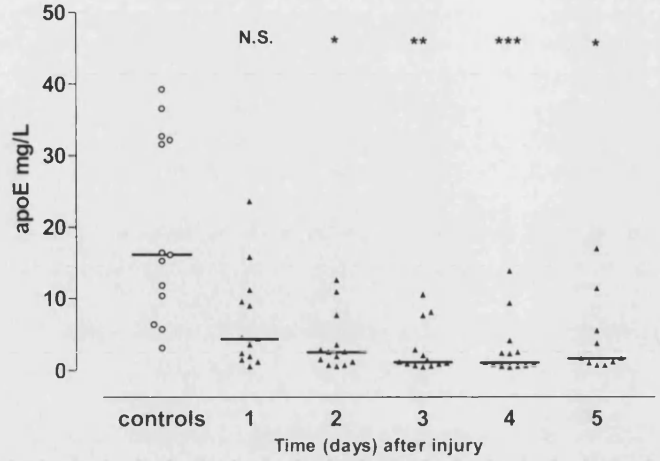


Figure 12B : CSF apoE/albumin ratio after TBI

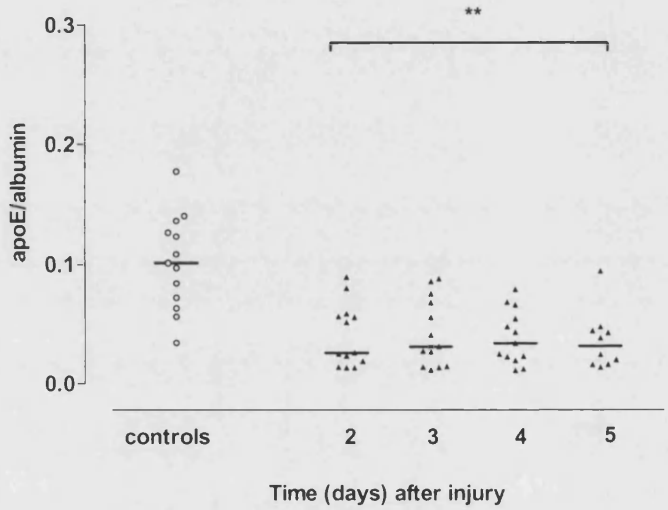


Figure 12C : CSF apoE/total protein ratio after TBI

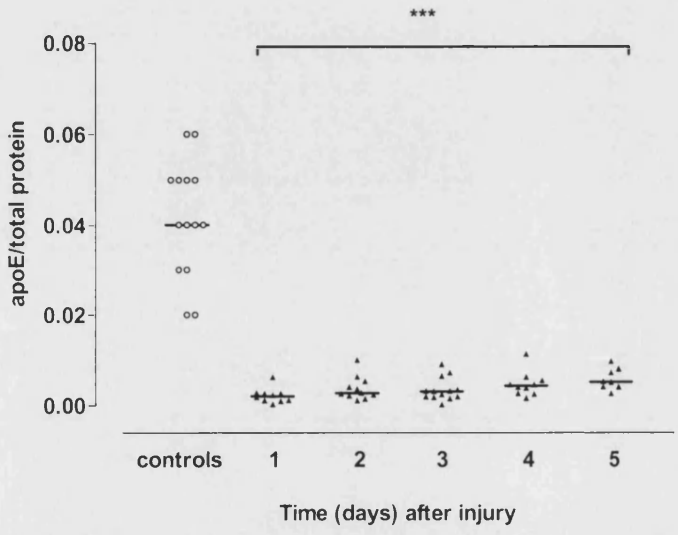


Figure 13 Time course for alterations of CSF apoE concentration and the ratio of apoE to albumin and total protein after SAH

Figure 13A: Serial column scatter plot of apoE concentration (mg/L of CSF) after SAH.

Figure 13B: Serial column scatter plot for ratio of CSF apoE to albumin concentration after SAH. Mean (± SD) CSF albumin concentration of control group was 135 (37) mg/L. Mean (± SD) albumin concentration in SAH CSF was: day 2 = 1815 (1723), day 3 = 531 (507), day 4 = 530 (499), day 5 = 618 (303), day 6 = 357 (260) and day 7 = 306 (262) mg/L.

Figure 13C: Serial column scatter plot for ratio of CSF apoE to total protein concentration after SAH. Mean (± SD) CSF total protein concentration of control group was 313 (112) mg/L. Mean (± SD) total protein concentration in SAH CSF was: day 2 = 2214 (1574), day 3 = 1750 (1074), day 4 = 1121 (747), day 5 = 1239 (1035), day 6 = 1163 (1141) and day 7 = 867 (572) mg/L.

Non-parametric ANOVA was used to compare the median concentration (represented by horizontal bar) of apoE in the CSF of the SAH patients at each time point with the control group median. No comparison was made between serial samples from the SAH group. Differences that are not statistically significant are summarised as ns (i.e. $p>0.05$), * represents $p<0.05$, ** represents $p = 0.01\text{-}0.001$, *** represents $p<0.001$. The CSF albumin, and total protein, concentration increased significantly after SAH and then decreased towards normal thereafter. The control CSF is from the ventricles of age matched subjects without acute brain injury and CSF profile within normal limits (i.e. no xanthochromasia, < 5 nucleated cells per ml of CSF, and normal biochemical profile including S100B etc).

Figure 13A : CSF apoE after SAH

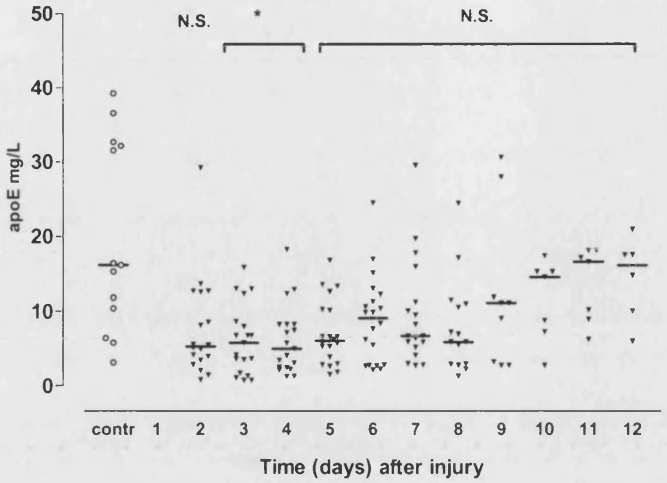


Figure 13B : CSF apoE/albumin ratio after SAH

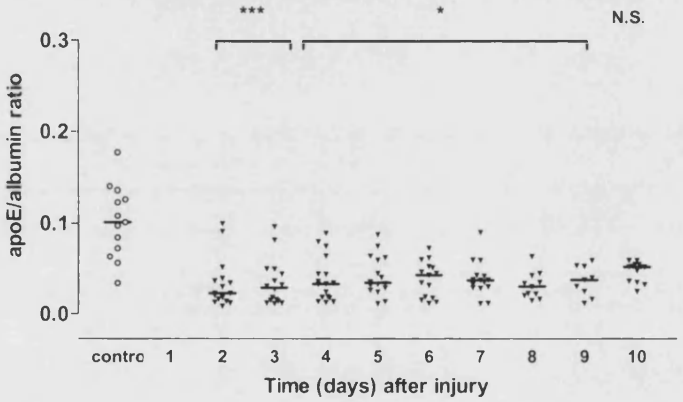
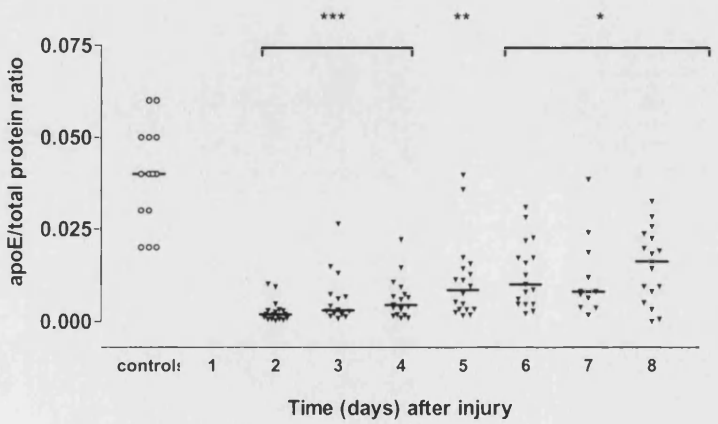


Figure 13C : CSF apoE to total protein ratio after SAH



Compared to controls, there is significantly increased total protein concentrations in TBI CSF ($p<0.001$) on day one after injury, but not on days two to five. After SAH, the increase in total protein is significant on days one to four ($p<0.001$), on days four and five ($p<0.01$), and days six and seven ($p<0.05$). After SAH the increase in total protein is not statistically significant from days eight to ten. After TBI, the apoE to total protein ratio is significantly decreased on days one to four ($p<0.001$), but not on day five. After SAH, the decrease in apoE total protein ratio is statistically significant on days two to eight ($p<0.001$ on days two, three and four; $p<0.01$ on day five; $p<0.05$ on day six and seven). See figures 12 and 13, panel C.

3.3.2.3 The change in CSF concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ with time after TBI and SAH

There is a significant decrease in CSF $A\beta_{1-40}$ concentration after TBI (day one $p<0.001$, day three $p<0.01$, day five $p<0.05$) and SAH (day three $p<0.001$, day seven $p<0.01$) compared to controls. After TBI, the decrease in $A\beta_{1-42}$ is not statistically significant on days one and three after injury, but is significant on day five ($p<0.05$). After SAH, $A\beta_{1-42}$ concentration is significantly decreased compared to the controls on day three ($p<0.01$) and day seven ($p<0.05$). See figure 14. The ratio of $A\beta_{1-40}$ is not significantly different from the control group ratio for all time points investigated in TBI and SAH CSF.

3.3.2.4 The change in CSF concentration of S100B and Tau with time after TBI and SAH

After TBI, there is statistically significant increase ($p<0.001$) in CSF S100B concentration compared to non injured controls for all the time points investigated. After SAH, S100B is significantly increased on days one ($p<0.001$) to nine, after which the increase is not statistically significant. See figures 15 and 16. After TBI, there is significant (day one $p<0.001$, days three and five $p<0.01$) increase in CSF Tau concentration compared to non injured controls. After SAH, the increase in Tau is significant ($p<0.001$) for all time points investigated. See tables 11 and 12.

Figure 14 Time course for alterations in CSF $A\beta_{1-40}$ and $A\beta_{1-42}$, concentration after TBI and SAH

Figure 14A: Serial column scatter plots of $A\beta_{1-40}$ concentration (ng/L of CSF) in control and TBI CSF.

Figure 14B: Serial column scatter plots of $A\beta_{1-42}$ concentration (ng/L of CSF) in control and TBI CSF.

Figure 14C: Serial column scatter plots of $A\beta_{1-40}$ concentration (ng/L of CSF) in control and SAH CSF.

Figure 14D: Serial column scatter plots of $A\beta_{1-42}$ concentration (ng/L of CSF) in control and SAH CSF.

Non-parametric ANOVA was used to compare the median concentration of Aβ peptides in the CSF of the TBI or SAH patients at each time point (represented by horizontal bar) with the control group median. Differences that are not statistically significant are summarised as N.S. (i.e. $p>0.05$), * represents $p<0.05$, ** represents $p = 0.01\text{-}0.001$, *** represents $p<0.001$. There was no significant difference between the ratio of $A\beta_{1-40}$ to $A\beta_{1-42}$ in control and TBI or SAH CSF.

Figure 14A : CSF $A\beta_{1-40}$ after TBI

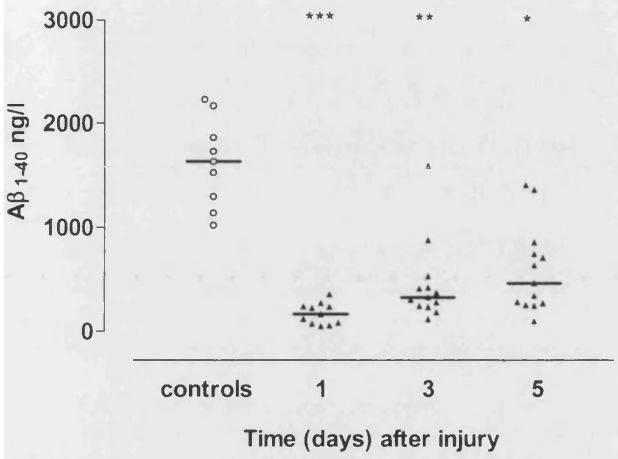


Figure 14B : CSF $A\beta_{1-42}$ after TBI

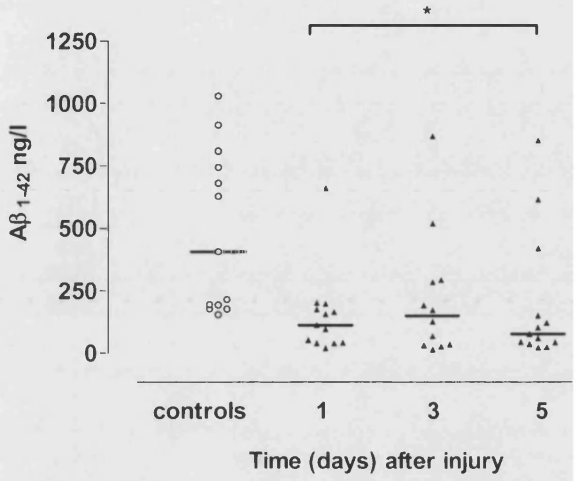


Figure 14C : CSF $A\beta_{1-40}$ after SAH

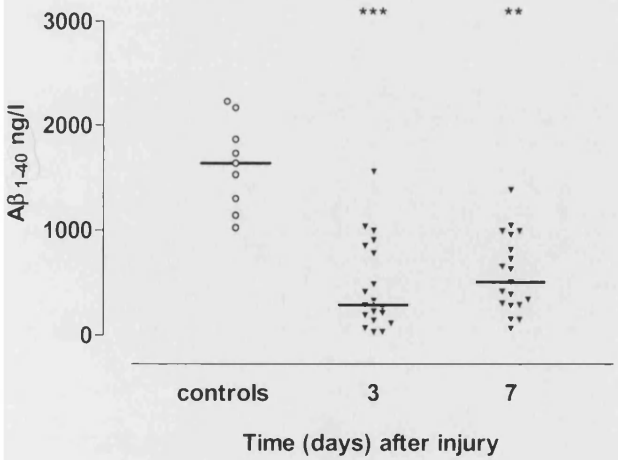


Figure 14D : CSF $A\beta_{1-42}$ after SAH

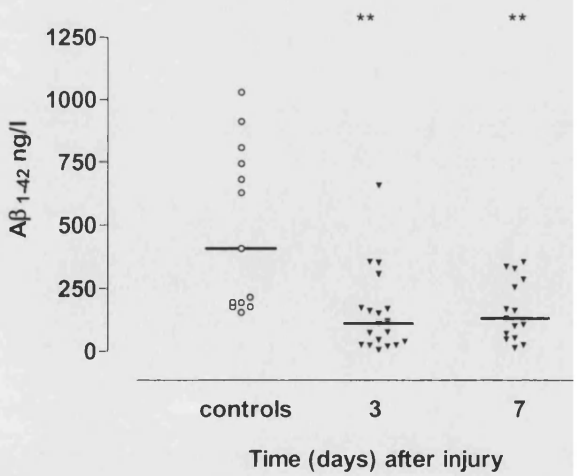


Figure 15 Time course for alterations in CSF S100B, and Tau concentration after TBI

Figure 15A: Serial column scatter plot of CSF S100B (Log concentration) after TBI and controls.

Figure 15B: Serial column scatter plot of CSF Tau (Log concentration) after TBI and controls.

Non-parametric ANOVA was used to compare the median concentration of S100B or Tau in the CSF of the TBI patients at each time point (represented by horizontal bar) with the control group median. S100B concentration of TBI CSF was elevated ($p = 0.01\text{-}0.001$) at each time point compared to the control group. Tau was also elevated (day 2: $p<0.001$, day 3 and 5: $p = 0.01\text{-}0.001$) compared to the control group.

Figure 15A : S100B in control and serial TBI CSF

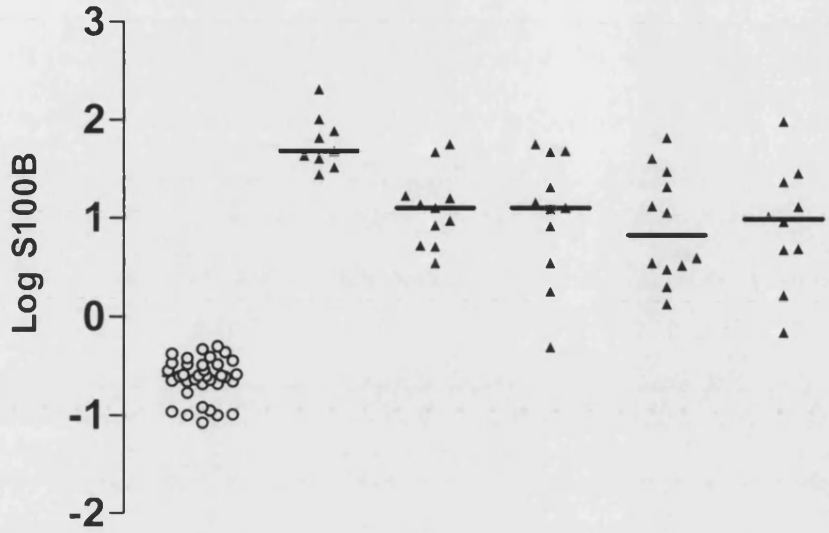


Figure 15B : Tau in control and serial TBI CSF

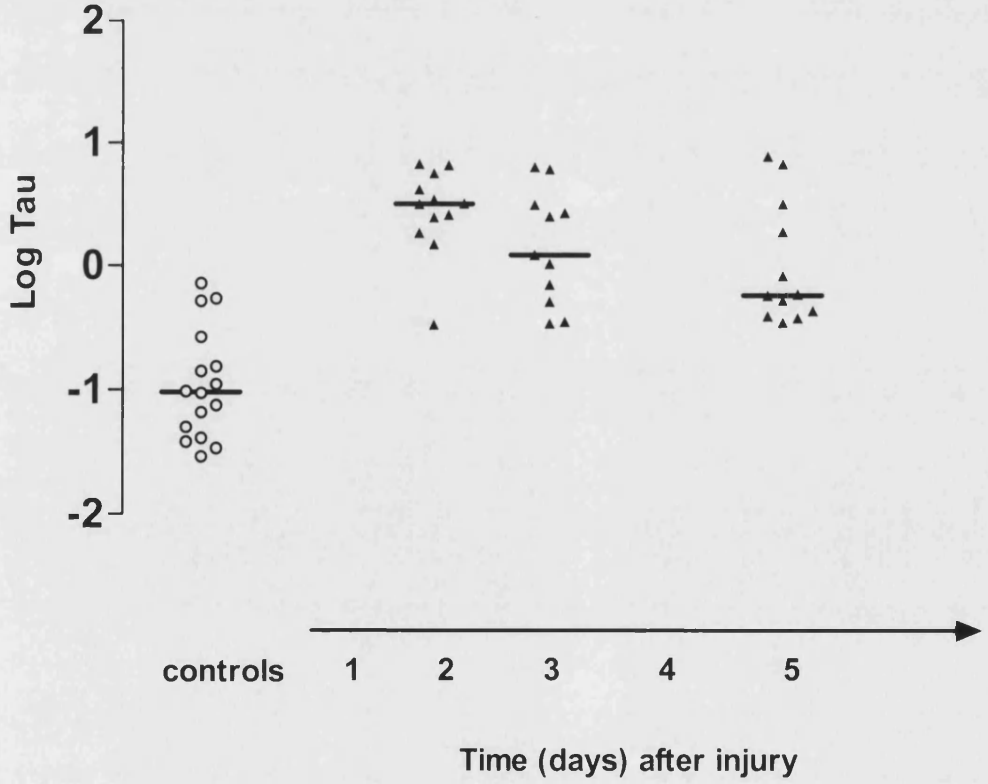


Figure 16 Time course for alterations in CSF S100B, and Tau concentration after SAH

Figure 16A: Serial column scatter plot of CSF S100B (Log concentration) after SAH and controls.

Figure 16B: Serial column scatter plot of CSF Tau (Log concentration) after SAH and controls.

Non-parametric ANOVA was used to compare the median concentration of S100B or Tau in the CSF of the SAH patients at each time point (represented by horizontal bar) with the control group median. S100B concentration of SAH CSF was elevated ($p = 0.01$-0.001) at each time point compared to the control group. Tau was also elevated ($p = 0.01$-0.001) at each time point compared to the control group.

Figure 16A : S100B in control and serial SAH CSF

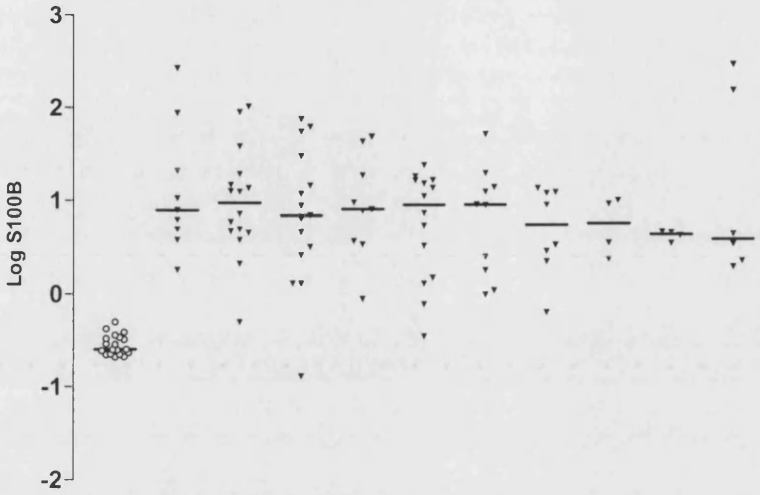


Figure 16B : Tau in control and serial SAH CSF

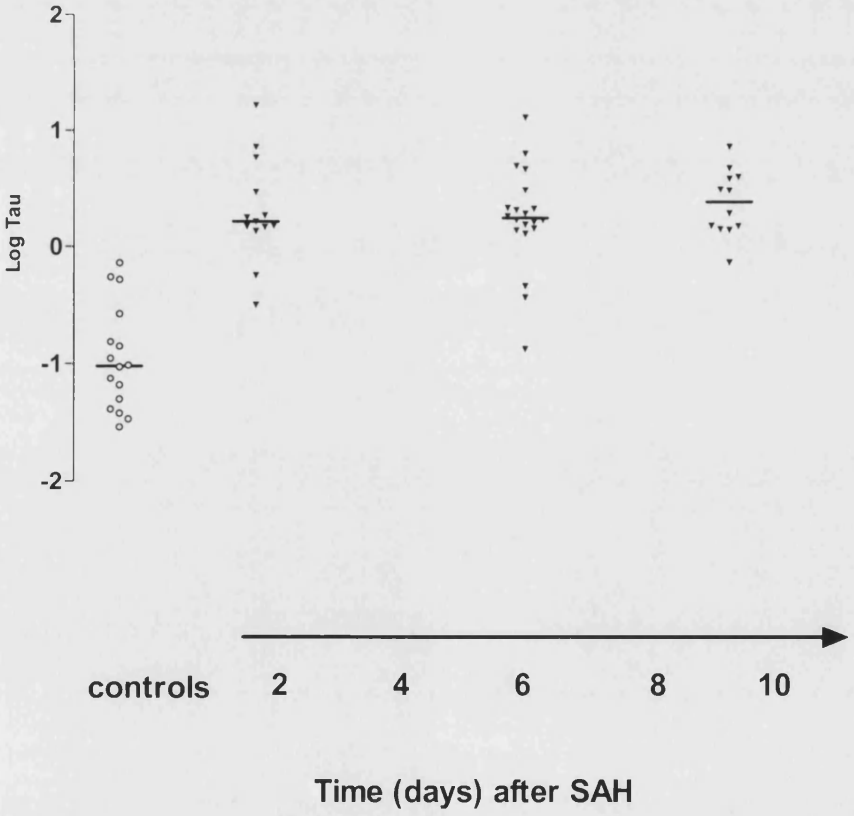


Table 11 CSF Tau and S100B in controls and serial TBI samples

Protein concentration Mean ± SD	control	Time after TBI				
		Day 1	Day 2	Day 3	Day 4	Day 5
Tau μg/L	0.19 ± 0.2	[a]	3.5 ± 2.0	2.3 ± 2.2	a	2.0 ± 2.6
S100B μg/L	0.26 ± 0.1	70.7 ± 55	17.5 ± 14.4	20.4 ± 20.1	16.3 ± 19.6	19.0 ± 18.2

[a] Not determined due to limited sample volume

S100B and Tau concentrations were significantly elevated after TBI for each time point compared to the control value using non-parametric ANOVA ($p<0.001$).

Table 12 CSF Tau and S100B in controls and serial SAH samples

Protein concentration Mean ± SD	control	Time after SAH				
		Day 2[b]	Day 3	Day 4	Day 5	Day 9
Tau µg/L	0.19 ± 0.2	3.4 4.4	[a]	2.7 ± 2.9	a	2.9 ± 1.9
S100B µg/L	0.26 ± 0.1	55.7 ± 45	19.7 ± 30.5	14.4 ± 17.6	9.6 ± 7.7	12.3 ± 15.3

[a] Not determined due to limited sample volume

[b] Limited SAH CSF day 1 after SAH.

S100B and Tau concentrations were significantly elevated after SAH for each time point compared to the control value using non-parametric ANOVA ($p<0.001$).

3.3.2.5 Time elapsed between brain injury and maximal change in protein concentration in CSF

The greatest decrease in apoE (apoE $_{min}$), compared to controls, occurred earlier after TBI (3.4 days) than apoE $_{min}$ after SAH (5.2 days) (P = 0.003, 95% CI: 0.4-3.3 days). The greatest decrease in $A\beta_{1-40}$ ($A\beta_{40min}$), compared to the controls, occurred 2 days after TBI, and is not significantly earlier than $A\beta_{40min}$ after SAH (3.9 days). The greatest decrease in $A\beta_{1-42}$ ($A\beta_{42min}$), compared to the controls, occurred 2.9 days after TBI, and is not significantly earlier than $A\beta_{42min}$ after SAH (4.2 days). The greatest increase in S100B (S100B ^{max}), compared to controls, occurred 2.2 days after TBI, and is not significantly earlier than S100B ^{max} after SAH (3.3 days). The greatest increase in Tau (Tau ^{max}), compared to controls, occurred 2.5 days after TBI, and is significantly (P = 0.008, 95% CI: 0.7- 4.3) earlier than Tau ^{max} after SAH (5 days). See figures 17.

After TBI there was statistically significant (r = 0.8, 95% CI: 0.44-0.94, P = 0.001) correlation between the magnitude of the reduction in CSF $A\beta_{1-40}$ concentration, and the time elapsed between injury and trough levels. Other maximal changes did not correlate significantly with time after injury.

3.3.3 The relationship between CSF protein concentration changes after TBI and SAH

Multiple correlation analysis found no statistically significant association between the minimum concentration of apoE in the CSF after TBI, and the maximal changes in other proteins. These data are presented in the form of a correlation matrix (contains r-values) in table 13 for TBI, and table 14 for SAH. There was significant correlation between trough apoE and $A\beta_{1-40}$ after SAH (r = 0.85, $p<0.0001$). See figure 18.

3.3.4 The relationship between CSF proteins, injury severity, and outcome

3.3.4.1 The relationship between CSF protein concentration and injury severity

The relationship between CSF protein concentration and injury severity was determined by generating a correlation matrix for Glasgow Coma Score and the maximal change in CSF concentration after TBI and SAH. This is summarised in table 15 for TBI, and SAH. After

TBI, $A\beta_{40min}$ correlated significantly (Spearman r = 0.69, P = 0.0123, 95% CI: 0.17-0.89) with injury severity measured using the GCS. It is estimated that approximately 47% of the variation in CSF $A\beta_{40min}$ is attributable to variation in severity of injury, or vice versa. There was no statistically significant correlation between injury severity and $apoE_{min}$ after SAH. This contrasts with the finding in the CSF samples analysed within three days of haemorrhage where apoE correlated with injury severity and outcome. From the scatter diagram of $apoE_{min}$ versus injury severity (and outcome), there are a greater number of low apoE concentrations in the less severely injured patients, and these low values occur more than three days after injury. Injury severity correlated with Tau^{max} (Spearman r = -0.8, P = 0.008, 95% CI:-0. 85 to -0.19) after SAH. Approximately 64 % of the variation in CSF Tau and 25% of the variation in apoE is attributable to variation in injury severity after SAH. There was no statistically significant correlation between the other proteins and best GCS. Comparing the proportion of patients in coma versus the proportion with a GCS>8 is not possible in the TBI group as they are all categorised as severely injured. Dichotomising this group into GCS 3-5, and GCS 6-8, identified no difference in the proportion of patients with S100B, or Tau, above the highest control value (all above the highest control), and there was no significant difference in CSF concentration between the injury severity subgroups. Similarly, for apoE, $A\beta_{40min}$, and $A\beta_{42min}$, there was no significant difference in the protein concentration, or proportions below the minimum control value, between severe injury subgroups. After SAH, injury severity according to the GCS ranged from 3 to 15. There was no significant difference in the proportion of patients with protein concentrations above or below the limits of the control values and the proportion in coma or with a GCS>8. However, there were no SAH patients in coma with CSF $A\beta_{1-42}$ concentration above the minimum control value, and the difference in proportions just failed to reach statistical significance (Fishers` exact test: p = 0.06). There was no statistically significant difference between the protein concentration of the SAH patients in coma versus those with GCS>8, although the difference in CSF Tau concentration between injury severity groups just failed to reach statistical significance (Mann-Whitney: p = 0.07).

Figure 17 Column bar graph comparing the time elapsed between brain injury and the maximal change in CSF protein concentration after TBI and SAH

Figure 17A: Column bar chart of time elapsed between brain injury and trough apoE, $A\beta_{1-40}$, and $A\beta_{1-42}$ levels after TBI and SAH. From the Mann-Whitney test, trough apoE and $A\beta_{1-40}$ levels occurred significantly earlier in TBI CSF compared to SAH CSF

Figure 17B: Column bar chart of time elapsed between brain injury and peak S100B and Tau levels after TBI and SAH. From the Mann-Whitney test, peak Tau levels occurred significantly earlier in TBI CSF compared to SAH CSF

Error bars represent the 95% confidence interval.

Figure 17A : Time elapsed between brain injury and minimum CSF concentration of apoE and Aβ peptides

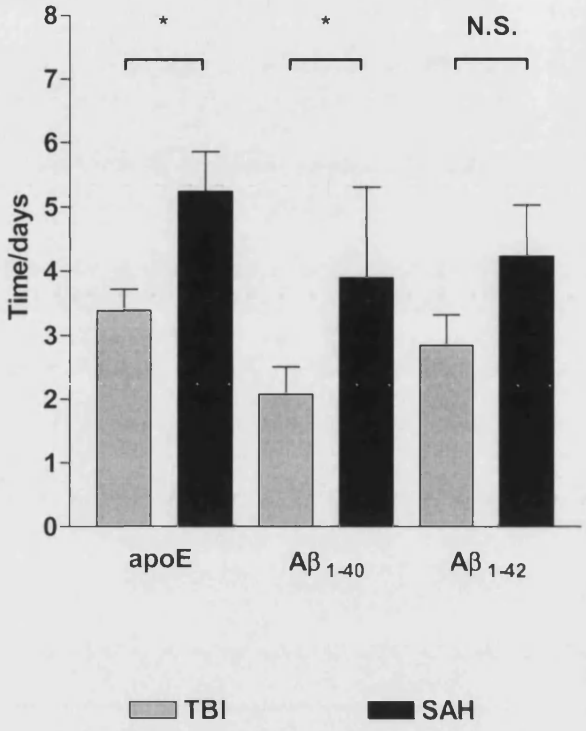


Figure 17B : Time elapsed between brain injury and maximum CSF concentration of S100B and tau

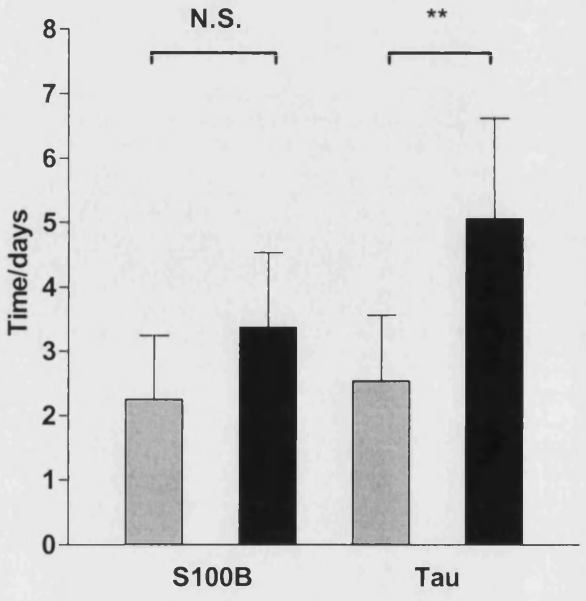


Table 13 Correlation matrix for protein concentration change after TBI

	$S100B_{max}$	Tau_{max}	$A\beta_{1-40\ min}$	$A\beta_{1-42\ min}$	$ApoE_{min}$
$S100B_{max}$	1	-0.18	-0.12	0.19	0.13
Tau_{max}	-0.18	1	0.09	0.07	0.55
$A\beta_{1-40\ min}$	-0.12	0.09	1	-0.05	0.08
$A\beta_{1-42\ min}$	0.19	0.07	-0.05	1	0.09

Table 14 Correlation matrix for protein concentration change after SAH

	$S100B_{max}$	Tau_{max}	$A\beta_{1-40\ min}$	$A\beta_{1-42\ min}$	$ApoE_{min}$
$S100B_{max}$	1	0.44	-0.45	-0.47	-0.34
Tau_{max}	0.44	1	-0.64	-0.60	-0.18
$A\beta_{1-40\ min}$	-0.45	-0.64	1	0.79	0.85
$A\beta_{1-42\ min}$	-0.47	-0.60	0.79	1	0.25

Figure 18 Relationship between trough CSF apoE and trough $A\beta_{1-40}$ after SAH

There is significant correlation between the trough concentration of apoE and $A\beta_{1-40}$ in the CSF after SAH.

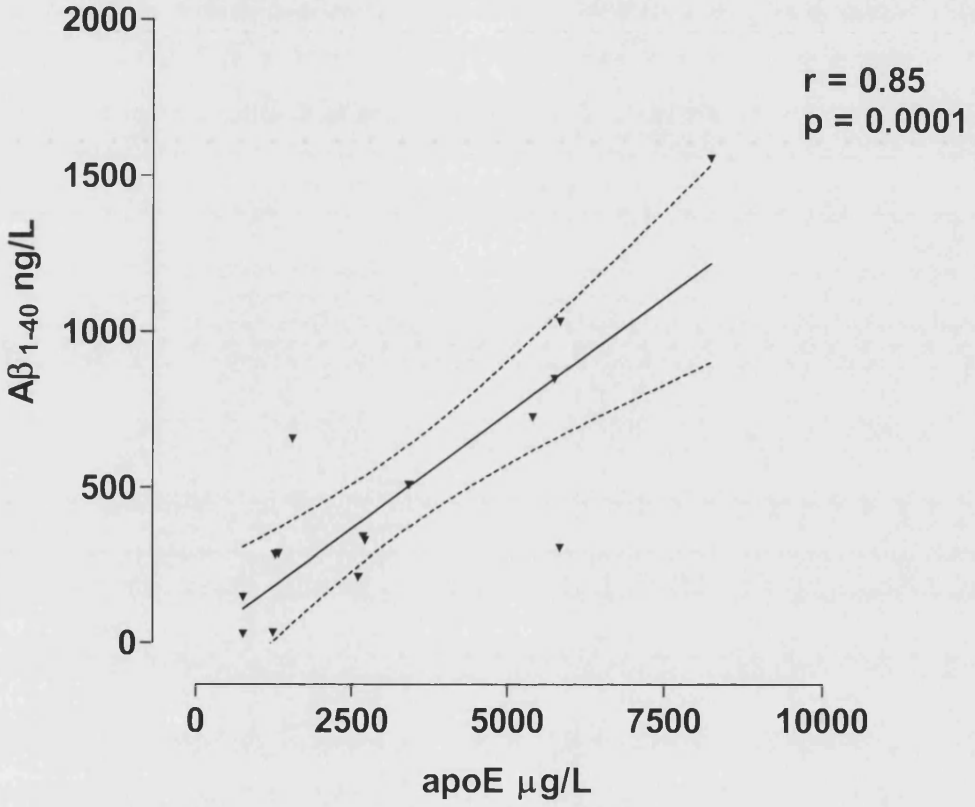


Figure 19 Relationship between CSF $A\beta_{1-40}$ and injury severity after TBI and CSF Tau and injury severity after SAH

The maximal change in CSF concentration after injury was plotted against Glasgow Coma Score (GCS) in the form of a scatter diagram for all of the proteins analysed. Dotted line represents the 95% CI of the slope of the regression line.

Figure 19A:Relationship between trough CSF $A\beta_{1-40}$ concentration and injury severity after TBI. All TBI patients had severe (GCS <8) injury.

Figure 19B:Relationship between peak CSF Tau concentration and injury severity after SAH.

Figure 19A : Relationship between trough CSF A β_{1-40} concentration after TBI and injury severity (GCS)

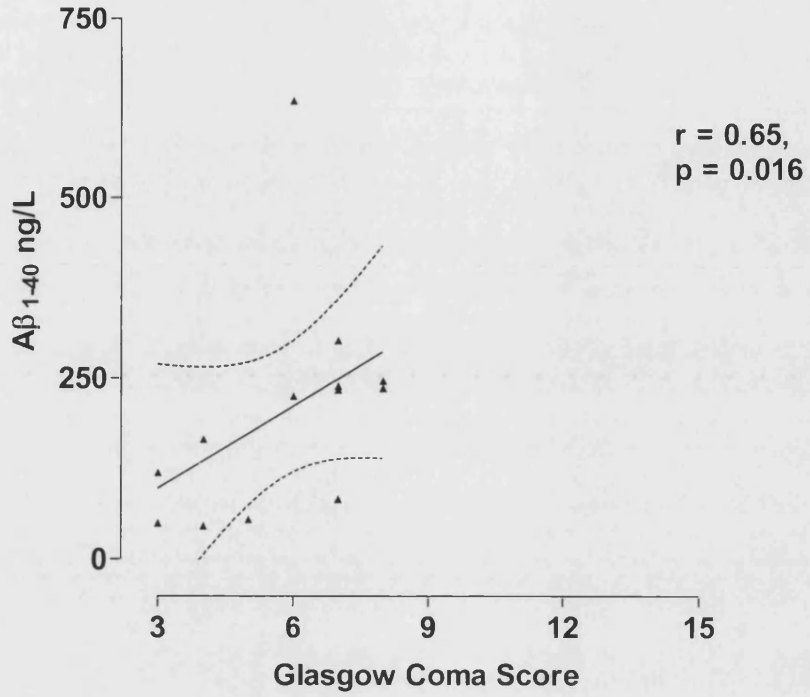


Figure 19B : Relationship between peak CSF tau concentration after SAH and injury severity (GCS)

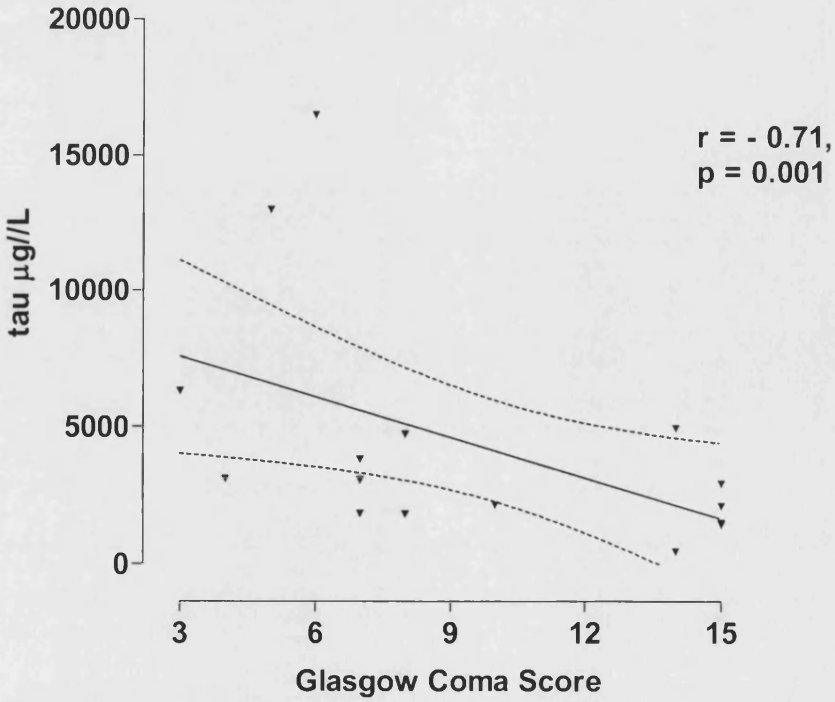


Table 15 Correlation matrix for maximal change in CSF proteins and GCS

	GCS TBI	GCS SAH
$ApoE_{min}$	-0.35	-0.021
$S100B_{max}$	0.135	-0.53
Tau_{max}	-0.25	-0.8
$A\beta_{1\text{-}40\ min}$	0.69	0.27
$A\beta_{1\text{-}42\ min}$	0.04	0.47
$Albumin^{max}$	-0.36	-0.37
$Total\ protein^{max}$	-0.58	-0.08

3.3.4.2 The relation between CSF protein concentration and clinical outcome

There was significant correlation (Spearman r = -0.89, P = 0.0013) between S100B ^{max} and clinical outcome (GOS) six months after TBI. It is estimated that approximately 80% of the variation in TBI CSF S100B is attributable to variation in clinical outcome, or vice versa. Three months after SAH, S100B ^{max} (Spearman r = -0.5, P = 0.029, 95% CI: -0.78 to - 0.04), Tau ^{max} (Spearman r = -0.63, P = 0.007, 95% CI: -0.85 to - 0.19), $A\beta_{40min}$ (Spearman r = 0.73, P = 0.015, 95% CI: 0.13 to 0.87), and $A\beta_{42min}$ (Spearman r = 0.84, P= 0.002, 95% CI: 0.34 to 0.93), correlated with the GOS. These data are summarised in table 16. Albumin and total protein did not correlate significantly with GOS. There was no significant correlation between injury severity (GCS) and outcome (GOS) after TBI. After SAH, GOS just failed to correlate significantly with GCS (p = 0.09). The head injured patients were more severely injured and a higher proportion had unfavourable outcomes after TBI then SAH (Fishers exact test p = 0.036). The proportion of patients with CSF protein concentration above or below the control limit was not significantly different between TBI patients with favourable versus unfavourable outcome. The concentration of the proteins in the CSF was not significantly different between outcome categories. After SAH, no patients with unfavourable outcome had $A\beta_{42min}$ above the minimum control CSF value. However, the proportion of SAH patients with CSF $A\beta_{42min}$ below the lowest control value just failed to reach statistical significance (Fishers exact test p = 0.07).

Table 16 Correlation matrix for maximal change in CSF proteins and GOS

	GOS TBI	GOS SAH
$ApoE_{min}$	-0.73	0.56
$S100B_{max}$	-0.89	-0.5
Tau_{max}	-0.00	-0.63
$A\beta_{1\text{-}40\ min}$	0.37	0.73
$A\beta_{1\text{-}42\ min}$	-0.17	0.84
$Albumin^{max}$	-0.41	-0.75
$Total\ protein^{max}$	-0.26	-0.60

Figure 20 Relationship between CSF $A\beta_{1-42}$, S100B, and Tau and clinical outcome after SAH

The maximal change in CSF concentration after injury was plotted against Glasgow Outcome Score (GOS) in the form of a scatter diagram. The dotted line represents the 95% confidence interval of the slope of the regression line. GOS 4 and 5 equate to favourable (independent) recovery (assessed three months after SAH); GOS 2 and 3 equate to dependent outcome; GOS 1 equates to fatal outcome.

Figure 20A:Relationship between trough CSF $A\beta_{1-42}$ concentration and clinical outcome after SAH.

Figure 20B:Relationship between peak CSF S100B (Log) concentration and clinical outcome after SAH.

Figure 20C:Relationship between peak CSF Tau (Log) concentration and clinical outcome after SAH.

Figure 20A : Relationship between trough CSF $A\beta_{42}$ concentration and clinical outcome (GOS) after SAH

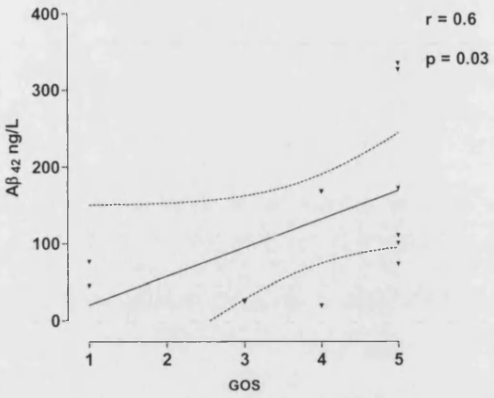


Figure 20B : Relationship between peak CSF S100B concentration after SAH and clinical outcome (GOS)

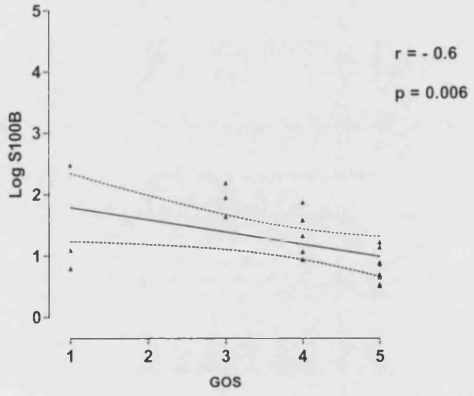
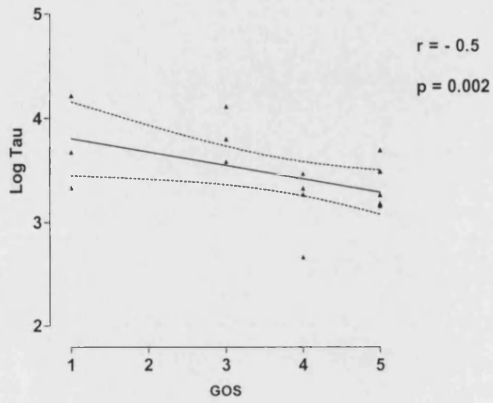


Figure 20C : Relationship between peak CSF Tau concentration after SAH and clinical outcome (GOS)



There were significant differences between the median concentrations of Tau^{max} (Mann-Whitney: p = 0.039), $A\beta_{40min}$ (Mann-Whitney: p = 0.014), and $A\beta_{42min}$ (Mann-Whitney: p = 0.011) in the outcome groups after SAH.

3.3.4.3 The relationship between the temporal changes in CSF protein concentration and injury severity and outcome

Within 48 hours of admission all TBI patients had a best GCS recording of eight or less (severe head injury). Injury severity was dichotomised into groups with GCS 3-5, and GCS 6-8. The proportion of patients in each severity group were then compared according to CSF levels above or below the minimum control value for apoE and Aβ, and maximum control Tau or S100B at each time point using Fisher's exact test. There was no statistically significant difference in the proportion of TBI patients with CSF protein concentrations above or below the control limits at each time point after injury. The SAH patients were dichotomised into groups of GCS 3-8, and 9-15, and analysed as above. Again there was no statistically significant differences in CSF protein concentration over time according to injury severity. The same analysis was undertaken for clinical outcome, dichotomising GOS into favourable outcome (GOS 4, 5), and unfavourable outcome (GOS 1,2,3). For S100B and Tau, there were no brain injury CSF values below the maximum value of the control group, which prevents the comparison of proportions. In these circumstances the threshold selected was the median of the brain injury values. Thus the contingency table comprised patients categorised according to favourable and unfavourable outcome (columns), and CSF S100B (or Tau) above or below the brain injury group median (rows). There was significantly increased relative risk of unfavourable outcome after SAH for patients with CSF S100B above the SAH group median concentration on day three (Fishers` exact test: p = 0.017, RR of favourable outcome: 0.23, 95% CI: 0.04-1.36) to day seven (Fishers` exact test: p = 0.046, RR of favourable outcome: 0.39, 95% CI: 0.12-1.25). The difference in proportions was not significant beyond one week after injury. There was a significant increase in the relative risk of unfavourable outcome after SAH for patients with CSF Tau above the SAH group median on days six (Fishers` exact test: p = 0.027, RR of favourable outcome: 0.24, 95% CI: 0.04-1.44) and day nine (Fishers` exact test: p = 0.048, RR of favourable outcome: 0.25, 95% CI: 0.05-1.36) after injury.

3.3.5 Summary of CSF protein concentration changes after TBI and SAH

The concentration of apoE in the CSF after TBI and SAH is substantially lower than that of non-brain injured controls. The reduction is apparent within three days of injury, and

persists for at least one week after injury. Other proteins increase in the CSF after acute brain injury with the exception of the Aβ peptides. For some proteins the magnitude of the maximal alteration in CSF concentration correlates with injury severity, and clinical outcome.

3.4 Characterisation of lipoprotein particles in control, TBI and SAH CSF

The first section of this chapter presents the results from the development of a robust ELISA method for the measurement of apoE in control and brain injury CSF. The previous two sections present the results of the quantification of apoE in the CSF of non brain injured controls, and patients with TBI and SAH using this ELISA. It was found that the concentration of some proteins in the CSF increased after TBI and SAH compared to controls. In contrast, the concentration of apoE (and Aβ) decreased. As apoE is a major component of CSF lipoprotein particles, studies were undertaken to investigate whether reduced concentration of the apoE protein in the CSF are paralleled by changes in the CSF lipoprotein particles after brain injury. This section presents the findings from the isolation and analysis of lipoprotein particles isolated from control, TBI, and SAH CSF.

Prior to the isolation and analysis of lipoprotein particles in the CSF of non-brain-injured controls and patients with SAH or TBI, the size exclusion chromatography column was calibrated using plasma lipoprotein particles of known size and composition.

3.4.1 Characterisation of size exclusion chromatography column

Plasma derived lipoprotein particles (VLDL, LDL, and HDL), were freshly prepared by sequential density ultracentrifugation, and mixed in equivalent proportions. A volume of 500μl was eluted at a rate of 0.25ml/min in fractions of 250μl. These fractions were assayed for apoB, total cholesterol (TC), and phospholipid (PL) as described in methods. In addition protein was assayed by measuring the absorbance of the fraction at 280nm. See figure 21.

Figure 21 Characterisation of size exclusion chromatography column using plasma lipoproteins

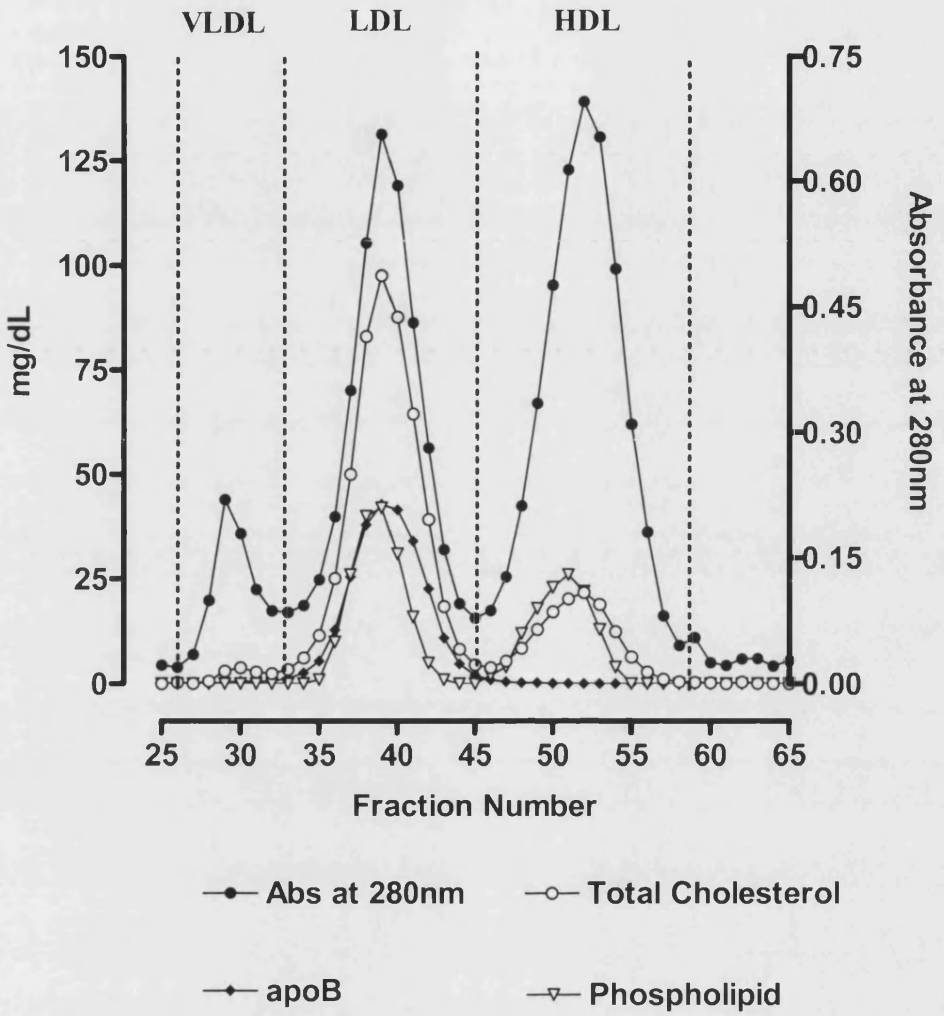
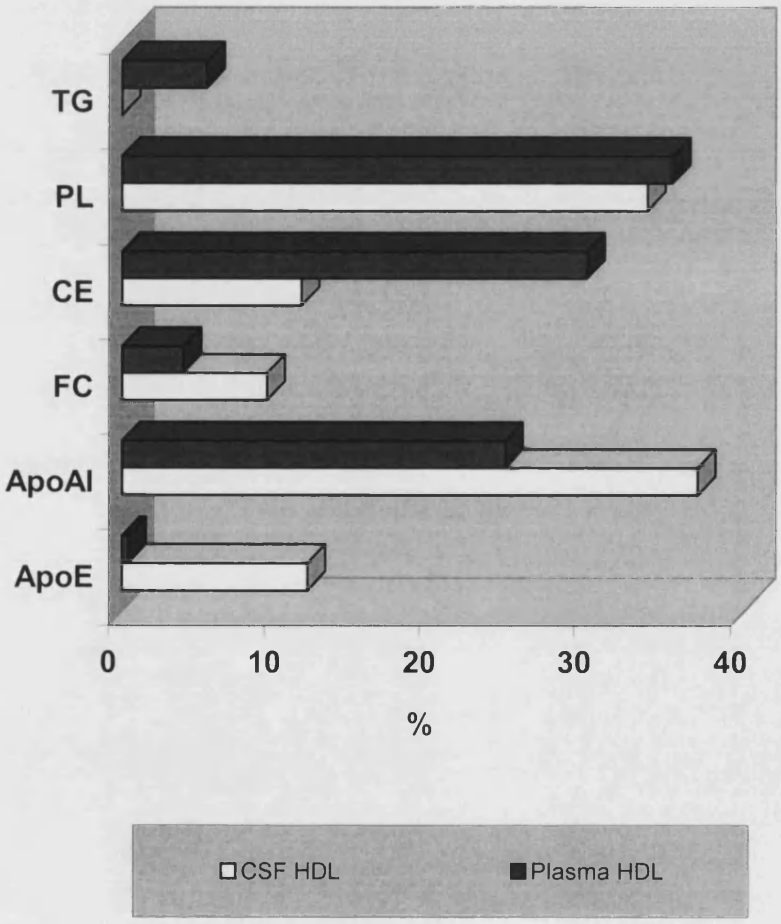


Figure 21 shows the plasma lipoprotein elution profile from a Sephadex 200 size exclusion column. VLDL ($d \leq 1.006$ g/ml) elutes from fraction 26-33, LDL ($d = 1.019$-1.063 g/ml) elutes from fraction 34-45 and HDL ($d = 1.063$-1.21 g/ml) elutes from fraction 46-58. The protein content of the fractions eluting from the column are monitored from the absorbance at 280nm.

Figure 22 Lipoprotein composition of Plasma and CSF HDL

Figure 22 displays the triglyceride (TG), phospholipid (PL), cholesterol ester (CE), free cholesterol (FC), apoAI and apoE composition (mg %) of plasma and CSF HDL. Plasma and CSF HDL differ substantially in their relative compositions as demonstrated in the bar chart. There is no triglyceride in CSF HDL. There is more apoE, apoAI, and free cholesterol in CSF HDL.



3.4.1.1 Lipoprotein particle profile of plasma

The elution profile of plasma confirmed the presence of three peaks comprising particles of differing size: VLDL eluted in fractions 26 to 33, LDL in fractions 34 to 45, and HDL in fractions 46 to 58. The fractions containing the LDL particle are clearly defined, as the only apoprotein in LDL is apoB.

3.4.1.2 Composition of plasma lipoprotein particles

The VLDL particle is rich in triglyceride (data not shown), and has relatively little protein, cholesterol or phospholipid. The LDL particle is characterised by the presence of apoB, and has relatively more cholesterol and phospholipid. The HDL particle has no apoB, is protein rich, and compared to the LDL particle has proportionately more phospholipid than cholesterol. The composition of plasma lipoprotein particles, contrasted with CSF lipoprotein particles, is summarised in table 17 and figure 22.

Table 17 Composition of CSF and plasma HDL lipoprotein particles

	Plasma HDL_2	**Plasma HDL_3**	**CSF HDL_1**	**CSF HDL_2**	**CSF HDL_3**
ApoE % (SD)	0.5 (0.3)	0.2 (0.2)	16.1 (8.8)	14.1 (6.6)	5.6 (7.6)
ApoAI % (SD)	15.2 (7.4)	34.2 (3.6)	13.8 (15.5)	42.8 (6.1)	54.7 (37)
FC % (SD)	4.9 (1.1)	3.0 (0.5)	13.8 (15.5)	4.3 (4.9)	10.1 (11.9)
CE % (SD)	34.3 (3)	25.5 (2.9)	20.1 (18.5)	14.4 (6.4)	nd
PL % (SD)	38.3 (3.0)	32.5 (1.2)	47.6 (15.3)	24.4 (2.9)	29.6 (23.1)
TG % (SD)	6.2 (1.2)	4.7 (0.8)	nd	nd	nd

FC = free cholesterol, CE = cholesterol ester, PL = phospholipid, TG = triglyceride,

nd = not detected. HDL_1 is rarely seen in human plasma.

3.4.2 Isolation and analysis of lipoprotein particles from control CSF

The minimum volume of CSF required for the isolation of CSF lipoprotein particles is 20 ml. The substantial volume required was generated by pooling 1ml aliquots of CSF from twenty-five consecutive control patients. A total of six control pools were generated in this manner from one hundred and fifty of the control CSF samples. These pools had no detectable apoB. The mean age of the patients was 32 years (median 25 years and range 16-65 years).

3.4.2.1 Lipoprotein particle profile and composition of control CSF

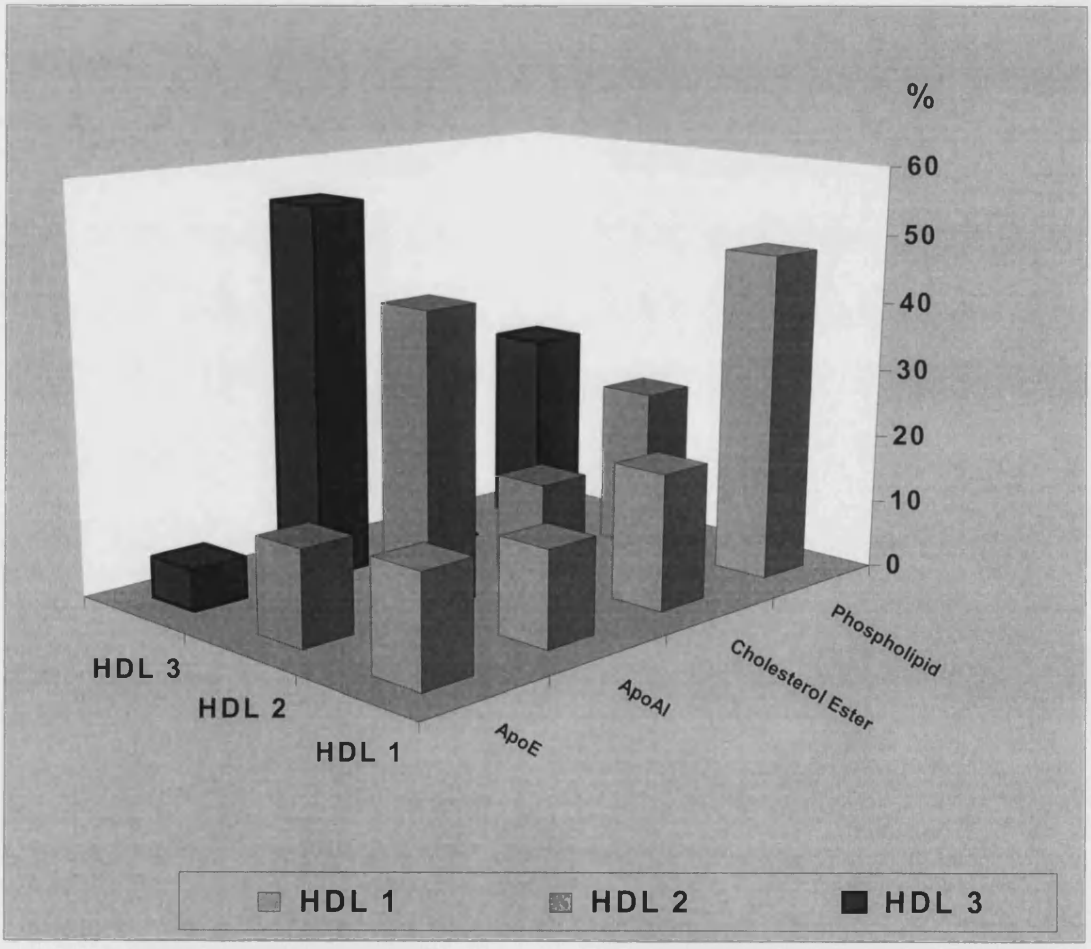
In contrast to plasma, the lipoprotein particles present in control CSF form one major peak. This peak occurs in fractions containing particles of similar size to the plasma HDL and LDL particles at the LDL/HDL boundary, which peaked at fraction 47. CSF does not contain apoB, thus these large CSF lipoprotein particles are known as HDL_1 (plasma has HDL_2 and HDL_3, but HDL_1 is rarely seen in plasma). Examination of the control apoE and apoAI elution profile indicates the presence of overlapping populations of particles containing both apoE and apoAI (fractions 45 to 52) and particles containing apoAI alone (fractions 50 to 60). Thus apoAI is present in both the HDL_1 and smaller HDL lipoprotein particle fractions and apoE is present mainly in the larger HDL_1 sized particle. See figure 24.

The larger CSF lipoprotein particles contain most of the phospholipid and cholesterol associated with the CSF lipoproteins. The molar ratio of apoAI to apoE in control CSF was approximately 1:3 and that of free cholesterol to phospholipid was approximately 1: 2.

Having calibrated the size exclusion chromatography column for the fractionation of CSF lipoprotein particles from control CSF, isolation and compositional analysis of TBI and SAH CSF was performed.

Figure 23 Lipoprotein composition of CSF HDL particles

CSF lipoprotein particles are unique in that they comprise HDL_1 sized particles, which are not normally present in human plasma. Analysis of the HDL subgroups in control CSF, as displayed in the bar chart clearly shows the differences between CSF HDL subgroups. The smaller HDL_3 have less apoE and cholesterol ester. In contrast apoAI predominates. The smaller HDL particles remodel through the action of the cholesterol ester forming enzyme LCAT, the activity of which is promoted by the abundant apoAI present on the smaller HDL particles. As the HDL accumulate cholesterol ester apoAI is replaced by apoE, which facilitates the transport of the cholesterol to target cells expressing the LDL family of receptors.



3.4.3 Isolation and analysis of lipoprotein particles from acute brain injury CSF

3.4.3.1 CSF lipoprotein particle profile and composition after SAH

After SAH the CSF contains plasma lipoprotein particles released at the time of haemorrhage. Compared to control CSF there was a ten-fold increase in total cholesterol ($p = 0.007$), and free cholesterol ($p = 0.001$) in SAH CSF. Importantly, there was no significant difference between the ratio of FC to TC in control and SAH CSF. The FC to TC ratio was constant across the lipoprotein profile. The phospholipid concentration of SAH CSF was five times higher than that of the controls ($p<0.05$). The molar ratio of free cholesterol to phospholipid increased from 1:2 in controls to 1:1 in SAH. The apoE to apoAI ratio decreased from 1:3 in controls to 1:13 in SAH. In SAH CSF these changes were accompanied by the appearance of a population of particles of very small size (fractions 58-68) that are rich in phospholipid and free cholesterol. There was a significant increase ($p = 0.04$) in the total area under the curve (AUC) for apoAI associated particles in SAH CSF compared to control subjects, but the difference in the total AUC for the apoE associated particles failed to reach statistical significance. These data are summarised in table 18. However, near the LDL/HDL boundary in SAH CSF there was a reduction in apoE AUC ($p = 0.13$) indicating a depletion of large apoE-containing lipoproteins. In SAH CSF the concentration of apoB was 1.5 ($\pm$ 0.5) mg/100ml; apoB was not detected in control CSF. Although the decrease in concentration of CSF LpE after SAH is not statistically significant (Mann-Whitney: $p = 0.86$), it is surprising that LpE does not increase in proportion with apoAI as they have similar proportions in the plasma. The ratio of LpAI to LpE is significantly lower after SAH (Mann-Whitney: $p = 0.026$). See figure 24.

Figure 24 Composition of lipoprotein particles fractionated from non-injured control and SAH CSF

The vertical dashed line indicates the elution intervals for LDL and HDL sized particles for the size exclusion column. Panels A and B correspond to total cholesterol, Panels C and D correspond to free (non-esterified) cholesterol, E and F correspond to phospholipid, G and H correspond to apoE, and panels I and J correspond to apoAI in non-injured controls and SAH patients respectively. After SAH, phospholipid, free cholesterol and apoAI levels increase and are associated with plasma VLDL, LDL and HDL sized particles. Large apoE-HDL particles are decreased and VLDL associated apoE appears after SAH. In addition very small lipoprotein particles appear in SAH CSF that are not present in control CSF. Profiles represent the mean of six control pools and CSF from six patients after SAH. CSF was obtained from six SAH patients (mean age 54, median 52, and range 26-65 years) admitted to the neurosurgical unit at the Institute of Neurological Science Glasgow, U.K., between March 2000 and February 2001. All SAH patients were in coma (Glasgow Coma Score $\leq$ 8), had an aneurysmal distribution of blood in the subarachnoid space on the admission CT (all Fisher grade III), and required insertion of an external ventricular drain for the treatment of acute hydrocephalus. The SAH CSF was obtained from the cerebral ventricles within three days of aneurysm rupture.

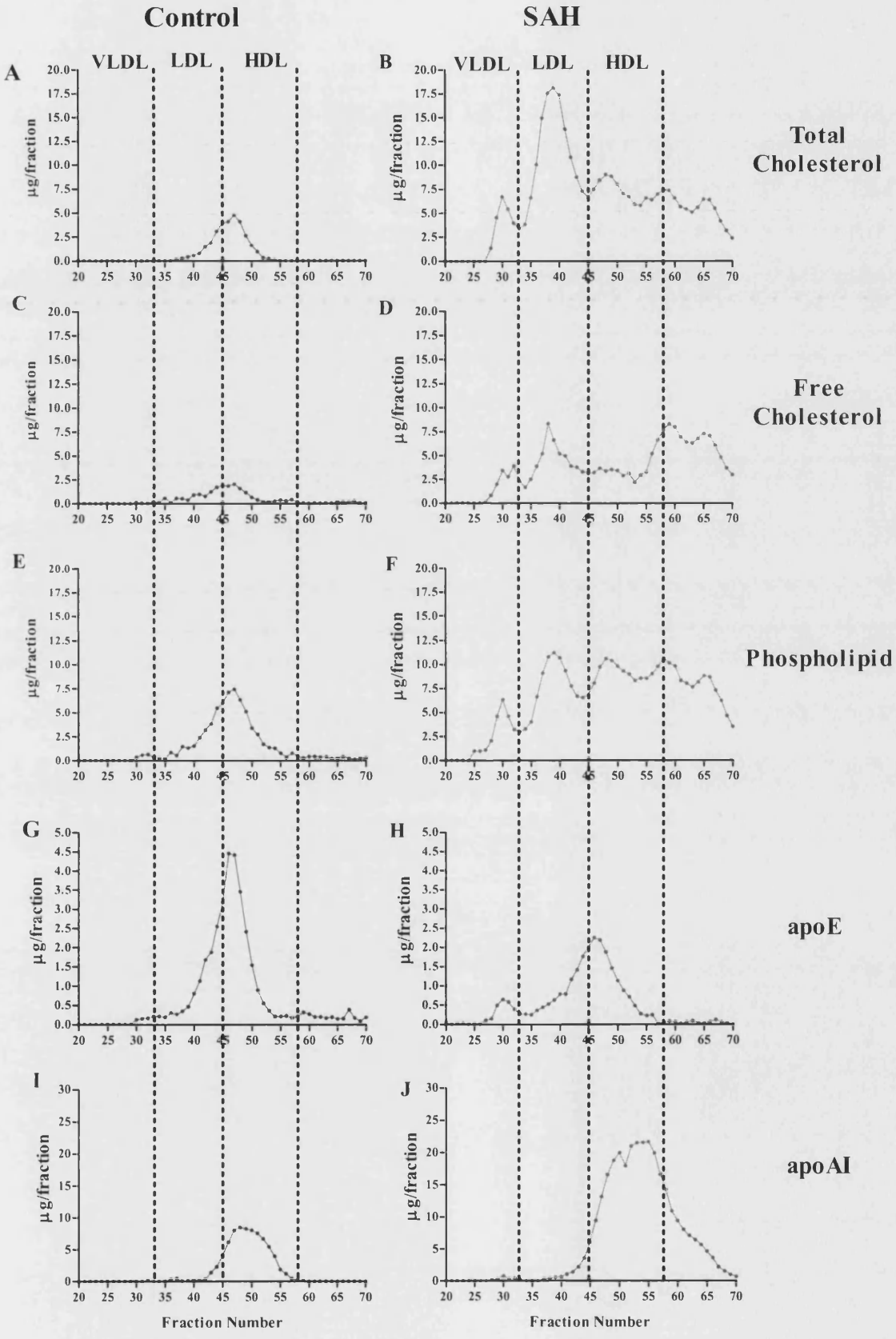

Control
SAH
A
B
C
D
E
F
G
H
I
J
VLDL
LDL
HDL
µg/fraction
Fraction Number
Total Cholesterol
Free Cholesterol
Phospholipid
apoE
apoAI

Figure 25 Composition of lipoprotein particles fractionated from non-injured control and TBI CSF

CSF from 150 patients was used to generate 6 non-injured CSF pools of sufficient volume for CSF lipoprotein particle fractionation by size exclusion chromatography. CSF from 27 patients with severe traumatic brain injury was pooled for comparison with the control pools. The vertical dashed line indicates the elution intervals for LDL and HDL sized particles for the size exclusion column. Panels A and B correspond to free (non-esterified) cholesterol, C and D correspond to phospholipid, E and F correspond to apoAI, and panels G and H correspond to apoE in non-injured controls and traumatic brain injury patients respectively. As observed in SAH CSF, there is a population of very small lipoprotein particles associated with free cholesterol, phospholipid and apoAI.

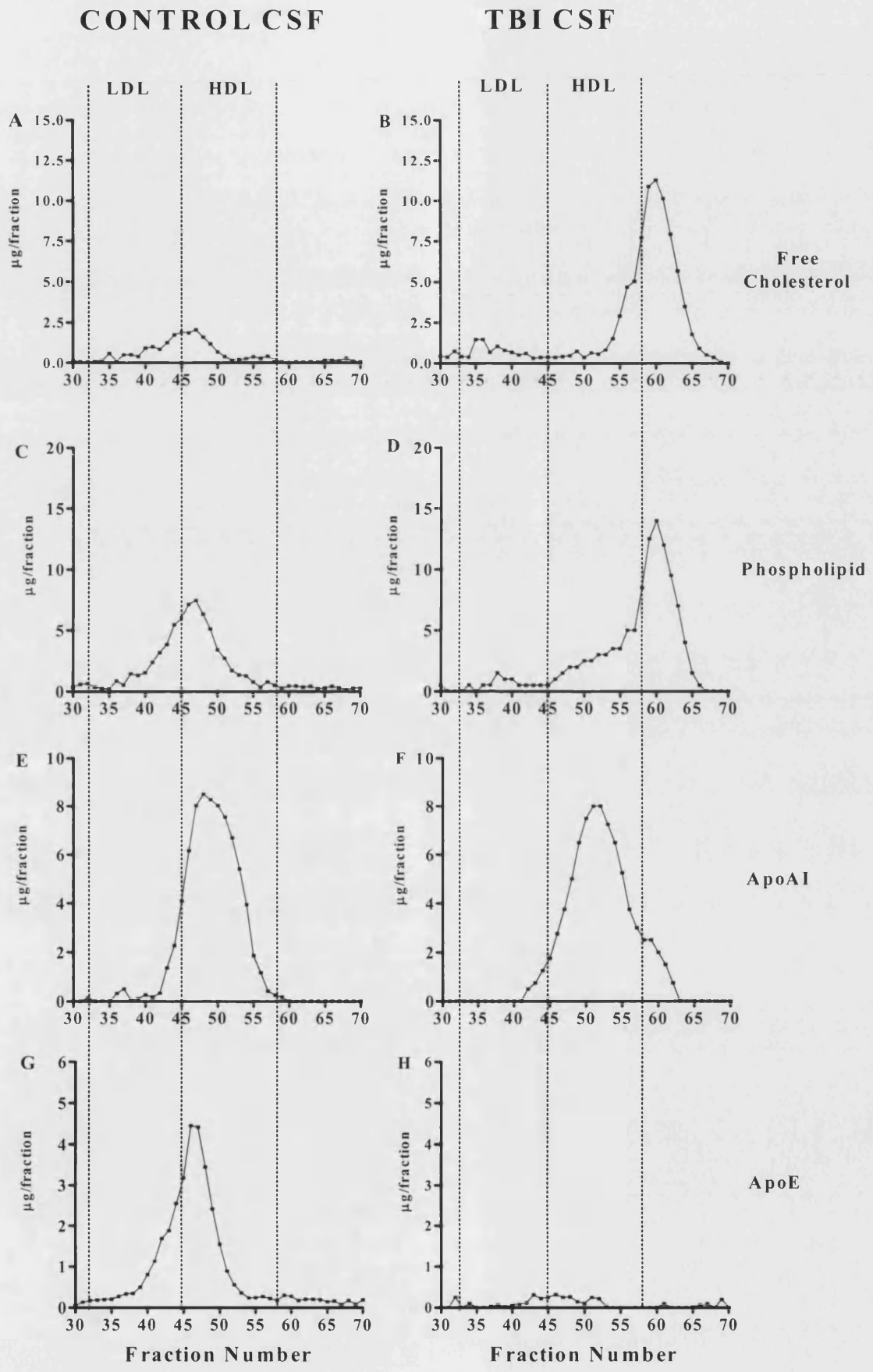

CONTROL CSF
TBI CSF
LDL
HDL
A
B
C
D
E
F
G
H
µg/fraction
Free Cholesterol
Phospholipid
ApoAI
ApoE
Fraction Number
Fraction Number

Table 18 Composition of lipoprotein particles in control and SAH CSF

Lipoprotein Component	Concentration mg/100ml of control CSF, [μM]	Concentration mg/100ml of SAH CSF, [μM]
Total cholesterol	0.13 ± 0.06, [3.4]	1.28 ± 1.12 [33.2] **
Free cholesterol	0.08 ± 0.04, [2.3]	0.78 ± 0.66, [20.2] **
Phospholipid	0.29 ± 0.08, [3.8]	1.33 ± 1.12, [17.2] *
ApoAI	0.30 ± 0.146, [0.1]	1.24 ± 0.99, [0.4] *
ApoE	0.14 ± 0.05, [0.04]	0.10 ± 0.03, [0.03]

Values are means ± S.D. Significance of difference: *$p<0.05$, **$p<0.001$ control and SAH means compared with unpaired t-test of log transformed data

Table 19 Composition of CSF lipoprotein particles in control and TBI CSF

Lipoprotein component	Concentration mg/100ml of control CSF, [μM]	Concentration mg/100ml of TBI CSF, [μM]
Total cholesterol	0.13 ± 0.006, [3.4]	0.29, [7.5] *
Free cholesterol	0.09 ± 0.04, [2.1]	0.36, [9.3] **
Phospholipid	0.29 ± 0.09, [3.8]	0.44, [5.7] *
ApoAI	0.30 ± 0.15, [0.1]	0.32, [0.1]
ApoE	0.14 ± 0.05, [0.04]	0.01, [0.004] **

Single TBI pool compared to six control pools using the one sample t-test.

*$p<0.05$, **$p<0.01$

3.4.3.2 CSF lipoprotein profile and composition after TBI

The volume of TBI CSF available for size exclusion chromatography was very limited. One pool comprising one millilitre of CSF from each of twenty-seven TBI patients (within three days of injury) was fractionated for analysis of the lipoprotein particles. In CSF from TBI patients, there was a five-fold increase in non-esterified cholesterol compared to control CSF ($p = 0.0001$). In addition, phospholipid containing particles increased significantly compared to control CSF ($p = 0.04$). The molar ratio of free cholesterol to phospholipid in TBI patients increased from 3:5 in controls to 11:5 in TBI. These changes were accompanied by the appearance of a population of particles of very small size (fraction 58-63) which were phospholipid and free cholesterol rich. Large apoE- HDL particles were depleted in the TBI CSF as evidenced by the shift to the right of the elution peaks for free cholesterol and phospholipid. There was no significant change in the apoAI associated particles of the TBI CSF pool compared to control pools, but apoE associated particles were significantly decreased (one sample t-test; $p = 0.002$). The molar ratio of apoAI to apoE in TBI patients increased from 2:1 in controls to 25:1 in TBI. There were no apoB containing lipoprotein particles in TBI CSF. These data are summarised in table 19 and figure 25.

3.4.4 Summary of characterisation of CSF lipoproteins after acute brain injury

CSF lipoproteins are distinctive from plasma lipoproteins. Substantial differences in the composition of the lipoprotein particles exist between control and acute brain injury CSF. After SAH, despite the substantial increase in lipoprotein particles originating from the plasma, there is a reduction in the quantity of apoE containing particles. After TBI, where there is a lesser release of lipoprotein particles originating from the plasma, there is even greater decrease in the apoE containing lipoprotein particles. The changes in the apoE containing lipoprotein particle composition parallel the changes identified in nascent CSF apoE concentration.

4 Discussion

4.1 Principal findings of the study

4.1.1 Protein concentrations in control, TBI and SAH CSF

The principal findings in relation to the proteins selected for investigation in this study are:

- The concentrations of apoE, $A\beta_{1-40}$, and $A\beta_{1-42}$ are significantly lower in the CSF of patients with TBI, and SAH compared to controls.
- The decrease in these proteins occurs within days of injury and persists for at least one week after injury.
- The decrease in protein concentration after acute brain injury correlates with injury severity and clinical outcome.
- The concentrations of S100B and Tau are markedly increased in the CSF of TBI and SAH patients compared to controls.
- The increase in these proteins occurs within days of injury and persists for at least one week after injury.
- The peak CSF concentration of Tau and S100B correlates with the severity of the brain injury, and the clinical outcome after SAH.

4.1.2 The analysis of lipoprotein particles in control, TBI and SAH CSF

The principal findings in relation to the lipoprotein particles present in the CSF of controls and patients with TBI and SAH are:

- CSF contains unique lipoprotein particles.
- The apoE lipoprotein particles were depleted from brain injury CSF compared to controls.
- CSF lipoprotein particles undergo remodelling after acute brain injury.
- Novel lipoprotein particles are present in brain injury CSF that are not present in control CSF.

4.2 Interpretation of results

4.2.1 Protein concentrations in control, TBI and SAH CSF

There are a number of possible explanations for the decrease in CSF apoE, and Aβ, after acute brain injury. These include increased clearance from CSF, decreased production, dilution effects, sampling artefact, and assay artefact or interference. Evaluation of the apoE ELISA determined that the assay has high recovery, sensitivity, and precision.

Importantly, the recovery of apoE added to control or brain injury CSF, and white matter homogenate was high, and there was no significant difference between brain injury and control CSF. Thus there appears to be no significant interference from substances present in the brain injury CSF that are not present in control CSF. There was no decrease in the apoE signal when CSF was assayed in the presence of increasing concentrations of human albumin, or the total protein used for the protein standard curve (comprising human albumin and gamma globulin) at concentrations recorded in the brain injury CSF. In addition, there was linearity in the assay over a range of CSF dilutions for control and brain injury CSF and these dilution lines were parallel with the apoE calibration curve. Thus, differences detected between control and brain injury CSF are not significantly affected by differences in the matrices at different analyte concentration. The lower limit of detection of the assay was substantially lower than the lowest recorded CSF apoE concentration. The precision of the assay, coupled to the simultaneous assay of controls and brain injury CSF decreased the chance that differences between cases and controls are due to inter or intra assay variation. The specificity of an assay is particularly important when high values are observed (which is not the case here) due to concern about antibody cross reactivity. The specificity of the assay has been extensively described by Stark et al, who found no significant cross reactivity with the major lipoproteins present in plasma. Both in their study and the present study, there was no significant difference between calibration curves obtained using apoE3, apoE4 or apoE2. (Starck et al. 2000) The potential that other variables, such as pH, are substantially different in brain injury CSF has not been investigated. Although this does not affect the ability to detect apoE added to brain injury CSF, there is a possibility that *in-vivo* apoE undergoes physiochemical alterations resulting in an apparent decrease in the CSF due to masking of epitopes. For example, the apoE may form stable complexes *in-vivo* with Aβ or components of the complement cascade. Formation of amyloid aggregate *in-vitro* appears to be pH dependent and the possibility that acidosis after brain injury contributes to depletion of apoE-Aβ from CSF *in-vivo* requires further investigation. (Atwood et al. 1998) Although only semi-quantitative, denaturing SDS PAGE correlated approximately with the ELISA findings in the samples analysed using this method, suggesting that the decreased signal in brain injury CSF is not attributable to differences in secondary and tertiary structure, or detergent sensitive binding interactions. In addition, the apoE ELISA assay dilution buffer contains detergent, which results in protein unfolding and removes associated cholesterol and lipid. There are non-antibody based methods for the quantification of apoE such as HPLC (derived from phenylalanine content of apoE) and capillary electrophoresis in SDS gels (SDS-CGE), but these were not utilised in the present study. (Schlenck et al. 1999) It has

not been possible to independently evaluate the amyloid beta assays due to the high cost of the assay. However, these commercial assay kits have been evaluated by others and have been used to assay amyloid beta in the CSF of patients with AD and controls, as well as other biological fluids. (Chishti et al. 2001; Kusiak et al. 2001) The manufacturers report high recovery of amyloid beta from plasma.

The possibility that apoE and amyloid beta are decreased in brain injury CSF compared to control CSF due to a systematic difference in CSF sample acquisition and processing requires evaluation. It has been demonstrated that the route of sampling influences the concentration of apoE such that the concentration is higher in ventricular CSF than CSF from the lumber subarachnoid space. CSF apoE is intrathecally synthesised accounting for the concentration gradient between ventricular and lumbar CSF. (Carlsson et al. 1991; Linton et al. 1991) The decrease in ventricular CSF apoE observed after acute brain injury is significant when compared to either control CSF obtained from the lumbar subarachnoid space or control CSF from the lateral ventricles. Thus the magnitude of the decrease in apoE concentration observed when brain injury CSF is compared to lumbar CSF represents an underestimate of the true decrease that would be observed when compared to the less often available ventricular control CSF. It has been demonstrated that continuous CSF drainage does not significantly reduce the concentration of apoE in non-brain injured controls over time. Thus, the loss of apoE from brain injury CSF is unlikely to be a consequence of brain apoE exhaustion due to external drainage. The high concentration of apoE in control ventricular CSF obtained via ventricular catheters suggests that there is no significant decrease in apoE due to sampling via this route. Furthermore, there was no significant difference in the apoE concentration before or after passage through a ventricular catheter *in-vitro*. The conditions for collection and storage are particularly important for hydrophobic polypeptides such as Aβ, which have a tendency to aggregate and adsorb onto surfaces. Thus the CSF collection tubes utilised in this study were of the polypropylene type as recommended by Hesse et al. (Andreasen et al. 2001; Hesse et al. 2000) Brain injury and control CSF samples were processed and stored in an identical manner, and underwent only one freeze-thaw cycle prior to simultaneous batch analysis. Analysis was performed within three months of freezing in the majority of cases, though serum or plasma apoE is reported to be stable when stored at –20 degrees centigrade for as long as three years. (Alsayed et al. 1990; Blum et al. 1980; Koffigan et al. 1987; Kottke et al. 1991; Phillips et al. 1983; Schiele et al. 2000b) The stability of apoE in the CSF samples used for this study is supported by the finding that the frozen CSF standard curve and QCs did not change significantly over the six-month period of apoE ELISA evaluation

and sample analysis. The consistent finding that CSF apoE is decreased in SAH CSF obtained from patients in the USA and Scotland provides supporting evidence that the differences in brain injury CSF and control CSF are not due to systematic differences in sample processing between the two units. The apoE concentration of CSF supernatant before and after centrifugation was not significantly different suggesting that the apoE was not lost from the supernatant in the initial centrifugation step. All samples once thawed were vortexed prior to sample dilution. The rapid freezing after sampling and addition of a protease inhibitor to all the CSF samples decreased the chance that apoE was lost due to increased proteolysis in brain injury CSF. Poly acrylamide gel electrophoresis of control and brain injury CSF did not identify apoE antibody immunoreactive fragments suggestive of apoE proteolysis.

The possibility that the decrease in CSF apoE is attributable to dilution is unlikely for the following reasons. First, in the acute phase after TBI, ventricular volume is often decreased such that CSF spaces including the basal cisterns, third and lateral ventricles may become completely obliterated. This observation often results in difficulty cannulating the ventricle, and is associated with raised intracranial pressure and unfavourable outcome. (Teasdale et al. 1984) Second, a substantial increase in CSF volume would be required to produce the scale of decrease in CSF apoE observed after TBI, which would not be tolerated within the fixed confines of the skull. Third, the brain tissue water content has been estimated to increase by less than ten percent after TBI. (Marmarou et al. 2000) Fourth, from the analysis of lipoprotein particles a significant decrease in LpE was observed, but not in LpAI, which would be expected if the effect was due to dilution alone. This strongly supports the concept that the observed decrease in apoE after TBI is specific to apoE. It is possible that dilution accounts for some of the decrease in CSF apoE observed after SAH, as acute hydrocephalus is not an uncommon finding. (van Gijn J. and Rinkel, 2001) However, there is still a substantial decrease in the relative amount of LpE to LpAI supporting the concept that apoE is selectively lost from the CSF after SAH as well.

Reduced production of apoE, by astrocytes, oligodendrocytes and microglial cells, in response to brain injury could potentially contribute to the decreased concentration observed in CSF after acute brain injury. However, failure of apoE production would not explain the decrease observed after SAH, where plasma apoE is likely to be released into the CSF. In vitro, pro-inflammatory cytokines, which are prevalent after acute brain injury, are reported to result in reduced astrocyte culture medium apoE concentration. (Baskin et al. 1997; Starck et al. 2000)

Soon after acute brain injury, the concentration of albumin (and total protein) increase substantially indicating substantial opening of the blood brain barrier. The effect is relatively transient given that the CSF concentration of albumin (and total protein) decreases progressively over subsequent days. During this time period, the ratio of apoE to albumin (or total protein) is disproportionately decreased suggesting that apoE is selectively depleted from the CSF. One would speculate that the brain has substantial capacity to clear apoE given that the concentration of apoE in plasma (which is released into the subarachnoid space or neuropil, at the time of brain injury, or haemorrhage) is an order of magnitude higher than in CSF. (Carlsson et al. 1991) Analysis of paired plasma and CSF samples has not been undertaken in this preliminary study. The possibility that apoE released into the CSF after injury is rapidly cleared to the plasma compartment requires further investigation.

An alternative explanation for the findings that apoE and Aβ are depleted in brain injury CSF is possible, for which there is indirect supporting evidence from studies of other forms of human brain injury, and experimental brain injury. It is hypothesised that selective retention and active uptake of apoE, and Aβ, takes place within the neuropil and subarachnoid space as part of the response of the brain to injury, and this results in diminished concentrations in the CSF. Such a concept would not be novel as there are several neurological disorders for which such a mechanism has been proposed to explain the observation that Aβ or apoE concentration are decreased in the CSF and increased in the brain parenchyma. For example, patients with AD have decreased concentration of Aβ in the CSF compared to controls, whilst the concentration of Aβ in the brain parenchyma is increased. (Andreasen et al. 1999; Andreasen et al. 2001; Fagan et al. 2000; Kuo et al. 1996; Mehta et al. 2000; Motter et al. 1995; Nitsch et al. 1995; Shoji et al. 1998; Tamaoka et al. 1997) Similarly, increased apoE immunoreactivity has been observed in AD brain, and the concentration of apoE is reported to decrease in the CSF of patients with AD. (Blennow et al. 1994; Hesse et al. 2000; Landen et al. 1996; Skoog et al. 1997) However, decreased apoE concentration in the CSF of patients with AD has not been consistently reported with some studies reporting increased concentrations. (Blain et al. 1997; Fukuyama et al. 2000; Lindh et al. 1997; Merched et al. 1997) Indeed in this study there was no significant difference between the concentration of apoE in the CSF of the control group and that of eighty-two patients investigated for dementia, although the number of patients in this group who will meet the criteria for definite AD is unknown at this time. In the present study, the concentration of apoE in the CSF of patients with definite MS was decreased, which is consistent with the observations of others. (Gaillard et

al. 1996; Rifai et al. 1987) These findings in CSF of MS patients are corroborated by the observation that apoE immunoreactivity is increased in macrophages and astrocytes located in regions of demyelinating MS brain. (Carlin et al. 2000) There is evidence from experimental brain injury studies, and human post mortem studies, that apoE is upregulated in the brain after injury, and participates in a co-ordinated response to acute brain injury. Neurons, which do not normally demonstrate immunopositivity to apoE, develop immunoreactivity after experimental transient global ischaemia, and acute subdural haematoma. In the subdural model intense immunoreactivity to apoE localises to the neuronal population adjacent to the haematoma. Possible explanations for this observation include the transport of apoE from the haematoma to injured neurons beneath the haematoma, increased de-novo synthesis of apoE by the injured neuron, or increased uptake of apoE released by other cells in the neuropil in response to injury. In the transient ischaemia model, intense apoE immunoreactivity is initially observed in astrocytes, which is later followed by apoE immunoreactivity in the population of neurons that are selectively vulnerable to ischaemia. (Horsburgh et al. 1997; Horsburgh et al. 2000b; Horsburgh and Nicoll, 1996) Although these temporal and cell type dependent changes suggest that apoE is involved in a co-ordinated response of the brain to injury, the actual role that apoE plays is not known. *APOE* knockout mice have delayed tissue clearance after cerebral infarction, macrophage related apoE expression being observed at the centre of infarcts in wild-type mice. (Kitagawa et al. 2001) There is evidence from experimental brain injury that apoE has a role as an anti-oxidant, or may be functioning as a lipid and cholesterol transporter recycling debris to injured cells. (Horsburgh et al. 2000a; Kitagawa et al. 2002; White et al. 2001a) These findings in experimental brain injury corroborate findings in human brain injury given the observation that apoE immunoreactivity is increased among patients who die after TBI, global ischaemia, and Herpes encephalitis. (Horsburgh et al. 1999a; Nicoll et al. 2001) Importantly, *APOE* dependent endocytic pathway alterations have been observed in the human hippocampus after fatal global ischaemia. (McColl et al. 2003) Thus there is a body of evidence to support the concept that brain apoE is up-regulated in response to injury. However, these studies do not explain the concept that apoE is decreased in the CSF after SAH where it is likely that substantial quantities of apoE are released into the subarachnoid space at the time of haemorrhage. There have been no systematic studies in post-mortem specimens from patients who die after SAH examining the distribution of apoE, or Aβ within the brain. The possibility that apoE is depleted due to binding with debris released after cell injury and haemolysis, and that this is a non-specific process facilitating debris disposal without recycling or functional significance has not been excluded.

In addition to a role in CNS cholesterol trafficking, apoE appears to promote the formation of extracellular fibrillary amyloid. Amyloid beta (especially $A\beta_{1-42}$) is hydrophobic and binding interactions between apoE and Aβ have been reported *in-vitro*. (LaDu et al. 1994; LaDu et al. 1995; Munson et al. 2000; Pillot et al. 1999) Studies using *APOE* and *APP* transgenic mice support the concept that apoE is pro-amyloidogenic. (Bales et al. 1999; Bales et al. 1997; Holtzman et al. 2000a) The finding from this study that after acute brain injury Aβ and apoE concentration decrease in the CSF may be explained by the formation of insoluble apoE-Aβ aggregates. These aggregates may precipitate *in vivo* or be rapidly cleared to the plasma compartment. Studies of post-mortem tissue from patients who die after TBI identify apoE in association with aggregates of Aβ in the neuropil consistent with this hypothesis. (Bales et al. 1997; Clinton et al. 1991; Gentleman et al. 1997; Graham et al. 1996; Graham et al. 1999; Horsburgh et al. 2000) Raby et al report an increase in Aβ concentration in the CSF of TBI patients. (Emmerling et al. 2000; Raby et al. 1998) CSF samples were analysed from six TBI patients (average age thirty-five years) and twenty-four post mortem controls (average age seventy eight years) raising concerns about sample size and control selection. The authors compare mean values from one hundred and five TBI samples raising the additional concern that the statistical significance reported is confounded by repeated measures. They report that the concentration of Aβ is significantly elevated compared to controls after TBI. However, Franz et al report findings in TBI CSF concordant with the present study. In 29 patients with severe head trauma, the concentration of $A\beta_{1-42}$ was significantly lower than that of controls, and low levels were associated with more severe outcome. (Franz et al. 2003) There are no published series reporting alterations in CSF Aβ after SAH, though they are reported to be unchanged after ischaemic stroke. (Hesse et al. 2000)

The decrease in apoE and Aβ observed in this study occurs within days of injury, and returns to normal approximately one week after injury. Due to the absence of CSF samples very soon (i.e. within hours) after injury it has not been possible to investigate the possibility that apoE is initially increased. Furthermore, the requirement to restrict external ventricular drainage to the minimum clinically indicated time period limits the analysis of CSF for longer than one week after TBI and two weeks after SAH. The absence of correlation between the trough in apoE, and $A\beta_{1-42}$, concentration and the time elapsed from injury to the time of maximal change suggests that these proteins are rapidly depleted from the CSF. One may speculate that the greater hydrophobicity of $A\beta_{1-42}$ would result in the rapid formation of insoluble aggregates. From the weak correlation between the time taken to reach trough $A\beta_{1-40}$ and the magnitude of the reduction we may speculate that Aβ

$_{1\text{-}40}$ is depleted in a time dependent process after TBI. The ratio of $A\beta_{1\text{-}40}$ to $A\beta_{1\text{-}42}$ did not change significantly after injury suggesting that the decrease in these peptides is not fragment length specific.

Analysis of the time course for the change in apoE and Aβ concentration enabled determination of the lowest value attained after injury and its relationship to injury severity and clinical outcome. There was significant correlation between injury severity and $A\beta_{40}$ after TBI and apoE after SAH. Although no deductions regarding causality may be inferred, we may hypothesise that more severe injury results in increased clearance of apoE and Aβ from the CSF. Alternatively, some other factor that is influenced by injury severity also influences the concentration of these proteins, or low CSF concentration results in more severe injury. There was significant correlation between clinical outcome and the lowest CSF concentrations of apoE and Aβ after SAH. Thus we may hypothesis that the degree of the decrease in apoE and Aβ concentration observed in the CSF soon after SAH determines some of the variation in clinical outcome three months later. Again, other confounding variables may account for the association. From the time course data, it is apparent that CSF apoE is significantly diminished by a lesser degree and for shorter time period after SAH then TBI. This may be due to the greater amount of plasma derived apoE released into the subarachnoid space after SAH compared to TBI. According to the GCS score, the TBI patients were more severely injured than the SAH patients. Thus, it is possible that the TBI patients have lower CSF apoE due to greater retention within the brain, or greater clearance to the plasma, or some other factor. In addition, hydrocephalus due to CSF outflow obstruction is more common after SAH then TBI, which may result in less rapid clearance of apoE from the CSF.

The observation that outcome after SAH was less favourable in patients with low CSF Aβ is intriguing suggesting that Aβ peptides may be important after SAH. Importantly apoE isoform dependent properties have been identified for Aβ peptides and these may be important after SAH. Firstly, vasoactive properties have been identified for Aβ peptides and promotion of endothelin-1 mediated vasospasm by Aβ appears to be apoE isoform dependent. (Paris et al. 1998) Secondly ischaemic susceptibility of transgenic APP mice appears to be dependent on apoE isoform, possibly by modulation of the inflammatory response induced by middle cerebral artery occlusion. (Koistinaho et al. 2002) Furthermore, Aβ appears to diffuse freely within neural tissue raising the possibility that Aβ cleared via the cerebral vasculature may become deposited within smooth muscle, as in Cerebral Amyloid Angiopathy, resulting in intimal narrowing and alterations in cerebral

blood flow. (McCarron et al. 1999; Meyer-Luehmann et al. 2003) Lastly, Aβ peptide induce free radical mediated oxidative stress and neuronal vulnerability appears to be *APOE* dependent. (Butterfield et al. 2002)

In addition to the decreased concentration apoE, and Aβ, in the CSF after acute brain injury, the time course for release of S100B, and Tau was investigated. These proteins were chosen as they are selectively expressed in astrocytes and neurons respectively, and are emerging as marker proteins for the processes of astrocytosis and axonal disintegration that occur after brain injury. Furthermore ELISA assays developed for use in CSF were readily available. There are few reports relating to the concentration of Tau in CSF from patients with TBI. (Zemlan et al. 2002; Zemlan et al. 1999) Zemlan developed an ELISA for cleaved Tau using monoclonal antibody generated by differential CSF hybridoma screening using CSF from TBI and control patients. CSF Tau was elevated one thousand fold in the fifteen TBI patients. Affinity purification identified a cleaved form of Tau in the TBI CSF, which consisted of the interior portion of the protein, containing the microtubule-binding domain. Although the mechanism and type of TBI (e.g. from the CAT scan) is not specified, the authors conclude that CSF Tau measurement may be useful for the quantification of axonal injury and disintegration. The concentration of Tau in the CSF of the patients investigated in this study increased by the same order of magnitude as in Zemlans` study. Importantly, the Tau assay kit used in this study is commercially available enabling other groups to add to this series. Total Tau has been reported to increase substantially after stroke, but there appears to be no increase in phospho-Tau, which is the predominant form, observed to increase in AD. (Blennow et al. 1995; Hesse et al. 2001; Sjogren et al. 2001) The assay used in this study assays total Tau concentration preventing estimation of the proportion of phospho-Tau released after acute axonal injury. Increased Tau immunoreactivity has been observed in the brains of patients with a history of mild chronic repetitive head trauma, being predominantly localised to perivascular regions. (Geddes et al. 1999) CSF Tau was significantly higher in SAH compared to TBI, and time from injury to maximal CSF Tau concentration was significantly longer. Although this may partly be explained by the longer period of ventricular drainage, it is also possible that after SAH there is greater microtubule disintegration and a more sustained release compared to TBI. This finding is surprising given that the TBI patient group was more severely injured according to the admission GCS, and unfavourable outcome was less prevalent. The detection antibody used in the ELISA kit is specific for CNS Tau, therefore, the high concentrations observed in SAH CSF are unlikely to result from cross reactivity with epitopes present in plasma. One explanation might be that injury severity, and

outcome, after TBI may not be due to the quantity of axonal loss, but the anatomical distribution of axonal loss. Alternatively, axonal loss may be greater after TBI compared to SAH, but recovery greater after SAH. Thus one might speculate that the less profound and shorter decrease in apoE observed after SAH facilitates the survival (and reconnection) of injured axons not destined to undergo complete disintegration.

As discussed in the introduction, S100B has emerged as a non-specific marker protein, which is elevated in the CSF of patients with a range of neurological disorders. It is not known if the high S100B observed in the CSF after acute human brain injury is due to an active response of the brain to injury, or a passive process whereby brain injury disrupts astrocytic (and oligodendrocyte) integrity releasing cell constituents into the extracellular space. The pattern of release in this study is similar to that reported by others, with maximum CSF S100B concentration occurring within two days of injury, followed by sustained levels for up to two weeks after SAH. (Persson et al. 1987) *In-vitro* S100B has concentration dependent paracrine effects that are trophic to developing or injured neurons at nanomolar concentrations, but at micromolar concentrations result in glial activation, upregulation of nitric oxide synthase (NOS), and apoptosis. (Delphin et al. 1999; Hu et al. 1997; Ikura and Yap, 2000) Several intracellular functions have been attributed to S100B including the regulation of cytoskeletal dynamics through disassembly of tubulin filaments, and inhibition of protein kinase phosphorylation. (Sorci et al. 1998) The role that S100B plays in acute brain injury is not yet established. The high concentration of S100B observed in the CSF after acute brain injury is in the micromolar range and precedes the release of Tau into the CSF. One may speculate that S100B released after brain injury acts as a signal for apoptotic cell death resulting in the disintegration of microtubules manifest by the release of Tau into the CSF. There was significant correlation between peak S100B concentration and clinical outcome after SAH and TBI, but not injury severity. One may speculate that high S100B concentration results in worse outcome, or merely reflect more severe damage, which itself results in a more severe outcome. The peak Tau concentration correlated significantly with both injury severity and clinical outcome after SAH. Thus, in the case of Tau, it seems likely that the increase in CSF Tau reflects the severity of the injury and this reflects in the poorer outcome for the patient.

4.2.2 The analysis of lipoprotein particles in control , TBI, and SAH CSF

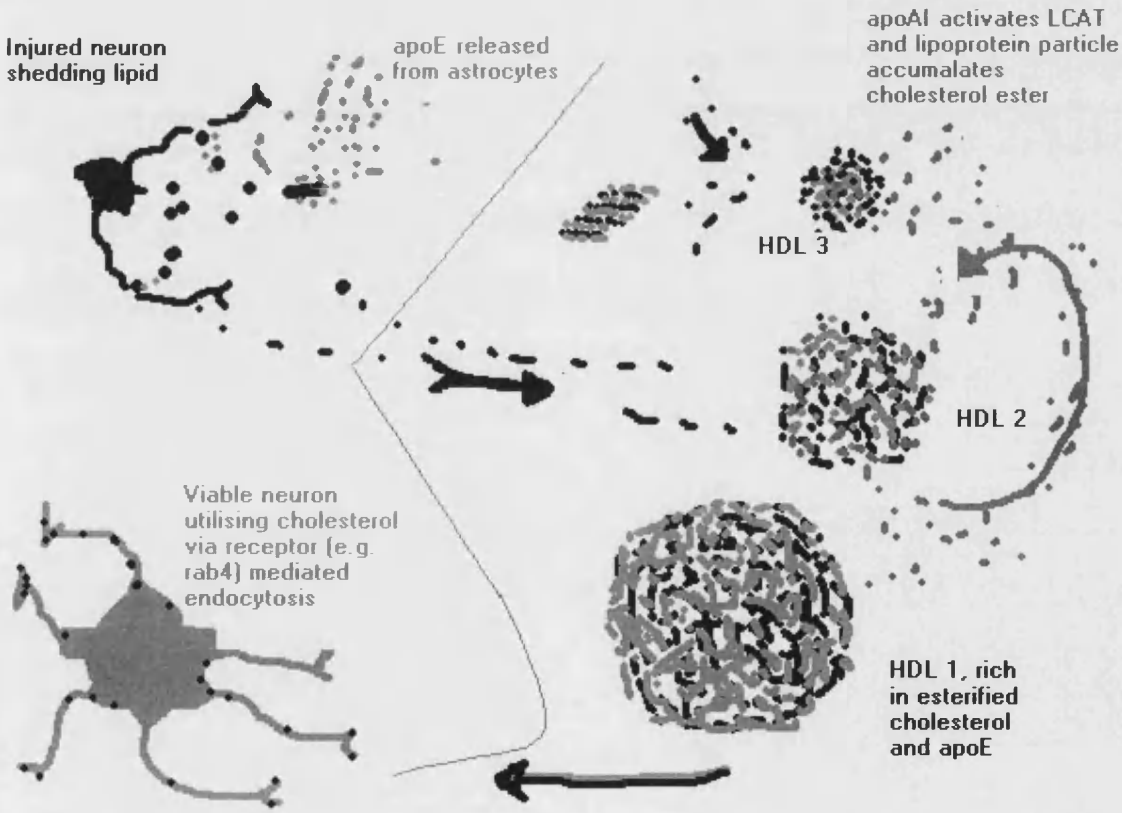
Size exclusion chromatography is now regarded as the optimal method for isolating Lps from the CSF, and in agreement with others, the current study identified a solitary peak of CSF Lps. (Guyton et al. 1998; LaDu et al. 2000; Montine et al. 1998; Montine et al.

1997) The CSF Lps contrast plasma Lps in several ways. First, due to the absence of apoB in the CNS, there are no apoB containing LDL Lps. Second, the triglyceride content of CSF is substantially less than that of plasma such that there is no detectable VLDL in CSF. Third, a unique CSF Lp of a size in the upper HDL and lower LDL range is present in CSF. These Lps are referred to as HDL_1 and are rarely seen in human plasma. The larger CSF Lps (HDL_1) are mainly apoE containing particles. The CSF LpEs are the "end-stage" cholesterol particle ready for receptor mediated endocytosis. ApoAI resides on the smaller Lps where it promotes the esterification of cholesterol through it's activation of LCAT. Thus the LpAs are in the process of expanding to form LpEs, the final cholesterol transport package. The concept that CSF lipoprotein particles remodel after injury is supported by the finding that there was no significant difference between the ratio of FC to TC in control and brain injury CSF and the FC to TC ratio was constant across the lipoprotein profile range.

After TBI, LpE virtually disappears from the CSF. In contrast to LpE, LpA did not decrease significantly after TBI, but the apoAI localised to a smaller population of Lps than those present in control CSF. Smaller sized apoAI particles are the preferred substrates for the formation of cholesterol ester by lecithin cholesterol acyltransferase (LCAT), which facilitates efficient reverse cholesterol transport. The majority of the increased lipoprotein cholesterol and phospholipid is associated with very small particles, the characteristics of which are still to be determined. We speculate that after TBI, CSF LpE is markedly decreased due to increased clearance via lipoprotein receptors of the ependymal layer lining the ventricular space, the astrocytes adjacent to the arachnoid space, the neurons in the adjacent cortex and the pial cells of the arachnoid itself. In support of this concept, *APOE* dependent endocytic pathway alterations have been identified in human hippocampus after global ischaemia. (McColl et al. 2003) There is evidence for cholesterol and phospholipid recycling within the peripheral and CNS after experimental injury, and apoE is believed to participate in this process along with other lipoproteins. (Poirier, 1994; White et al. 2001a) The finding that CSF LpE is diminished after TBI might also be explained by the formation of insoluble aggregates with the $\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides. *In-vitro* binding between LpE and the Aβ peptides could be investigated using affinity chromatography to determine whether LpE is absorbed onto a column surface to which Aβ peptide has been immobilised. Whilst *APOE* genotype does not appear to influence the lipid composition of LpE, there is evidence to support differential binding between CSF lipoproteins comprising different apoE isoforms. (Fagan et al. 2000; Rebeck et al. 1998) Alternatively LpE cleared to the plasma compartment is likely to be rapidly remodelled to LDL via peripheral CETP, and cleared via the liver.

Figure 26 Schematic illustration of cholesterol recycling after acute brain injury

Lipid released from neurons undergoing necrotic or programmed cell death release cholesterol and other lipids. The debris combines with apoE and rafts of lipid and apoE remodel through the action of lecithin cholesteryl ester transferase to form high density lipoprotein (HDL) particles. Initially the particles are rich in apoAI as it promotes the activity of LCAT. Once the particle has maximally accumulated cholesterol ester, apoAI disengages, and the apoE rich particles form. The apoE rich particle may be taken up via receptor mediated endocytosis by cells expressing receptors such as rab4, rabantin-5, and the LDL family of receptors. The cholesterol ester may then be eliminated from the CNS via the 24S-hydroxycholesterol pathway for excretion via the liver, or be utilised for repair within the CNS.



After SAH the CSF Lp profile is more complex due to the presence of plasma Lps released into the subarachnoid space at the time of haemorrhage. There is almost one order of magnitude more cholesterol in the CSF after SAH compared to controls. After SAH, when plasma Lps are released into the subarachnoid space, one would expect both LpE and LpA to increase in the CSF as their concentration in plasma is an order of magnitude higher than in the CSF. It is surprising that while the concentration of LpA in the CSF after SAH increases compared to control CSF, the concentration of LpE in SAH CSF is lower than that observed in controls. Thus as with TBI, apoE containing Lps are cleared from the CSF compartment after SAH. In addition, as observed from the elution profile of TBI CSF, SAH CSF also has small (estimated from elution profile to be < 7 nm) apoAI-containing lipoprotein particles. We speculate that these small lipoprotein particles will be discoidal in shape and are formed when rafts of phospholipid and free cholesterol are sheared from neuronal membranes after neuronal cell death. (LaDu et al. 1998) Thus the excess lipid released into the neuropil after cell death may combine with apoAI and through reverse cholesterol transport involving apoE is delivered to neurons involved in synaptogenesis and sprouting. (LaDu et al. 2000)

4.2.3 Limitations of the study

There are a number of limitations to this study. First, due to limited availability of CSF in brain injured patients, the study population is small, selective, and derived from more than one centre. Small sample size limits the power to relate differences in marker protein concentration to differences in injury type, injury severity, *APOE* genotype and clinical outcome. The patient sample in this study is restricted to patients for whom there is a clinical indication for ventriculostomy. After TBI, intraventricular pressure monitoring tends to be performed in patients at the severe end of the clinical spectrum, and these patients tend to have poorer outcome. The small sample size coupled to the positively skewed nature of the severity of injury prevents analysis comparing more than two variables by multiple regression. Thus, it is not possible to correct for factors known to influence outcome after brain injury, such as age, temperature, hypoxia, hypotension, pupillary response to light and the presence of hemiparesis, brain stem reflexes and the lesions identified on CT scanning. Furthermore, selection bias limits the generalisability of the findings to patients with less severe injury who do not undergo ventriculostomy. The SAH group, though also too small for multiple regression analysis, did include less severely injured patients and consequently the clinical outcome was less positively skewed. Significant correlations were identified in the SAH patients between CSF parameters and

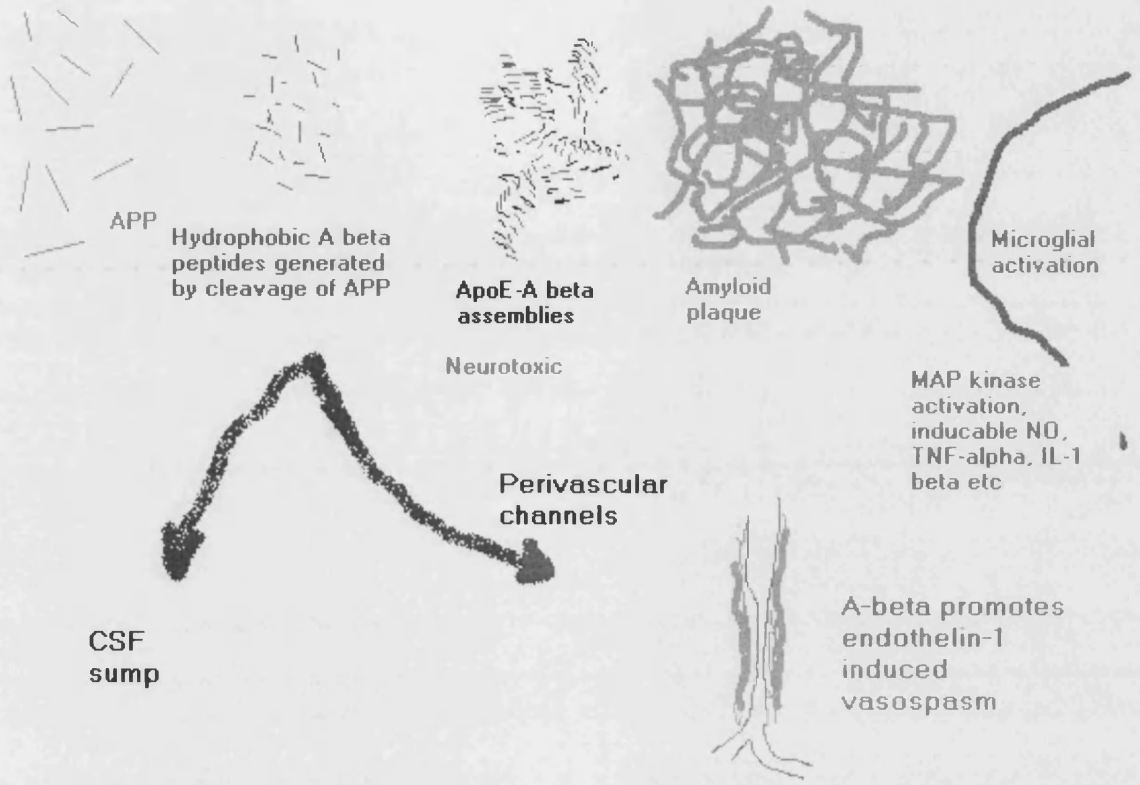
both injury severity and clinical outcome. Injury severity, assessed using the Glasgow Coma Score, after SAH or TBI did not correlate with clinical outcome in this study.

Second, assaying the concentration of a substance in the ventricular or lumbar CSF of healthy normal controls is not ethical, due to the invasiveness of the procedure, and the associated risks. Thus the control group was selected from patients with suspected shunt dysfunction, hydrocephalus, or a suspected neurological disorder for whom there was a clinical indication for CSF analysis. The control patients did not have acute brain injury, had normal conscious level and ICP, and the CSF indices were within the normal range. A substantial number of control CSF (less than five cells per cubic millilitre) samples were analysed and these were categorised according to the indication for CSF analysis and biochemical indices. From the analysis of these subgroups it was possible to estimate the influence of category type upon the concentration of apoE in the control CSF. Patients with a diagnosis of definite MS were found to have significantly lower CSF apoE concentration compared to the other subgroups. However, there was no significant difference between the apoE concentration of the other subgroups. On the basis of this finding, patients with suspected or definite MS were not used as controls. Although there was significant correlation between the concentration of albumin in the CSF of the control group and age, there was no significant correlation between CSF apoE and age. The association between the ratio of apoE to albumin in the CSF of the control group and age just failed to reach statistical significance. Control CSF for the determination of control values for Tau and Aβ were selected from the population of control CSF specimens such that they matched the age distribution of the brain injured patients, and had albumin and total protein concentration within the ninety-fifth centile of the larger control population. The values for the various protein concentrations determined in the control group of this study compared reasonable favourably to those reported by others given the variations that exist between groups with regard to control selection, methodology, and random variation. In the present study the apoE concentration of control CSF was 12.5 ± 5.5 mg/L. This is higher than that reported by Carlson (7.5 ± 3.2), Pirttilla (5.6 ± 1.7), Blennow (5.1 ± 2.7), Hesse (4.5 ± 2.7), Skoog (3.8 ± 1.7), Song (4.1 ± 1.7) and Merched (0.8 ± 0.3). (Blennow et al. 1994; Carlsson et al. 1991; Hesse et al. 2000; Merched et al. 1997; Pirttila et al. 2000; Skoog et al. 1997; Song et al. 1997) Importantly, in the present comparative study brain injury and control CSF was assayed simultaneously. Thus it is the relative, and not absolute, apoE concentration that are important to this study. The validity of the CSF controls is underpinned by the normality in terms of total protein concentration and low albumin (absence of plasma contamination). Furthermore, the concentration of astrocytic and

neuronal markers, S100B and Tau respectively, were similar in the controls used in the present study when compared to values in the published literature. The concentration of S100B in control CSF was 0.39 μg/L, almost identical to that reported in controls by Green et al (0.4). (Green et al. 1997) The concentration of Tau in control CSF was 190 pg/ml which compares favourably to that reported by Green et al (198 pg/ml) and Vandermeeren (120 pg/ml). (Green et al. 1999; Vandermeeren et al. 1993) In relation to the Aβ peptides assayed, published values for the concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ in "normal" CSF are approximately 10 ng/ml and 1 ng/ml respectively. (Andreasen et al. 1999; Kanai et al. 1998; Mehta et al. 2000; Motter et al. 1995; Shoji et al. 1998) Again though the absolute values obtained from the controls in the present study are less than these published values, the relative difference between control and brain injury CSF is striking.

In addition, a critical limitation arising from the analysis of CSF stems from the uncertainty as to how closely the composition of ventricular or subarachnoid CSF reflects that of the extracellular space. Studies in human brain injury comparing ventricular CSF and extracellular fluid (ECF) concentrations (determined using microdialysis catheters) of metabolites, amino acids, and proteins are at an early stage. Ventricular CSF most likely reflects global release via the CSF, and excludes molecules transported via perivascular and other channels. There is evidence in the literature that Aβ diffuses relatively freely through neural tissue, and may be deposited in periarterial interstitial fluid drainage pathways in AD. (Meyer-Luehmann et al. 2003; Preston et al. 2003; Weller et al. 2000; Weller et al. 1998) A preliminary microdialysis study identified substantially higher concentrations of S100B in the ECF of brain injured patients compared to ventricular CSF suggesting very high local ECF concentration or some other factor (personal communication). With respect to apoE, ECF concentrations may be relatively low due to receptor mediated uptake and an intracellular location. Thus the present study at best presents indirect *in-vivo* evidence and should be interpreted accordingly.

Figure 27 ApoE- amyloid aggregate hypothesis



The function of APP is unknown, but under normal physiological conditions APP is cleaved by secretase enzymes to form hydrophobic peptide fragments (Aβ). Aβ diffuses within neural tissue and is conveyed by bulk flow to the CSF of the ventricular system (the CSF sump) or via perivascular channels to blood vessel walls. In Cerebral Amyloid Angiopathy (CAA) Aβ accumulates in vessel walls for unknown reasons. In AD Aβ colocalises with apoE in amyloid plaque. $A\beta_{1\text{-}40}$ is the predominant form in those with AD in possession of the APOε4 allele. Amyloid aggregates appear to be neurotoxic resulting in dystrophic neurites and activation of the inflammatory response. As AD develop and advances, the concentration of Aβ in the CSF diminishes as Aβ accumulates in the cortex as Aβ aggregate. This process is promoted by apoE with possession of the APOε4 allele having the greatest amyloid promoting effect.

4.3 Future investigations and potential clinical relevance

Although these data demonstrate alterations occurring in the CSF of patients with acute brain injury, the extrapolation that these finding reflect the response of the brain to injury may not be justified. This is at best indirect evidence to support a role for these proteins in the response of the brain to injury. Key areas that require further investigation are as follows. First, the possibility that apoE, and Aβ, are depleted from the CSF due to increased clearance to the plasma compartment requires investigation. Peripheral insults such as acute myocardial infarction are associated with alterations in plasma lipoprotein and lipid profiles, which may persist for months after the initial insult. (Ryder et al. 1984) Marked alterations in lipoprotein particles have been observed in the plasma of patients undergoing a systemic inflammatory response to bacterial infection. Total plasma HDL decreases in these patients due to the decrease in plasma apoA HDL; plasma apoE HDL actually increases. (Barlage et al. 2001) After ischaemic stroke (where *APOE* appears not to influence outcome) the investigators of the Northern Manhattan Stroke Study reported that lipoproteins remained stable during the acute phase. (Kargman et al. 1998) Such studies have not been undertaken after TBI and SAH. Under normal circumstances the plasma to CSF ratio for apoE is approximately ten to one, resulting in a steep gradient across the intact *blood brain barrier.* In order for apoE and LpE to decrease in the CSF due to increased transfer to the plasma compartment there would have to be a substantial decrease in plasma apoE to reverse this gradient even under conditions of impaired *blood brain barrier* integrity.

A relatively simple approach would be to administer labelled apoE into the CSF before experimental brain injury and determine the distribution of the labelled apoE thereafter. Cannulating the ventricles or cisterna magna of rodents subjected to experimental brain injury presents significant challenges due to scale, but has been performed in rodents as small as mice enabling studies to be conducted on transgenic mice. When apoE was infused into the lateral ventricle of *APOE* knockout mice, however, apoE was not detected by immunocytochemistry. (Horsburgh et al. 2000a) Labelled apoE could also be introduced to the plasma compartment prior to experimental brain injury, and the anatomical distribution determined after injury and in controls. The attraction of experimental brain injury is the ability to control for, and limit, variation in biological factors, and the type or size of injury to a degree that is often not possible in human studies. Furthermore, histological and biochemical examination of the brain tissue is feasible, and the administration of agents is often acceptable. The disadvantage of

experimental brain injury is that findings from experiments performed in the controlled environment of the laboratory on different species may be of limited relevance to humans.

Second, the hypothesis that apoE and Aβ decrease in the CSF as a result of increased retention and uptake by the brain could be explored further in humans by a variety of means. For example comparison could be made between the concentration of apoE in the CSF, brain tissue, and plasma during surgery after brain injury. For example, CSF is often drained from the subarachnoid space for the purpose of brain relaxation during aneurysm surgery, and brain tissue may be resected for the purposes of access to the aneurysm neck (e.g. gyrus rectus). These patients would have been fasted for general anaesthetic reducing the concerns relating to gastric ulceration. CSF is less often accessible at the time of surgery after TBI due to raised intracranial pressure, but it might be feasible to determine the concentration of apoE in contused brain resected after trauma. Controls are a problem in this type of study, though relatively normal brain may be resected during the course of epilepsy surgery. Alternatively, *in vivo* measurement of the tissue concentration of smaller proteins and peptides could be attempted using microdialysis but would require the development of dialysis membranes with high recovery of the protein. Methods for imaging these proteins *in-vivo* may be on the very distant horizon. (Bacskai et al. 2001; Fox and Rossor, 2000) In addition, CSF and brain tissue could be obtained from autopsy cases and comparisons made between patients who die after acute brain injury and those dying from other causes, provided consent can be obtained for tissue retention for the purpose of medical research. Studies of this nature may be confounded by changes that occur post mortem particularly in cases where there is a time delay between death and autopsy during which time brain liquefaction occurs. Further immunocytochemical studies could be performed on archive brain injury tissue to supplement previous studies with focus upon the distribution of apoE, and CNS lipoprotein receptor immunoreactivity after SAH. A limited study undertaking semi-quantitative apoE determination in archival head injury material found no increase in apoE levels compared to controls. (Horsburgh et al. 2000)

The end-point of clinical research is improved outcome for patients. The question as to whether novel treatment strategies targeting brain apoE, Aβ, or cholesterol, have the potential to improve patient outcome after acute brain injury requires definitive evidence to support their involvement in the response of the brain to injury. This study provides some novel preliminary evidence supporting a role for these molecules in the response of the brain to injury, but large scale systematic studies would be required, in addition to the post mortem and experimental brain injury studies outlined above, to establish such a role.

Placebo controlled randomised trials of neuroprotective agents have proved expensive and of no demonstrable benefit to brain injury patients. (Maas et al. 1999) The challenges that beset the neuroprotection studies apply equally to large-scale observational studies. One particular criticism of the neuroprotection trials was the failure of the investigators to monitor the physiological effect to which the agent was targeted, focussing mainly on clinical outcome as the treatment outcome measure. In addition, it had not been established that a process, which may be demonstrable in experimental brain injury, actually occurred in the various types of human brain injury, nor at what time point the process was occurring. In future studies there is likely to be increased use of physiological and biochemical monitoring to determine treatment effects. This may include intracranial pressure monitoring and trend analysis, brain oximetry and cerebral blood flow studies, and biochemical monitoring using microdialysis and or positron emission tomography or magnetic resonance spectroscopy. In addition there is the potential that proteins relevant to acute brain injury could be serially monitored in CSF, microdialysis dialysate, plasma, and urine. These could potentially help classify and quantify the response of the brain to injury in terms of secondary processes such as cytoskeletal disruption, astrocytosis, neuronal apoptosis or necrosis, microglial activation and the inflammatory response. The less invasive the means of monitoring such proteins (e.g. plasma and urine versus CSF or microdialysis) the less is the associated risk, and the wider the acceptability. However, biochemical monitoring of plasma (and CSF) reflect global changes in the response of the brain to injury, obscuring valuable information relating to focal injury, which is an important component to brain injury. Microdialysis does give biochemical information about focal events, but there may be uncertainty relating the position of the microdialysis membrane to the intended area of monitoring. Currently available dialysis membranes have acceptable recovery of molecules with a molecular weight below 12KDa. Plasma monitoring has a number of other disadvantages. First, there is a time delay during transit of the protein from the brain to the plasma via the CSF and cerebral venous sinuses, making the correlation of plasma concentration with acute cerebral events such as raised intracranial pressure or ischaemic insults problematic. Second, dilution and metabolism in the peripheral compartment, coupled to the possibility of shared epitopes between the CNS protein and peripheral proteins, requires the assay to have high sensitivity and specificity. The substantial advantage of plasma monitoring is that monitoring can continue over a much broader time window than is acceptable for the invasive methods. Furthermore, plasma monitoring of protein release after brain injury may enable monitoring of patients who are not severely injured, in whom invasive measures are inappropriate. Patients with so-called mild and moderate head injury are often managed with less therapeutic

intervention than those with severe head injury. However, it has recently been recognised that these so-called mild and moderate head injuries constitute the substantial majority of community based disability after head injury. (Thornhill et al. 2000) This raises the question as to whether this patient group should be more aggressively targeted for pharmacological intervention. Whilst imaging techniques such as MRI may facilitate the non-invasive stratification and monitoring of structural alterations after mild and moderate head injury, there are limitations due to availability and injury classification. Stratification of injury severity and monitoring of secondary processes after injury may be feasible by assaying brain derived proteins released into the plasma compartment after injury. There are a number of published series reporting increased plasma S100B after acute brain injury, but no large scale studies and no studies reporting increased plasma Tau. (Anderson et al. 2001; Biberthaler et al. 2001; Herrmann et al. 2001; Petzold et al. 2002; Raabe et al. 1999a; Raabe et al. 1999b) Furthermore, there are no published series reporting alterations in Aβ, apoE, or cholesterol after acute brain injury. Thus whilst CSF studies such as this provide insight into the response of the brain to injury they are of limited utility with regard to large scale observational studies.

There are several key questions relating to the finding that apoE, and Aβ, is decreased in the CSF in response to injury, the answers to which may be clinically relevant:

- If the apoE is decreased in the CSF because it is retained and taken up by the brain after injury, is the apoE beneficial or harmful?
- If the apoE is decreased in the CSF because it is lost to the plasma compartment, is this effect beneficial or harmful?
- Would treatment strategies targeting the depletion of apoE from the CSF improve clinical outcome after acute brain injury?

Whilst there is evidence that apoE has cholesterol and lipid recycling functions, and antioxidant activity, there is also evidence that apoE promotes amyloid formation. The relative importance that these postulated functions have upon outcome must be determined before undertaking studies targeting CNS apoE after brain injury. Evidence from experimental brain injury using *APOE* transgenic mice, which exhibit impaired neuronal plasticity after injury, identify increased apoE immunoreactivity in association with clearance of lipid and cholesterol from the site of injury. The observed effect is not dependent of *APOE* genotype and therefore does not explain the observed difference in recovery observed in TBI and SAH patients of different *APOE* genotype. (White et al. 2001a; White et al. 2001b) The failure to determine differences in functional recovery after experimental injury in many studies limits estimation of the relevance of changes observed histologically. Although intraventricular infusion of apoE appears to decrease

oxidative stress in experimental brain injury, the failure to determine such an effect on functional recovery, limits interpretation of such findings in terms of potential clinical benefit. There are a number of preliminary studies that could be performed in vitro. First, the toxicity of lipoprotein particles, isolated from the CSF as described in this study, to serum free human cell cultures of neurons and glial cells could be determined. Importantly effects upon Aβ and cholesterol metabolism should be assessed. Second, the effect of insults such as hypoxia, added Aβ, or cholesterol to such cultures could be evaluated. Third, the effect of cholesterol lowering or apoE modifying agents could be assessed upon these cultures under different conditions. ApoE labelled with a fluorescent marker could be added to the culture medium to monitor apoE trafficking in response to injury.

The studies with *APP* and *APOE* transgenic mice support the concept that apoE is amyloidogenic and that *APOE* ε4 is more so than *APOE* ε3. These findings, coupled to the finding of increased amyloid deposition in the brains of patients who die after TBI, provide compelling evidence to support an isoform dependent interaction that is important after TBI. However, studies with *APP* transgenic mice suggest that amyloid deposits may be cleared after experimental brain injury. (Bales et al. 1997; Nakagawa et al. 1999; Nakagawa et al. 2000) Furthermore, studies of long-term survivors of TBI, who die for other reasons, do not identify increased numbers of Aβ deposits in TBI patients compared to controls. (Macfarlane et al. 1999) Although these studies suggest that Aβ deposition after TBI may be reversible, they do not provide evidence that Aβ deposition results in worse outcome after TBI. The data identifying increased prevalence of Aβ deposits in the brains of *APOE* ε4 patients who die after TBI could be interpreted as suggesting that patients who have the *APOE* ε4 allele have more deposits and are more likely to die after TBI. (Roses and Saunders, 1995) The clinical relevance of Aβ deposition and clearance after TBI stems from the recent finding that Aβ deposits are cleared from *APP* transgenic mice immunised with Aβ. (Dodart et al. 2002a; Games et al. 2000) Importantly, in these studies, evidence of improved function in immunised animals was presented. Clinical trials assessing Aβ immunotherapy in AD have currently been halted due to adverse effects underlining the requirement to understand more about human apoE and Aβ biology. (Schenk et al. 2000) Intriguingly, a recent case report identified post-mortem findings in the brain of an AD patient who died after Aβ immunization (AN-1792) similar to those observed in the transgenic mice studies. (Nicoll et al. 2003)

This study suggests that cholesterol containing LpE is selectively depleted from brain injury CSF. Relatively little is known about brain cholesterol flux in the normal human

brain, and even less in relation to chronic and acute brain injury. One may speculate that the LpE is decreased in the CSF due to increased uptake by the brain. Thus cholesterol and other debris released from injured cells undergoing necrotic or apoptotic death may be recycled to viable cells with an ongoing cholesterol demand. It is also possible that excessive cholesterol release after injury is potentially toxic, and the response of the brain to this biochemical hazard is to clear the excessive cholesterol to the plasma compartment for metabolism in the liver and clearance via the gastro-intestinal tract. In the CNS cholesterol is converted to 24S-hydroxycholesterol for excretion via the liver. There are reports that 24S-hydroxycholesterol is increased in the plasma of patients with AD, and it is speculated that this reflects increased cholesterol turnover due to increased neurodegeneration in the brains of AD patients. It is also reported that this effect is greatest in AD patients who posses the *APOE* ε4 allele. (Lutjohann et al. 2000; Papassotiropoulos et al. 2000) Intriguingly, patients who are brain dead and patients with advanced AD have decreased plasma concentration of 24S-hydroxycholesterol. (Bretillon et al. 2000) The increased interest in cholesterol and AD stems from the key role that apoE plays in cholesterol homeostasis and the recent finding that patients treated with Statins have a decreased prevalence of AD. (Jick et al. 2000; Wolozin et al. 2000) If it can be shown that cholesterol is an important determinant of outcome after acute brain injury, and high brain cholesterol levels adversely affect outcome then treatment of acute brain injury patients with Statins that decrease brain cholesterol levels may improve outcome. The effect of Statins could be monitored centrally via microdialysis or via the CSF as in this study or in the plasma via 24S-hydroxycholesterol, which would be expected to decrease. The attraction of such a study in patients with acute brain injury is that Statins have well characterised safety profiles, and are administered parentally. Cholesterol has also been implicated in Aβ deposit formation through *in-vitro* evidence supporting the concept that the cholesterol status of the cell membrane lipid bi-layer is an important determinant for the activity of the APP cleavage enzyme α-secretase. (Bodovitz and Klein, 1996) Furthermore, in vitro evidence suggests that cholesterol depletion due to Statins results in decreased amyloid deposition in hippocampal neurons. (Simons et al. 1998) Prior to any consideration of the use of cholesterol lowering agents in the context of acute brain injury, the relationship between brain or plasma cholesterol status and outcome must be established. After ischaemic stroke, low cholesterol appears to be associated with worse recovery, and low plasma cholesterol is a risk factor for haemorrhagic stroke. (Dyker et al. 1997; Iso et al. 1989; Leppala et al. 1999; Vauthey et al. 2000) Thus after acute brain injury it may be more beneficial to supplement the brains` depleted cholesterol-apoE (LpE) status by the infusion of HDL.

5 Conclusions

After traumatic brain injury and subarachnoid haemorrhage there is a profound decrease in the concentration of apolipoprotein E in the cerebrospinal fluid compared to that found in controls. The decrease in the apolipoprotein E concentration is paralleled by a decrease in the apolipoprotein E class of lipoprotein particles in the cerebrospinal fluid. The decrease in apolipoprotein E is paralleled by a decrease in the concentration of amyloid-beta. The decrease in apolipoprotein E and amyloid-beta correlate with the severity of injury and clinical outcome. The concentration of marker proteins for astrocytes and neurons are increased in the cerebrospinal fluid after traumatic brain injury, and these correlate with the severity of injury and clinical outcome.

These findings provide new indirect evidence to support the concept that apolipoprotein E plays a role in the response of the brain to injury. These findings are consistent with those from other studies suggesting that apolipoprotein E participates in a co-ordinated response to clear or recycle debris that results from brain injury. In addition, the finding that both apolipoprotein E and amyloid-beta decrease in the cerebrospinal fluid after injury is consistent with the concept that apolipoprotein E and amyloid-beta form insoluble aggregates *in-vivo*. From the finding that the concentration of apolipoprotein E in the cerebrospinal fluid of patients with subarachnoid haemorrhage is lower in those with more severe clinical outcome, one may hypothesise that increased clearance adversely affects recovery. This effect is not detected at later time points suggesting that low concentrations of apolipoprotein E in the cerebrospinal fluid are critical soon after injury.

This study takes the first steps to find *in-vivo* evidence to support a role for apolipoprotein E in the response of the brain to injury. From this study emerge a number of avenues for future investigation using post-mortem material, experimental brain injury, and further clinical studies. The justification for the further investigation of the relationship between apolipoprotein E, amyloid-beta deposition, and cholesterol homeostasis after acute brain injury, is the recent identification of novel treatment and prevention strategies for chronic neurodegenerative disorders. Thus amyloid-beta immunization or cholesterol lowering agents may have a role after acute neuronal injury. In addition, if low cerebrospinal fluid apolipoprotein E results in worse outcome, this begs the question as to whether apolipoprotein E supplementation by administration to the plasma or cerebral ventricle would improve outcome?

6 Appendix

6.1 Summary of issues to address when obtaining consent

6.1.1.1 For all samples

- The sample will be treated as a gift
- The donor has no right to a share of any profits that might arise from research using the sample.
- Who will be responsible for custody of the sample (host institution/funding body).
- What personal information will be used in the research.
- The arrangements for protecting the donor's confidentiality.
- If the research might reveal any information of immediate clinical relevance, this will be fed back.
- Arrangements for feeding back or obtaining access to individual research results, if any, and for informing participants of the outcome of the research.
- Consent to access medical records, if required.
- Specific consent for any genetic tests, if required.

6.1.1.2 If the sample is to be stored for possible secondary use

- The types of studies the sample may be used for and the diseases that may be investigated
- Possible impact of secondary studies on the interests of donors and their relatives.
- Means of accessing information on secondary studies, if appropriate.
- Secondary studies will have to be approved by an ethics committee.
- Consent to share samples with other uses.
- Consent to commercial use, and an explanation of the potential benefits of commercial involvement, if appropriate.

6.2 Patient information sheet and consent form

6.2.1.1 Information sheet for patients and their relatives participating in the apoE CSF study

Thank you for considering taking part in this study. Before reading any further, could I remind you that you are under no obligation to participate in this study and if you should decide to participate you are able to discontinue at any stage.

6.2.1.2 What is the study about?

We are doing research into a brain protein called apoE. ApoE may be involved in the recovery process following brain injury. You may not have had a brain injury but the analysis of your fluid is important to us for comparison with those who have had a brain injury. Relatively little is known about this protein but it may have a role in recovery after brain injury and the type of protein we inherit may influence this process. We hope this research will provide useful information that may benefit patients in the future.

6.2.1.3 What does the study involve?

We require your permission to undertake research on a sample of brain fluid (cerebrospinal fluid or CSF for short). This fluid would normally be discarded. The tests we would like to do involve the study of the apoE protein and the DNA (genetic material) of the gene that produces the protein. No extra procedure is involved but we do require your permission to keep the fluid and perform the tests. It would also be helpful for us to store the sample for future analysis of other proteins and genes that may be related to brain injury.

6.2.1.4 Who will have access to the data?

The information from this investigation will be stored on a computer that is not linked to other systems such as the Internet so will only be known to the research team here in Glasgow. The results of this study will at a later date be published in medical journals so that other doctors and researchers can share this knowledge but it will be presented in such a way that patient confidentiality is preserved. If you have any further questions please do not hesitate to contact me. If I am not available at that time then please leave a name and number so I can call you back. Thank you for your consideration. Andrew Kay BSc MBBS FRCS. Wellcome Trust Clinical Research Fellow. University Department of Neurosurgery. Tel: 0141 201 2047

6.2.1.5 Consent form for CSF study

I consent to residual cerebrospinal fluid (CSF) being used for analysis of the protein apoE including the determination of the APOE gene type.

YES/NO

I consent to storage of residual CSF and analysis of other proteins and genes that may be involved in brain injury.

YES/NO

I understand that I am under no obligation to participate in this study and that I may discontinue at any stage.

YES/NO

I have read the patient information sheet and understand that any questions relating to the study may be discussed with a member of the research team.

YES/NO

I understand that information arising from this research may at a later date be presented in medical journals and that my anonymity and confidentiality is assured.

YES/NO

Name (Please print)

Signature

Date

6.3 ApoE ELISA

In detail, 5 µl of neat rabbit polyclonal anti human apoE antibody (DAKO ref. no. A0077), was added to 25ml of 0.02 mol/l Citrate buffer pH9.6 and mixed. 100 µl of the diluted antibody solution was aliquotted to each well of a 96 well ELISA plate (Nunc-immuno plate, Maxisorp ref. no. 43945A) and the plate incubated overnight at 4°C. After overnight incubation the plate was washed four times with 300 µl phosphate buffered saline (PBS) pH 7.3. After four washes the plate was blotted dry. 150 µlof fresh blocking buffer (2% Bovine Serum Albumin in PBS pH 7.3) was aliquotted into each well, and the covered plate was incubated at 37° C for one hour. The CSF sample/antibody diluent (dilution of equal volume of blocking buffer with PBS pH 7.3) was prepared, and CSF samples diluted

one volume in three hundred. The standard curve (100 μg/l, 50 μg/l, 25 μg/l, 12.5 μg/l, 6.2 g/l, 3.1μg/l, apoE) which had previously been prepared as a batch, aliquotted, and stored at -80°C, was thawed along with the internal quality controls. After incubated at 37° C for one hour the ELISA plate was washed four times with 300 μl of wash buffer (PBS pH 7.3/Tween 20) and blotted dry. The standard curve and diluted CSF samples were then added in duplicates of 100 μl to the ELISA plate. The plate was covered and incubated for one hour at 37° C. The goat anti human apoE antibody (CHEMICON ref. no. AB497) was diluted in assay diluent by adding 12.5 μl of the goat anti human apoE antibody to 25ml of assay diluent. After incubation of the standards and samples, the ELISA plate was washed four times as previously described. The diluted goat anti human apoE antibody was added to each well (100 μl) and the plate was covered for incubation at 37° C for one hour. The rabbit anti goat peroxidase (HRP) conjugate was prepared by adding 5 μl of rabbit anti goat IgG HRP conjugate to 25ml of assay diluent. After incubation at 37° C for one hour, the plate was washed as previously described. The diluted HRP conjugated (100 μl) antibody was aliquoted into each well, and the plate covered for incubation at 37° C for one hour. The HRP substrate was prepared and kept in the dark at room temperature until required. After incubation at 37° C for one hour, the plate was washed. In addition to the usual four washes with PBS/tween the plate was washed two times with 300 μlof PBS pH7.3, and blotted dry. 14 μl of 30% H_2O_2 was added to the 20ml of substrate solution and mixed. 100 μl of this solution was aliquotted into each well and incubated at room temperature for 15 minutes. The colour reaction was stopped by adding 50 μl of 1M hydrochloric acid to each well. The absorbance was determined at 492nm and 405 nm using a plate reader. The concentration of apoE was determined for each sample relative to the standard curve by plotting the standards and fitting a polynomial regression line, from which sample values mathematically derived. The internal quality control assayed on each plate comprised a CSF pooled from 100 patients CSF samples, and plasma pooled from 100 plasma samples. These were prepared, frozen, and stored at the same time as the standard curve.

6.3.1.1 Reagents required for one plate (usually do 2 or more)

96 well Nunc Maxisorp plate

1 g BSA, fraction V (SIGMA, ref. A 7030)

1 g Glycine (Merck, ref. 104201)

1 ml Tween 20 (Sigma, ref. P1379)

1 compound σ-phenylenediamine (Sigma, ref. P8412)

10 ul H_2O_2 30 % (Merck, ref. 107298)

1 g Na_2CO_3 (Merck, ref. 106392)

1 g $NaHCO_3$ (Merck, ref. 106329)

25 g Na_2HPO_4, 2 H_2O (Merck, ref. 106580)

20 g NaCl (Prolab, ref. 27810295)

3 g KH_2PO_4 (Merck, ref. 104873)

5 ml H_2SO_4 (Merck, ref. 100731)

0.5 g Citric acid (Merck, ref. 818707)

5 ul Rabbit polyclonal antibody anti human apoE (DAKO, ref. A0077)

5 ul Goat polyclonal antibody anti human apoE (CHEMICON, ref. AB 497)

5 ul Rabbit polyclonal antibody anti goat IgG conjugated with peroxidase (DAKO, ref. PO449)

A variety of standards are available e.g. Human recombinant apolipoprotein E3 or E4 or E2 from PANVERA, CHEMICON, or DADE BEHRING.

6.3.1.2 Stock Solutions

(Volumes calculated for a 96 well plate)

- R1 Sodium Carbonate 0.20mol/l -store for 1 month at +4°C

Na_2CO_3 0.53 g

H_2O 25 ml

- R2 Sodium Hydrogen Carbonate 0.2 mol/l- store for1 month at +4°C

$NaHCO_3$ 0.42 g

H_2O

- R3 Carbonate Buffer 16 mmol/l-Bicarbonate 34 mmol/l pH 9.6- Store 1 week at +4° C

Sodium anhydride solution 0.2 M (R1) 2 ml

Sodium hydrogen carbonate 0.2 M (R2) 4.25 ml

H_2O 16 ml

If pH>9.6, add R2 until pH=9.6

If pH<9.6,add R1 until pH=9.6

Make up to total volume of 25 ml

- R4 Disodium Phosphate 10/15 mol/l, NaCl 1.54 mol/l

Na_2HPO_4, $2H_2O$ 23.76 g

NaCl 18 g

H_2O 200 ml

Warm to 40°C to dissolve

Store at room temperature for 1 week

- R5 Potassium Phosphate 1/15 mol/l-Store 1 week at +4°C

KH_2PO_4 2.27 g

H_2O 250 ml

- R6 Citrate Buffer 0.04mmol/l pH 5.8- Store 1 week at +4°C

Citric acid 0.192 g

Na_2HPO_4, $2H_2O$ 0.538 g

H_2O 20 ml

Adjust to pH 5.8 as necessary

H_2O 25 ml

- R7 Sulphuric Acid 1 mol/l -store 6 week at room temp

H_2SO_4 1,47 ml

H_2O 25 ml

- R8 Coating Polyclonal Rabbit Ab (Ab1)- 1/5005, 118 ng/well in carbonate buffer

- R9 Phosphate disodium solution 1/15 mol/l, NaCl 0.154 mol/l

Phosphate disodium solution 10/15 M NaCl 1.54 M (R4) 100ml

H_2O 1000 ml

- R10 PBS buffer 1/15 mol/l, NaCl 0.154 mol/l pH 7.4

Solution of mono-potassium phosphate 1/15 M (R5) 150 ml

Solution of disodium phosphate 1/15 M, NaCl 0.154 M (R9) pH 7.4

- R11 BSA with 2% glycine 7.5 g/l

BSA (Sigma, ref. A7030) 1g

Glycine (Merck, ref. 1.04201) 0.375 g

PBS buffer1/15 M, NaCl 0.154 M pH7.4 (R10) 50 ml

- R12 Sample/Ab diluent

Blocking reagent (R11) 35 ml

PBS buffer 1/15 M, NaCl 0.154 M pH 7.4 (R10) 35 ml

- R13 Wash Solution

PBS 1/15 M, NaCl 0.154 M pH 7.4 (R10) 500ml

Tween 20 (Sigma, ref. P1379) 0.5 ml

- R14 goat polyclonal anti human apoE -1/2 000

- R15 HRP anti goat IgG- 1/5000

- R16 Substrate (OPD at 2.8 mg/ml)

Citrate buffer 0.04 mol/l pH 5.8 (R6) 10.5 ml

H_2O_2 30% (MERCK, ref. 107298) 7 ul

σ-phenylenediamine, HCL (SIGMA, ref. P8412, 30 mg)

6.3.1.3 Summary of Steps

- Coat

Polyclonal Dako (118 ng/well) 100 µl/well

Seal with sticking paper and incubate for >17 hours at 4°C

Prepare solutions R9 R10 R11 R12 R13

- Wash-PBS

Wash 3 times with PBS 1/15 M pH 7.4 (R10) 300µl/well

Remove wash buffer

- Block

Block solution (R11): 150 µl/well

Incubate for 1 hour in a well-humidified room at 37°C

- Prepare dilutions and standard curve

Dilute samples; thaw standard curve and QCs

- Wash

Wash 3 times with wash solution (R13) 300 µl/well

- Add samples and standard curve

Aliquot 100 µl/well

- Capture

Seal plate and incubate for 2 hours at 37°C in humidified room

- Incubate with detection antibody

Prepare R14 (final dilution Ab2 1/2000)

3 washes R13 300 µl/well

Remove wash

R 14 100 µl/well

Incubate plate for 1 hour at 37°C in humidified room

- Incubation with HRP Ab

Prepare R15 (Ab3 diluted to 1/5 000)

3 washes (R13) 300 µl/well

Dry

R15 at 4°C

Incubate plate at 37 °C for 1 hour in humidified room

- Develop colour reaction

Prepare R16

4 washes (R13) 300 µl/well

2 washes with PBS Buffer (R10) 300 µl/well

Dry

Substrate (R16) 100 µl/well

<30 mins at room temperature

- Stop reaction

Sulphuric acid 1 mol/l (R7) 100 µl/well or 50 µl/well of 1M HCL

Read absorbance at 492 nm.

6.4 Albumin rocket electrophoresis

- Electrophoresis buffer

Barbitone buffer pH =8.6/I = 0.075

Per litre: Na-Barbitone 13.14g, Barbitone 2.07g, Ca-lactate 0.58g.

Dissolve the Na-Barbitone in half the final volume of H_20, add Barbitone and mix until dissolved. Dissolve the Ca-lactate and make up to final volume. Preserve by adding 1ml/L of 5% w/v thymol in isopropanol (anti-bacterial).

- Stain

2g Comassie Brilliant Blue G250 dissolved in 1L of Methanol (350): glacial acetic acid (100): water (550) by volume. Ethanol may substitute methanol.

- Destain

Methanol (350): glacial acetic acid (100): water by volume. Ethanol may substitute methanol.

- Gel

50mL electrophoresis buffer, 1.5g polyethylene glycol 6000 (PEG6000), 0.5g ME agarose.

Dissolve in boiling water. Gel dimensions 190mm x 159mm x 1.5mm thick gel (47mL).

- Antibody

Poly- goat anti-human albumin (Inctar, Lot No. 918743)

Cool gel to 65 ° C before adding antibody

Concentration = 200 µl in 50 ml

CSF neat 5µl/well

- Electrophoresis

Power 150V/25mA. Run overnight, but can be run in 3 hours.

- Assay

Remove gel and press under filter paper x 15-30 minutes. Wash in normal saline x 15-30 minutes. Repress and dry. Stain for 15 minutes.

- Destain

- Dry with hairdryer

Enlarge with photocopier x 200

Measure with ruler for calibration curve.

Albumin standard = 50mg/mL dilute down 40 / 30 / 20 / 10 / 5

6.5 Sequential preparation of VLDL, IDL, LDL, HDL_2 and HDL_3 by ultracentrifugation

6.5.1.1 IDL 1.019 g/ml (Containing VLDL).

4 ml Plasma + 0.32 ml d = 1.182g/ml mix and overlay with 1.68 ml of 1.019g/ml. Centrifuge at 35,000 r.p.m. overnight in 50.4 rotor. Remove and discard the top 2ml.

6.5.1.2 LDL 1.063 g/ml

To remaining 4 ml add 1.47 ml d = 1.182 mix and overlay with 0.53 ml d = 1.063g/ml. Centrifuge at 35,000 rpm overnight in 50.4 rotor. Remove and keep the LDL in the top 2 ml if 4 ml of Plasma was spun.

6.5.1.3 HDL_2 1.125 g/ml.

To the remaining 4 ml add 1.3 ml d = 1.3104 g/ml + 0.7 ml of d = 1.125 g/ml. Centrifuge at 35,000-rpm for 48hr in 50.4 rotor. Remove and keep the top 1 ml. (Discard next 2ml).

6.5.1.4 HDL_3 1.21 g/ml.

To remaining 3 ml add 3ml of d = 1.3104 mix. Centrifuge at 35,000-rpm for 48hr in 50.4 rotor. Remove and keep the top 1ml.

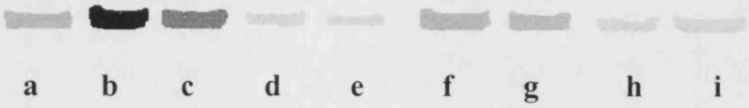
6.6 Brain tissue preparation

Unfixed post-mortem brain tissue was obtained from control subjects courtesy of Dr Axel Petzold and the MS Society Tissue Bank at the Institute of Neurology, Queen's Square. The mean post-mortem interval was 26.9 (1-40) hours. Snap-frozen blocks of control tissue (0.5-1 g wet weight) were cut and re-suspended at 1:5 g/mL in Tris-HCL buffer (100 mM Tris, pH 8.1 with 1% Triton X-100). Samples were homogenised on ice by sonication, triturated 3 times through 19 and 21 gauge needles and spun at 20,000g. In order to separate myelin protein, di-iso-propyl (1:5,000) was added. After extensive mixing the mixture was spun at 20,000g. The supernatant was covered by a myelin layer. A needle

was put through the myelin layer and the supernatant drawn up into a 1ml syringe. A protease inhibitor cocktail containing AEBSF for inhibition of trypsin and chymotrypsin; aprotinine for inhibition of trypsin, chymotrypsin, plasmin, trypsinogen, urokinase and kallikrein; leupeptin for inhibition of calpain, trypsin, papain and cathepsin B; statin for inhibition of aminopeptidases; pepstatin A for inhibition of acid proteases and E-64 for inhibition of cysteine proteases was added in a dilution of 1:100 to supernatant. After dilution into aliquots of 1:1000, 1:5,000, 1:10,000, and 1:100,000 the samples were stored at -70°C.

6.7 CSF PAGE

Results from 4% PAGE and immunoblotting of brain injury and control CSF samples.



a = control CSF: [apoE] ^{ELISA} = 7.1 mg/L

b = control CSF: [apoE] ^{ELISA} = 31 mg/L

c = control CSF: [apoE] ^{ELISA} = 18 mg/L

d = TBI CSF: [apoE] ^{ELISA} = 0.5 mg/L

e = TBI CSF: [apoE] ^{ELISA} = 0.2 mg/L

f = TBI CSF: [apoE] ^{ELISA} = 10.5 mg/L

g = TBI CSF: [apoE] ^{ELISA} = 5.5 mg/L

h and i = white matter homogenate: [apoE] ^{ELISA} = 0.5 and 0.4 mg/L respectively

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