UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Association between cytokine gene polymorphisms and clinical outcome after traumatic brain injury

by

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ABSTRACT

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ASSOCIATION BETWEEN CYTOKINE GENE POLYMORPHISMS AND CLINICAL OUTCOME AFTER TRAUMATIC BRAIN INJURY

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Clinical outcome after traumatic brain injury (TBI) is variable and cannot easily be predicted. There is increasing evidence to suggest there may be genetic influences on outcome. Cytokines play an important role in mediating the inflammatory response provoked within the central nervous system after TBI. I hypothesise that polymorphisms within cytokine genes may alter gene transcription and thereby alter the magnitude and duration of the inflammatory response after TBI, which in turn may affect clinical outcome.

DNA from buccal swabs has previously been collected from two separate cohorts totalling 1463 patients treated after TBI. A clinical database for these patients has been collated, including details of their injuries, outcome (GOS) after 6 months (n=1067, identified prospectively) or outcome 15-25 years after injury (n=396, identified retrospectively). Apolipoprotein E polymorphisms have previously been determined in these patients and association with outcome after TBI explored. Eleven single nucleotide polymorphisms (SNPs) in five cytokine genes were identified from the literature as having potential influence on outcome after TBI; -238 and -308 in TNFA, -174, -572 and -597 in IL-6, -889 in IL-1A, -31, -511 & +3953 in IL-1B and -509 and -800 in TGFB. After assessment of the DNA samples a DNA pre-amplification technique (GenomiPhi, Amersham Biosciences) was identified to enable multiple SNPtyping assays to be performed on a liinited DNA resource. A number of techniques were evaluated in order to determine SNPtypes. The LightTyper high throughput detection system (Roche) was selected and assays were designed and optimised for all eleven SNPs listed above. The SNPtypes of each patient sample were then determined.

In the prospective cohort (n=1067) the TNFA -308 SNP was associated with outcome 6-months after TBI (p=0.009), with the rarer A allele associated with a poorer outcome. None of the other SNPs demonstrated an association with 6-month outcome when considered individually. Patients who had composites of the TNFA -308, IL1A -889 and IL1B +3953 SNPtypes were prone to poorer outcome. The TNFA GA haplotype was also associated with 6-month outcome (p=0.036). The IL1A -889 SNP was associated with the occurrence of seizures (p=0.049) and raised intracranial pressure (p=0.01) after TBI, whilst the IL1B -31 SNP was associated with severe infections (p=0.027).

In the retrospective cohort (n=396) the TNFA -308 SNP (p=0.018) and the GA haplotype (p=0.021) were also found to be associated with 'long term' outcome. Two SNPs (IL1A -889 and IL1B +3953) and a haplotype (TTCT) in the IL-1 gene cluster were associated with a change in functional ability (as assessed by the GOS) between 6-month and 'long-term' assessments. When possessed together the rarer alleles at TNFA -308 and IL1A -889 significantly increased the risk of a decline in function (p=0.03).

Organotypic hippocampal slice cultures (OHSCs) were used to investigate the potential neurotoxic or neuroprotective effects of IL-6 and apolipoprotein E in parallel in-vitro studies, in order to explore underlying mechanisms. IL-6 exerted a protective effect in an OHSC model of ischemia but not in an excitotoxicity model. In the same models significantly less damage was sustained by cultures treated with apolipoprotein E3 as compared to those with E4 (p=0.04, ischaemia; p=0.003, excitotoxicity).

An association between cytokine gene SNPs and outcome after TBI has been demonstrated. This suggests that neuroinflammation after TBI does have an impact on clinical outcome and TNFα plays a crucial role in the process. These findings will require validation in other cohorts and the true biological effect of the SNPs must be confirmed.

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ABBREVIATIONS

Within this thesis the following abbreviations have been used;

AD	Alzheimer's disease
apoE	apolipoprotein E protein
APOE	apolipoprotein E gene
ARMS	amplification refractory mutation system
BBB	blood brain barrier
CBF	cerebral blood flow
CI	confidence interval
CNS	central nervous system
CPP	cerebral perfusion pressure
CSF	cerebrospinal fluid
CT	computed tomography
DABCYL	4-dimethylaminoazobenzene-4''-carboxylic acid
DNA	deoxyribonucleic acid
DOP	degenerate oligonucleotide primer
FITC	fluorescein-5-isocyanate
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Scale
HWE	Hardy Weinberg equilibrium
ICP	intracranial pressure
IL-1	interleukin-1
IL-1α	interlukin-1α protein
IL1A	interleukin-1α gene
IL-1β	interleukin-1β protein
IL1B	interleukin-1β gene
IL-6	interleukin-6 protein
IL6	interleukin-6 gene
MADGE	multiple array diagonal gel electrophoresis
MHC	major histocompatibility complex
NGF	nerve growth factor
NMDA	n-methyl-d-aspartate
OGD	oxygen glucose deprivation
OHSC	organotypic hippocampal slice culture
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RTA	road traffic accident
SNP	single nucleotide polymorphism
TBI	traumatic brain injury
TGF-β	transforming growth factor-beta protein
TGFB	transforming growth factor-beta gene
TNF-α	tumour necrosis factor-alpha protein
TNFA	tumour necrosis factor-alpha gene

Chapter 1

General Introduction

1.1 Introduction

Central nervous system (CNS) injury results from many pathological processes, including vascular (thrombo-embolic and haemorrhagic) events, inflammation, infection, metabolic disturbance and trauma. The damage caused is often irreversible, and may result in death or serious disability. Patients who survive serious brain injury usually require long-term care and impose a considerable burden on society. However, on an individual basis, it is difficult to predict outcome. It is important to be able to establish prognosis both for patient information and planning for future needs and services. Prognostic factors may also provide clues to the underlying mechanisms that leave the brain more or less susceptible to damage and repair.

Over recent years the role of genes, and gene polymorphisms, in terms of their role in disease susceptibility and outcome has been increasingly explored. This approach is now being extended to investigate the outcome after traumatic brain injury.

1.2 Traumatic Brain Injury

1.2.1 Pathology of head injury

Traumatic brain injury (TBI) can be defined as brain injury after trauma from an external source. Most commonly this occurs as a result of road traffic accidents

Injury Type	Number (%)
Motor vehicle occupant	295 (30)
Pedestrian	126 (13)
Other RTA	87 (9)
Work	63 (6)
Assault	53 (5)
Domestic	122 (12)
Sport	30 (3)
Fall under influence of alcohol	121 (12)
Other	99 (10)

Table 1.1 – Cause of head injury, as reported in the European Brain Injury Consortium Survey of Head Injuries (Murray *et al.*, 1999)

(RTAs), falls or physical assault (Murray *et al.*, 1999). Injuries to the brain can be classified according to a number of systems. The damage occurring after head injury may be defined as either a *primary* injury (usually resulting from the biomechanical effects of an impact with the skull and underlying brain parenchyma) or *secondary* (delayed) injury, resulting from biological processes provoked by the initial trauma. Alternatively injuries may be defined as focal (affecting a specific region of the brain) or diffuse (affecting many or all regions of the brain).

Primary brain injuries may include:

Skull fracture, which may be sub-divided in to depressed or non-depressed fractures, which can result in disruption of the underlying blood vessels, cortical laceration and cranial nerve damage.

Surface haematomas; haemorrhage between the cortical surface of the brain and the overlying skull which may occur with or without skull fracture. Extradural haematomas are most often associated with skull fractures, usually as a result of arterial laceration. Subdural haematomas may occur after disruption of so-called 'bridging veins', running between the cortical surface and the overlying meninges, and, in the context of trauma, have a high mortality. There may also be haemorrhage into the subdural space from contused cortex, due to the disruption of small cortical veins.

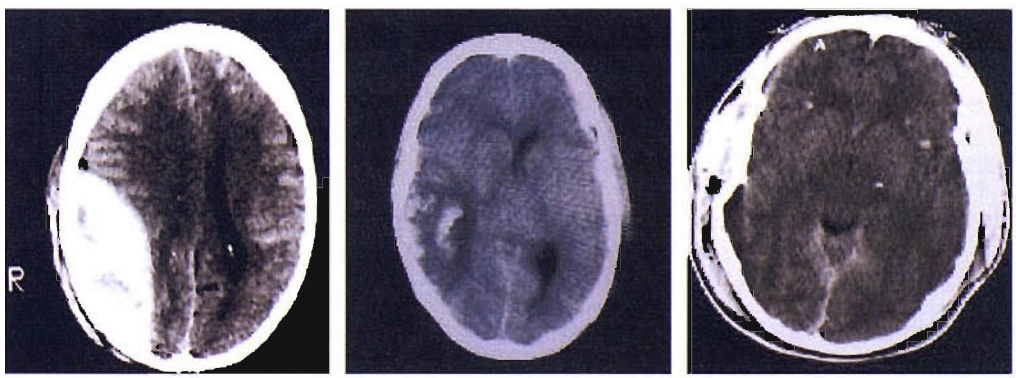


Figure 1.1 – CT images demonstrating the variety of pathologies seen after TBI. From left to right; extradural haematoma, right parietal haemorrhagic contusion, multiple petechial haemorrhages, associated with diffuse axonal injury.

Intracerebral haemorrhage; bleeding within the brain substance, as a result of injury to larger, deeper, cerebral vessels. The frontal and temporal lobes are most frequently affected.

Other haemorrhage; bleeding may also occur within the subarachnoid space due to laceration of small vessels within this space. If there is sufficient bleeding communicating hydrocephalus may ensue. Hydrocephalus may also occur after intraventricular haemorrhage. However, after TBI, intraventricular haemorrhage is uncommon but is usually indicative of a severe injury where it does occur.

Contusion; superficial areas of haemorrhage and brain swelling, often resulting from damage sustained as the brain strikes the surrounding skull during sudden deceleration, due to the inertia between the brain and skull.

Diffuse Axonal Injury (DAI); damage to the white matter in the cerebral hemispheres and the brainstem, resulting from rotational forces experienced during head injury. Microscopically there is evidence of diffuse injury to axons, characterised by axonal retraction balls, microglial stars and degeneration of white matter fibre tracts (Gennarelli *et al.*, 1982).

Parenchymal disruption; occurs in cases of penetrating head injury. This may be most severe in gunshot injuries due to the dissipation of energy from high velocity missiles.

Secondary injuries may involve:

Brain swelling/oedema; Brain oedema occurs when there is an increase in extracellular fluid within the brain parenchyma, resulting in increased tissue volume. Both cytotoxic and vasogenic mechanisms may play a role (Kimelberg, 1995). Vasogenic oedema develops due to increased permeability of the blood brain barrier (BBB) due to a combination of mechanical injury and tissue necrosis, causing disruption of capillary endothelial cells. This leads to an increase in interstitial fluid. In contrast, cytotoxic oedema is characterised by neuronal and glial cell swelling and is independent of BBB integrity. After TBI cells become increasingly permeable,

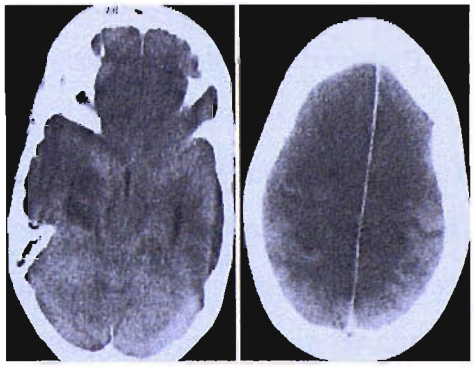


Figure 1.2 – CT images demonstrating diffuse cerebral oedema. In the left-hand image the basal cisterns are effaced and the lateral ventricles compressed, whilst on the right there is a loss of definition between the gyri and sulci.

Images from Unterberg *et al.*, 2004

through various mechanisms, to Na^+ and K^+, which in turn leads to failure of ATP-dependent ion channels and increasing cell swelling. It is becoming increasingly apparent that although vasogenic mechanisms play an important role in the development of oedema in tissue surrounding distinct pathology, such as haematomas, cytotoxic mechanisms are responsible for diffuse brain oedema (Unterberg *et al.*, 2004).

Hydrocephalus; occurs as a result of blood within the cerebrospinal fluid (CSF) space. This may cause obstruction of CSF flow within the ventricular system itself or by preventing absorption at the arachnoid villi.

Raised intracranial pressure; is an important determinant of cerebral perfusion pressure (CPP) after TBI. CPP itself is important in determining cerebral blood flow (CBF), which is critical for meeting the oxygen demands of the injured brain, and thereby preventing secondary ischaemia. The Monro-Kellie hypothesis states that the sum of the intracranial volumes of blood, brain, CSF and any other components (such as a haematoma) must remain constant, and an increase in one of these must be offset by a decrease in another, or else intracranial pressure will rise. As we have seen trauma acts to increase pressure through oedema, haematomas and hydrocephalus, amongst others. Another important cause is increased blood volume resulting from a loss of normal cerebral autoregulation.

Brain displacement/herniation; As a result of brain swelling or space occupying haematomas areas of brain may be forced under the falx or through the tentorial

incisura where midbrain compression may occur. The cerebellar tonsils may be forced into the foramen magnum, causing compression of the medulla (a process known as a 'cerebellar cone' or 'coning').

Primary injuries generally occur at the time of trauma and are not preventable. Many of the secondary injuries occur as a result of biological processes that evolve over the minutes and hours after the initial injury. These processes may be amenable to therapy and may alter outcome in the clinical setting.

1.2.2 Outcome after head injury

Worldwide, head injury is a major cause of morbidity and mortality. In the UK one million people present to Accident & Emergency units every year having suffered a head injury (Teasdale, 1995; Report of the working party on the management of patients with head injuries, 1999). Approximately half are children under the age of sixteen. The death rate is 9 per 100,000 per year, 1% of all deaths, but accounts for almost 20% of all deaths of people under 35 (Teasdale, 1995; Report of the working party on the management of patients with head injuries, 1999).

The morbidity from head injury is significant, with impairment of many human functions. These include neurological, cognitive, behavioural and social deficits. It has been estimated that the lifetime cost of providing care to a person who has suffered a severe TBI in the United States approaches $2 million, although this does not include lost earnings and the cost to social services (NIH Conses Statement, 1998).

Head injuries are usually classified according to severity, from mild to severe. The majority of mild injuries do not require specific treatment while severe injuries, if not fatal, require expert treatment and often result in significant disability even after treatment. The severity of injury can be classified according to the Glasgow Coma Scale (GCS) (Teasdale & Jennett, 1974) (see Appendix B1), with approximately 90% being mild, 5% moderate and 5% severe (Kay & Teasdale, 2001). Mild to moderate injuries can result in long-term disability but this is often determined by quality of rehabilitation. After severe injury outcome is more dependent on medical

intervention and treatment (Finfer & Cohen, 2001). In these patients factors including mechanism of injury, patient age and physiological markers such as hypotension on admission adversely affect outcome (Demetriades *et al.*, 2004).

The outcome from head injury is variable and cannot be predicted purely on the basis of clinical features or radiological findings (Teasdale & Graham, 1998; Graham *et al.*, 2000). It has been observed that apparently similar patients will have different outcomes despite similar injuries and clinical treatment. A study by Thornhill *et al.* (2000) showed that age of over 40, pre-existing physical disability and a history of brain illness were independent predictors of poor outcome after mild brain injury. However 35% of patients without any of these factors still failed to achieve a good outcome. It has therefore been proposed that genetic factors may influence outcome after head injury and there is now evidence to support this theory (see Section 1.5).

In studies of five-year outcome after head injury there is evidence for a general improvement in function although a decline is noted in some individuals (Masson *et al.*, 1997; Hammond *et al.*, 2004). However five years post injury is still not long enough to detect any decline later in life. Prospective studies of long-term outcome are sparse but do seem to suggest there is a decline (Corkin *et* al., 1989; Plassman *et* al., 2000). In addition there is a large body of evidence supporting the theory of traumatic brain injury as a risk factor for Alzheimer's disease (Mortimer *et al.*, 1991; Guo *et al.*, 2000; Fleminger *et al.*, 2003), and a late decline in function after a previous head injury might therefore be expected.

1.3 Neuroinflammation after brain injury

After injury an inflammatory reaction is provoked. Inflammation within the brain differs from inflammation within other organ systems of the body. The brain is protected by the 'blood-brain barrier', a complex arrangement of endothelial cells lying on the basal lamina of the cerebral vasculature (Petty & Lo, 2002), which prevent large molecules and cells from entering the brain. After traumatic brain injury the innate immune response within the CNS is activated via the complement cascade (Ember & Hugli, 1997) and other inflammatory mediators such as cytokines, chemokines, prostanoids and kinins. Rapid activation of glial cells, mainly

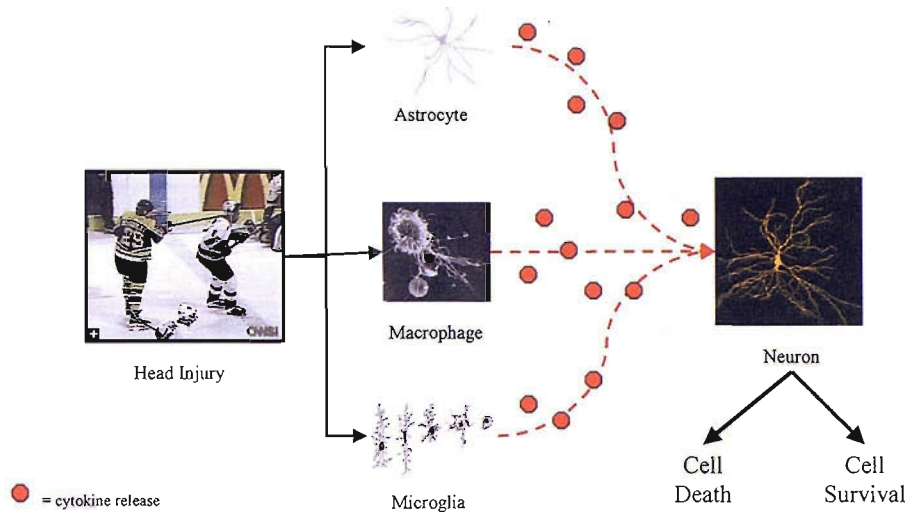


Figure 1.3 – Overview of cells involved in cytokine release after head injury.

Adapted from Allan & Rothwell, 2003.

microglia, may be augmented by polymorphs, macrophages and T- lymphocytes that are able to cross a compromised blood-brain barrier (Holmin *et al.*, 1998). These additional cells serve to exacerbate and perpetuate the inflammatory response within the CNS by the production of further inflammatory mediators. Blood brain barrier disruption is not a requirement for neuroinflammation post TBI, however. Experimental studies have clearly demonstrated rapid glial activation after injury without evidence of BBB breakdown (see below).

In the periphery the inflammatory response is generally one that is beneficial, providing a mechanism to promote tissue survival, repair and recovery, providing it is properly controlled and de-activated. In the CNS things are not so clear, as the inflammatory response may promote, for instance, the formation of brain oedema, an outcome that becomes detrimental if this causes a rise in intracranial pressure. It would be useful to identify which elements of the response were beneficial, which were detrimental and which become detrimental if they become unregulated or the

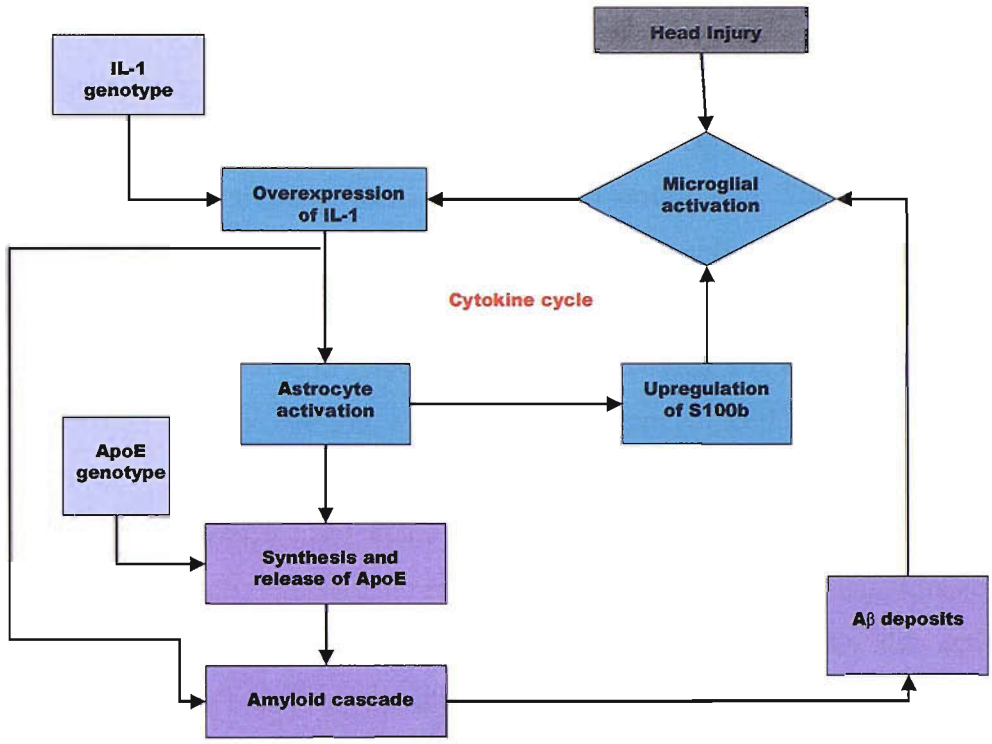


Figure 1.4 – An illustration of the 'cytokine cycle' and the possible influences of cytokine polymorphisms on this cycle, together with a possible interaction with the amyloid cascade.

Redrawn from Gentleman *et al.*, 2004.

response is prolonged. This is not straightforward as each element does not act alone but interacts with other elements to produce a response that may be beneficial or detrimental in the short or long term. Indeed it has been proposed that microglial activation triggers a 'cytokine cycle' (Gentleman *et al.*, 2004) in which the initial cytokine response is beneficial but prolonged activation upregulates other pathways that result in later cognitive decline – see Figure 1.4.

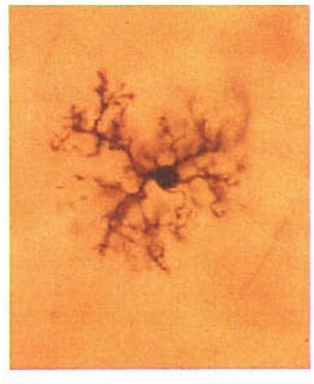


Figure 1.5 - Image of an activated microglia

Microglia

Microglia are small elongated bipolar glial cells, with a number of branches at their extremities. These extremities branch into a number of processes. They

represent 10-20% of the glia within the CNS and are uniformly distributed (within both grey and white matter) (Berry & Butt, 1997). Microglia act as the brain's intrinsic immune system and play an important role in the process of inflammation after TBI. Damaged neurons are able to signal their distress to surrounding microglia, thereby activating them (Polazzi & Contestabile, 2002; Streit, 2005). Even in their 'resting' state microglial processes are highly active in the uninjured brain but after injury these processes rapidly converge on the site of injury (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Microglial activation is also characterised by the transformation of the cell into a proliferative phagocytic cell, capable of secreting a number of cytokines. Microglial activation is generally perceived to be a neuroprotective response after acute injury. Activated microglia are capable of producing IL-1, which regulates the release of other inflammatory mediators such as TNF-α and IL-6 from the microglia. TGFβ is also produced by the activated microglia, promoting neural regeneration (see Section 1.9.4).

Cytokines

Of all the mediators of inflammation within the brain possibly the best understood are the cytokines (Allan & Rothwell, 2003). Cytokines are a group of low-molecular weight polypeptides, including the interleukins, chemokines, tumour necrosis factors and interferons. They are present in low levels in the normal brain, but levels increase after trauma, mediating both neuroprotective and neurotoxic effects (Hopkins & Rothwell, 1995; Rothwell, 1997; Lenzlinger *et al.*, 2001).

Cytokines are not purely mediators of inflammation but have many other interactions. Central neurotransmission may be modified by cytokines. Second messenger systems interact with cytokines, altering calcium homeostasis and neurotransmitter release, both of which may affect brain response to injury. Neuronal growth, development and survival are also cytokine dependent, with some cytokines promoting cell growth or survival but others exerting neurotoxic effects. Often these effects are dose dependent.

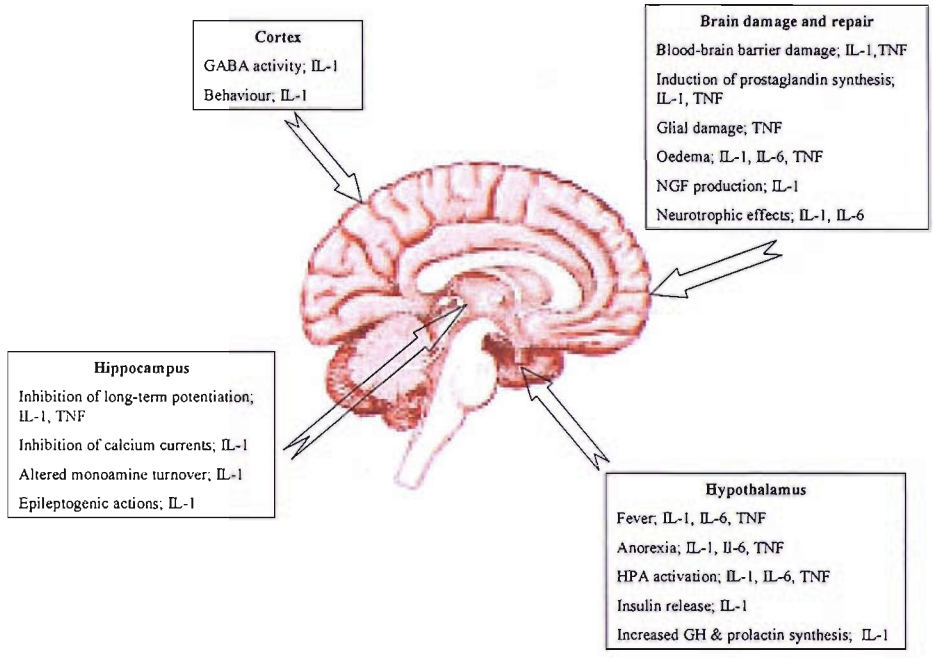


Figure 1.6 – Cytokine actions within the brain.

Adapted from McKeating and Andrews, 1998.

The importance of the microglia in the inflammatory response was outlined above. As well as being a primary source of cytokines they are also a primary target, releasing many neuroactive substances in response to cytokine stimulation. Neurotrophins and growth factors provide neuroprotection but substances such as NO, free radicals and proteases are neurotoxic. In response to injury the final outcome in terms of neuronal survival will depend upon the cytokine release profile and the substances released in response to those cytokines.

Cytokine genes are polymorphic (i.e. contain DNA sequence alternatives within normal individuals in certain populations- see Section 1.4) and these polymorphisms may alter the cytokine profile after injury. This change may be more or less neuroprotective, with resulting effects on clinical outcome. The effects of cytokine gene polymorphisms have been demonstrated in a number of disorders of the central nervous system. Polymorphism of TNF-α is associated with a 7-fold increase in the relative risk of death from cerebral malaria (McGuire *et al.*, 1994). Interleukin-1 (IL-1) polymorphisms may affect susceptibility to Alzheimer's disease (AD) (Nicoll *et*

al., 2000; Grimaldi *et al.*, 2000) and influence long-term prognosis in multiple sclerosis (Mann *et al.*, 2002). IL- 6 has been associated with AD (Pola *et al.*, 2002a) and increased risk of multi-infarct dementia (Pola *et al.*, 2002b), the latter suggesting a role in mechanisms of brain ischaemia. Cytokine polymorphisms are also associated with a number of systemic conditions, such as outcome after sepsis (Ma *et al.*, 2002) and the risk of gastric carcinoma (El-Omar *et al.*, 2000). Evidence suggests that the association with gastric carcinoma is mediated by variations in the inflammatory response to infection of the gastric mucosa by *Helicobacter Pylori*.

Apolipoprotein E and Neuroinflammation

Outcome after TBI may also be influenced by other non-cytokine factors that encourage or inhibit neuronal repair and regeneration. Apolipoprotein E (apoE) is a 34 kDa protein facilitating lipid transport and metabolism within the brain. It plays an important role in the processes of neuronal maintenance and repair. After injury apoE is upregulated within the brain (Horsburgh & Nicoll, 1996; Horsburgh *et al.*, 1997; Schauwecker *et al.*, 1998; Horburgh *et al.*, 1999), with most deriving from astroglia (Kamada *et al.*, 2003). ApoE also reversibly binds to various neurotrophic factors that are expressed in response to TBI (Gutman *et al.*; 1997). The apolipoprotein E gene (APOE) is polymorphic and two nucleotide changes within the coding region of the genes result in three different isoforms being produced. A cysteine residue is replaced with arginine at positions 112 and 158 of the protein. The three different isoforms are thus; E2 (112 cysteine: 158 cysteine), E3 (112 cysteine: 158 arginine) and E4 (112 arginine: 158 arginine). The important role of apolipoprotein E in the repair and maintenance of neurons, and its effects on outcome after head injury, is discussed in section 1.6. In the context of Alzheimer's disease it is known that chronically activated microglia may contribute to the neurodegenerative process (Streit *et al.*, 2004) and apoE may modulate this glial activation in an isoform-specific pattern (Laskowitz *et al.*, 2001, Lynch *et al.*, 2001). The apoE4 isoform is less effective at downregulating inflammatory cytokines than the E3 isoform. Therefore, through the medium of the microglia, apoE isoforms may influence the levels of cytokines produced after TBI, and thus influence outcome. Recent studies seem to confirm this, as a small peptide derived from the apoE receptor-binding region (enabling it to cross the blood-brain barrier) is able to reduce

TNF-α RNA levels after TBI and also improve functional outcome (Lynch *et al.*, 2005). In view of the previous studies showing an association between apoE isoforms and outcome after TBI and this putative mechanism for neuroprotection it is important to bear in mind the APOE genotype when assessing the impact of cytokine genes. It is also necessary to consider the role of polymorphisms within the promoter region of *APOE* that are in a position to influence the magnitude of *APOE* expression after injury.

1.4 Genetic sequence variation and the importance of SNPs

Within the human genome there is naturally occurring sequence variation, 90% of which is single nucleotide polymorphism (SNP) (Collins *et al.*, 1998). But what exactly are SNPs? Brookes (1999) defined them as 'single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in some population(s), wherein the least frequent allele has an abundance of 1% or greater.' The manner in which polymorphisms arise is diverse, and the distribution and frequency at which they occur varies (Salisbury *et al.*, 2003). A SNP arises due to a single base mutation. Single base polymorphisms were first identified when the mutation resulted in the creation or deletion of a restriction site. Digestion of the relevant piece of DNA with the correct digestion enzyme could distinguish the different alleles by the creation of different sized fragments. These polymorphisms are known as 'restriction fragment length polymorphisms' (RFLPs). As technology has advanced it has become possible to identify SNPs that do not create restriction sites by utilising DNA probes or direct sequencing.

The precise frequency of SNP occurrence within the genome is still unclear, as there is significant variation across DNA regions. Certainly SNPs are the most common genetic polymorphisms; some have estimated that there may be as many as 15 million across the whole genome (Botstein & Risch, 2003). Other polymorphisms exist in the form of 'microsatellites' and 'minisatellites' (tandem repeats, for example). Microsatellites rarely occur within exonic regions and are unusual within promoter regions. Any detected association between a microsatellite and outcome is therefore usually attributed to linkage disequilibrium.

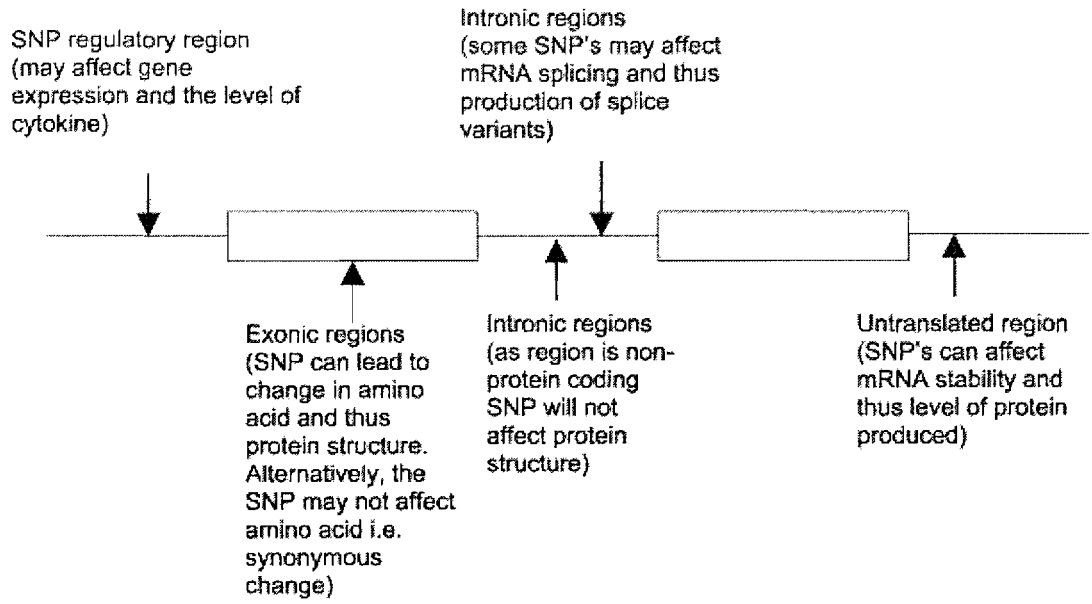


Figure 1.7 – Possible positions of SNPs and their resultant effects.

From Ollier, 2004.

Due to their frequency SNPs are important candidates to test as functional or causal mutations for disease susceptibility or severity. SNPs are found throughout the genome, in intronic, exonic and promoter regions. A SNP in a coding region may have a direct effect on the transcribed protein, whilst one in a promoter region can influence gene expression.

If a SNP contributes an increased risk of a particular disease occurring then that SNP should be found at a higher frequency in patients with the disease than those without. Similarly if a SNP contributes to a better or worse outcome it will be present at a higher frequency in patients who do better or worse. By targeting genes with known biological activity, and investigating SNPs within the coding or promoter regions (which are more likely to have a functional effect) associations may become apparent.

In this work I shall identify SNPs by their base position within the gene, relative to the transcription start site. Transcription starts at position +1, so SNPs lying within the promoter region will have a negative position number.

During my study I aim to investigate a number of cytokine, and non-cytokine factors. These factors are known to play a role in inflammation or neuronal repair within the CNS and are encoded by genes containing a number of SNPs.

1.5 Genetic association studies

Genetic association studies have become an increasingly popular way of examining the involvement of genetic variation in a variety of complex diseases. Many of the relevant studies in the field of TBI are discussed in Section 1.6. Association studies test whether a particular polymorphism (or allele) occurs more frequently in cases than non-cases. If an association should become apparent then this indicates that the polymorphism is within the 'susceptibility' locus, or in linkage disequilibrium* with the susceptibility locus. In either case identification of the association enables detection of people at risk of developing the given condition. Genetic susceptibility to complex conditions is in most cases likely due to many genes, which have small effects, rather than single genes. This, and the large number of identified SNPs within the genome, has led to a proliferation of association studies. However conclusions from these studies should be drawn with care.

Many of the associations claimed within the literature have not proved to be reproducible. Hirschhorn *et al.* (2002) found that of 166 possible associations that had been studied on at least three occasions only 6 were reproduced at least 75% of the time. The explanation for this may fall into three broad categories (see Newton-Cheh & Hirschhorn, 2005); 1) initial studies report a Type I error (false positive) which is then subsequently, and correctly, not replicated. 2) a correctly identified association is not replicated because follow-up studies are underpowered – a Type II error (false negative). 3) a true association in one population does not hold true in a second because of differing genetic and environmental backgrounds. It is likely that false-positive associations account for the majority of cases where an association cannot be replicated (Ioannidis *et al.*, 2001). False positives may arise for a number of reasons (Brookes & Prince, 2005; Newton-Cheh & Hirschhorn, 2005). A

* Linkage disequilibrium is the non-random association of alleles from two or more loci on a single chromosome. Haplotypes formed from these loci will occur more or less frequently than would be expected if haplotypes were formed randomly.

sufficiently large sample size must be used in order to attain sufficient power to detect effect sizes. In addition samples should be as genetically homogeneous as possible. Inadvertent sample stratification may occur in populations with a lot of demographic variation. Technical errors within the process of genotyping can also result in association errors, as only a small number of errors may mask a small gene effect. When performing studies it is important to ensure the phenotypic data is consistent, both within and between studies. Lastly the inappropriate use of statistical tests, such as the incorrect application of p-values, may result in incorrect interpretation of results. Cordell & Clayton (2005) have succinctly summarised some of the appropriate statistical methods for use in association studies.

False negative results commonly occur due to underpowered samples. Where the genetic variable contributes only a small effect, as is often seen in complex disease, sample sizes must be quite large (often greater than 1000 participants) to achieve significance at the level of $p<0.05$ (Newton-Cheh & Hirschhorn, 2005). Genotyping error and missing data will also mean there may be bias towards the null hypothesis. When comparing studies the possibility of sample heterogeneity must be considered. This may occur if a genetic variation manifests its effects only on a particular genetic or environmental background of many. Thus a polymorphism associated with outcome in one racial group may not be replicated in a different racial group.

Despite the many factors outlined above that appear to limit the usefulness of genetic association studies they actually serve to make clear the requirements for any future studies. Studies must be undertaken in large homogeneous cohorts. Genes/polymorphisms must be chosen with care, with special attention given to genes and SNPs with known functional relevance and effect. Genotyping must be accurate and reliable. The correct statistical tests must be employed and appropriate significance values reported.

1.6 Genetic influence on outcome after head injury*

The potential association of apolipoprotein E gene (APOE) polymorphism with head injury outcome was postulated almost ten years ago (Nicoll *et al.*, 1995). As discussed in section 1.3 apolipoprotein E is a key protein facilitating lipid transport and metabolism within the nervous system and has a role in neuronal maintenance and repair. It exists in three isoforms, coded by three alleles (ε2, ε3 and ε4) that result in amino acid substitutions within the protein. A small study by Sorbi *et al.* (1995) showed the ε4 allele of APOE was a poor prognostic indicator for post-traumatic coma. A preliminary study of 93 patients by Teasdale *et al.* (1997) demonstrated a significant association between APOE ε4 and poor outcome after head injury. Patients with the ε4 allele were more than twice as likely to have an unfavourable outcome 6 months post head injury than those without. Subsequent studies have shown a similar association, although in all cases the subject numbers have been small. Friedman *et al.* (1999) reported only 3.7% of patients with the ε4 allele had a good recovery compared with 31% of patients without. It should be noted that only patients who survived their injury and were referred for rehabilitation were included in this study. Outcome of patients who were able to complete a course of neurorehabilitation after TBI was also found to be associated with APOE genotype (Lichtman *et al.*, 2000). Overall and motor recovery, as assessed by the Functional Independence Measure, were both significantly lower in patients who possessed the ε4 allele than those without. In addition to this, Crawford *et al.* (2002), whilst demonstrating poorer outcome in ε4 patients, found memory post TBI was worse. In their study 110 active and veteran American military personnel were assessed using a number of memory and cognitive measures. Although all patients displayed impaired performance those with the ε4 allele were significantly worse.

A more recent study of outcome after head injury involving a much larger number of patients (n=984) (Teasdale *et al.*, 2005) has shown a much smaller difference in outcome between ε4 carriers and non-carriers. However the effect of ε4 carriage was

* This section has been modified from work published in the journal 'Current Opinion in Critical Care' (Waters & Nicoll, 2005).

more marked in children and young adults than at an older age. This emphasises the need for studies of this type to involve large numbers of patients.

An important point in studies of genetic variation is the ethnicity of the population. Chiang *et al.* (2003) extended the findings of the above studies to a Chinese population, in whom the ε4 allele is relatively uncommon in comparison to European and North American populations. 100 consecutive patients admitted with TBI were prospectively followed until 6 months post injury. More than twice as many patients with ε4 had an unfavourable outcome at that stage than those without (52.6% v 24.1%).

The APOE ε4 allele is most frequently seen in people of African descent. The only study to have used a cohort of exclusively African subjects is that of Nathoo *et al.* (2003). In a sample of 110 black Zulu-speaking patients with cerebral contusions, demonstrated by CT scanning, there was a non-significant trend between the ε4 allele and outcome. This may be because other gene interaction or expression is important in African populations or because of gene-environment interactions that modify the response to TBI in this population.

The ε4 allele adversely affects outcome after only a mild head injury. Sundstrom *et al.* (2004) were able to perform a series of neuropsychological tests on a small cohort of individuals both pre- and post-head injury. Those with the ε4 allele performed significantly worse in three tests post-head injury, whilst those without the ε4 allele performed no different. This confirms the findings of an earlier study (Liberman *et al.*, 2002) which showed ε4-carriers had lower neuropsychological outcome scores than non-ε4 carriers at 3 and 6 weeks post mild TBI. A more recent study of 90 patients (Chamelian *et al.*, 2004), including patients who suffered mild to moderate TBI, did not find any association between the presence of the ε4 allele and poor outcome.

Amyloid β-protein (Aβ) (a protein that can form plaques within the brain and is involved in the pathogenesis of Alzheimer's disease) when present in the brains of patients with severe head injury (Roberts *et al.*, 1994) is associated with the APOE

ε4 allele (Nicoll *et al.*, 1995). Of potential relevance to this there is a synergistic effect between the ε4 allele and a prior head injury in patients with Alzheimer's disease (Mayeux *et al.*, 1995). It is therefore important to see whether the ε4 allele affects long-term outcome. Results from longer-term follow-up (Millar *et al.*, 2003), studying patients 15 to 25 years after head injury, are available. Although confirming a late decline in function after TBI a relationship to APOE genotype was not shown. The mean age of the cohort was still too young to assess the risk of Alzheimer's disease. This raises the possibility that ε4 only exerts short term effects, such as by worsening the initial injury, or that other genes have a greater importance in determining long term outcome.

It is still unclear why ε4-carriers should have poorer short-term outcomes. The ε4 allele may predispose to pathologies that result in worse outcomes. After trauma patients with the ε4 allele are prone to larger intracranial haematomas (Liaquat *et al.*, 2002) and seizures (Diaz-Arrastia *et al.*, 2003), both of which may adversely affect outcome. It is suggested that APOE ε4 promotes production of Aβ aggregates, cytoskeleton alterations and oxidative damage (Horsburgh *et al.*, 2000), whilst also impairing the ability of the brain to repair and regenerate.

Polymorphisms within the promoter region may increase APOE expression and exacerbate the response to TBI. A recent small study (Lendon *et al.*, 2003) investigated 3 promoter polymorphisms. A poorer outcome was found in -219 G/T carriers, but this may be confounded by evidence of linkage disequilibrium between this polymorphism and the ε4 allele.

There is increasing interest in how neuroinflammation, and especially cytokines, may affect outcome after TBI. To date there is little information attempting to correlate cytokine gene polymorphism with outcome. A recently published study showed a significant association between the IL-1β +3953 polymorphism and six month outcome after head injury (Uzan *et al.*, 2005). However the patient numbers were small (n=69) so further studies are required to confirm this finding. Minambres *et al.* (2003) were unable to demonstrate a role for the –174 G/C interleukin-6 (IL-6)

promoter polymorphism (which is known to affect IL-6 levels) in influencing survival at 6 months.

After TBI progressive neuronal death has been demonstrated (Smith *et al.*, 1997). This may account for some of the late decline in function demonstrated by Millar *et al.* (2003). Many of the neurons die by the means of apoptosis. The p53 gene is a major regulator of apoptosis, and changes in its expression have been found after TBI (Lu *et al.*, 2000). Martinez-Lucas *et al.* (2005) have shown an association between Arg/Arg genotype of the Arg72Pro polymorphism in p53 and worse outcome after TBI. However this was only apparent at the time of discharge from the Intensive Care Unit, and did not persist when 6-month outcome was examined.

In summary, the majority of published literature supports the view that the ε4 allele of APOE adversely affects outcome after TBI in the first 6 months post injury. Its effects over a much longer period are less clear. The evidence for a role for other genes is beginning to appear but further work is required before any conclusions can be drawn.

1.7 Aims

Clinical outcome after traumatic brain injury may be determined by secondary processes occurring after the initial injury. An important component of the secondary process is the inflammatory response that is provoked. The magnitude and duration of this response may thus determine clinical outcome. The response is mediated by a group of cytokines and the production of these cytokines may be altered by polymorphisms within their genes. The aim of this thesis is to investigate whether polymorphisms within genes that play a role in the neuroinflammatory response affect clinical outcome after head injury. If it can be demonstrated that cytokine genes have an influence on outcome after head injury then this may have important implications for further understanding of the inflammatory response to brain injury, for prognosis after injury and possibly future treatment of patients who sustain head injury.

1.8 Hypothesis

At the outset of the project I propose the following hypothesis:

Cytokine gene polymorphisms, by influencing the magnitude and duration of the inflammatory response, influence clinical outcome after traumatic brain injury.

1.9 Study Plan

In order to test my hypothesis I will identify a number of cytokine gene polymorphisms that the current evidence suggests may have a role in determining the magnitude or duration of the neuroinflammatory response after TBI. These SNPs will then be determined in a cohort of patients who have sustained TBI. Within the same cohort measures of clinical outcome will be used to identify any associations between the SNPs and outcome after TBI.

1.10 Patient Database

A database of clinical outcome measures in a large number of patients who sustained traumatic brain injury, together with DNA samples, exists in Glasgow. Access to this material has been agreed. The database has been collected over a number of years and is a valuable resource for the study of genetics on outcome after head injury, as DNA samples exist for all of the patients in the database. The database has been utilised in previous studies of APOE genotype and head injury outcome (Millar *et al.*, 2003; Teasdale *et al.*, 2005). Six-month outcome measures are available for the larger cohort of 1067 patients. In a smaller cohort (n=396) neuropsychological assessments are available after much longer follow-up (mean 18 years). At the time of DNA collection consent was given for its use in future studies of genetic influence on outcome. A unique 'P' number that links the sample with the corresponding patient record in the clinical database identifies each sample. By utilising this valuable database it will be possible to test my hypothesis and establish any cytokine gene/SNP associations.

1.10.1 Six-month follow-up patients

Patients were recruited from consecutive admissions to the Institute of Neurological Sciences, Glasgow. This is the neurosurgical centre for the West of Scotland. All patients (n=1067) had sustained a head injury and were admitted between 1996 and 1999.

During admission clinical data was prospectively collected from patient records. This included the patient's demographic features and age, the cause of injury, clinical severity of brain damage in the acute stage as indicated by the Glasgow Coma Scale (Teasdale & Jennett 1974) and pupil reaction. The findings on CT scanning were classified according to the system of Marshall *et al.* (1991) and operative findings were obtained from case notes. The admission data is shown in Table 1.2.

In order to assess outcome patients were contacted by letter or telephone six months after their head injury. A structured questionnaire was used to score the Glasgow Outcome Scale (GOS). See Appendix B2 for more information on the GOS.

1.10.2 'Long-term' outcome patients

This group comprises 396 patients who were treated at the Institute of Neurological Sciences, Glasgow between 1968 and 1985. They were known to have survived for at least six months post head injury and were subsequently traced via hospital records and General Practitioners. Early clinical data was obtained in a similar manner to the six-month follow-up patients. Six-month outcome was assessed using the GOS.

	Non-ε4 carriers	ε4 carriers	All
Total	660 (67.1%)	324 (32.9%)	984 (100%)
Sex			
Male	536 (67.3%)	261 (32.7%)	797 (81%)
Female	124 (66.3%)	63 (33.7%)	187 (19%)
Cause			
RTA	156	80	236
Assault	146	76	222
Fall	308	148	456
Other	50	20	70
Age (yrs)			
Mean	35	35	35
SE	0.8	1.2	0.7
Range	<1 to 93	<1 to 86	<1 to 93
Initial severity of brain injury			
GCS at A&E			
3-8	191	76	267 (28%)
9-12	121	57	178 (19%)
13-15	332	181	513 (54%)
Pupils reactivity			
One or Both	603	299	902 (92%)
Neither	57	25	82 (8%)
CT scan pattern			
diffuse injury			
1 Normal	154	86	240 (24%)
2 Lesions	261	120	381 (38%)
3 Swelling	40	21	61 (6%)
4 Swelling and Shift	6	3	9 (1%)
Mass lesion	192	92	287 (29%)
No CT	4	2	6 (0.6%)

Table 1.2 - Characteristics of acutely head-injured patients related to possession of *APOE* ε4.
Courtesy: Teasdale *et al.*, 2005.

Long-term outcome was assessed with a panel of neuropsychological tests to detect cognitive decline. Pre-morbid intellectual function was estimated with the National Adult Reading Test (NART). Current function was established with the Mini Mental State Exam. Memory was assessed with sub-tests of the Wechsler Memory Scale (WMS) and the Graded Naming Test. Executive and attentional processes were assessed with the Stroop Neuropsychological Screening Test, Verbal Fluency (letter and category generation) and the Digit Span test from the WMS. Self-reported symptoms of depression, distress and psychiatric illness were assessed with the Beck Depression Inventory, General Health Questionnaire and Symptom Check List-90 respectively. 'Overall' outcome was assessed with the GOS.

1.11 Cytokine genes

As discussed in Section 1.3 cytokines are a diverse group of peptides that mediate neuroinflammation. I have targeted cytokines likely to exert functional effects after TBI for further study. When assessing the published literature for evidence of functional effects of cytokines and their polymorphisms I have given special attention to studies examining their effects after TBI, their effect after other CNS insults which provoke inflammation (such as stroke) and also their influence on chronic neurodegeneration such as occurs in Alzheimer's disease. This has enabled the determination of 11 target SNPs across four cytokine genes.

1.11.1 Tumour Necrosis Factor-α

Tumour Necrosis Factor-α (TNF-α) is a classical pro-inflammatory cytokine, playing an important role in initiating and controlling the inflammatory response. TNF-α is produced by all cell types within the CNS, although primarily by microglia and astrocytes. Initially synthesized as an inactive pre-cursor, pro-TNF-α, it is cleaved to its active form by the enzyme TNF-α convertase. The active form can then bind to a member of the TNF–receptor superfamily, which includes two high affinity receptors, TNFR1 and TNFR2 (see Allan & Rothwell, 2001; Locksley *et al.*, 2001).

TNF-α is known to be upregulated in the CNS after TBI (Shohami *et al.*, 1994; Fan *et al.*, 1996; Gourin & Shackford, 1997). In clinical studies raised levels of TNF-α in plasma and cerebrospinal fluid (CSF) have been demonstrated in patients after TBI (Goodman *et al.*, 1990; Ross *et al.*, 1994) as well as in patients with Alzheimer's disease (Tarkowski *et al.*, 1999), encephalitis (Grimaldi *et al.*, 1991) and meningitis (Waage *et al.*, 1989).

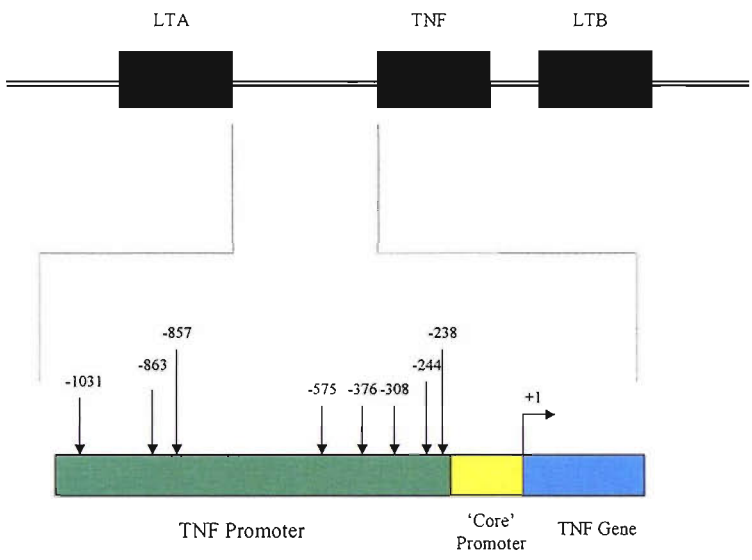


Figure 1.8 – schematic demonstrating the TNF promoter polymorphisms on chromosome 6p21. LTA=lymphotoxin-α, LTB=lymphotoxin-β, the adjacent genes to TNF. Adapted from Bayley *et al.*, 2004.

It had been thought that the actions of TNF-α were generally neurotoxic. In hippocampal slice cultures TNF-α induces cell death in a dose dependent manner (Zhao *et al.*, 2001) and exacerbates ischaemic injury (Barone *et al.*, 1997, Wilde *et al.*, 2000). In rodents inhibition of TNF-α reduces damage after head injury (Shohami *et al.*, 1996) and ischaemia (Nawashiro *et al.*, 1996; Barone *et al.*, 1997). However there was also a body of evidence suggesting TNF-α may have some neuroprotective effects. TNF-α has been demonstrated to protect neurons from excitotoxic damage (Cheng *et al.*, 1994) and to promote neuronal regeneration (Schwartz *et al.*, 1991).

More recent studies have now shown that TNF-α has a biphasic mode of action with an early neuroinflammatory effect followed by a late neuroregenerative action. Studies of TNF gene knock-out mice have clearly shown that deficiency of TNF-α is beneficial early after trauma but causes deleterious effects in the longer term (Scherbel *et al.*, 1999; Stahel *et al.*, 2000). If this holds true in humans then the effect of polymorphisms in the promoter region of the TNF gene may therefore be of vital importance in determining outcome after head injury. If they were to cause a reduction in TNF-α gene expression in the early phase or augment expression over a longer course then better outcome may result.

The TNF-α gene (TNFA) is located at chromosome 6p21.3, amongst the cluster of MHC genes (see Figure 1.8). This region is highly polymorphic, with multiple SNPs within the TNF promoter region (see Figure 2.1). The -238 (G/A) and -308 (G/A) SNPs within the promoter region have been most extensively studied and a database of disease associations (both positive and negative) is available (Bidwell *et al.*, 1999; Bidwell *et al.*, 2001; Haukim *et al.*, 2002). Studies have suggested the -308 SNP causes a significant alteration in transcription (Kroeger *et al.*, 1997; Wilson *et al.*, 1997), but this is not a universal finding. The -238 SNP has not been shown to significantly alter transcription, but when combined as a haplotype with the -308 polymorphism associations have been detected (Collins *et al.*, 2000; Culpan *et al.*, 2003).

1.11.2 Interleukin-1

The interleukin-1 (IL-1) 'family' (reviewed in Rothwell & Luheshi, 2000) consists of three proteins, IL-1α, IL-1β and IL-1ra. IL-1α (mostly membrane-bound) and IL-1β (the secreted form) are agonists that exert similar, if not identical, actions via a single cell surface receptor (IL-1R1). IL-1ra is a highly selective antagonist of the same receptor. IL-1α and IL-1β are formed as precursors, pro-IL-1α being biologically active whilst pro-IL-1β requires cleavage by caspase-1 (expressed by microglia) to become active (see figure 1.9). All ligands and receptor components are present at low levels in the CNS (Vitkovic *et al.*, 2000). After CNS injury IL-1 ligand and

receptor levels are increased. Microglia are the main source but with some contribution from astrocytes and neurons subsequent to microglial activation.

The actions of IL-1 appear to promote both acute and chronic effects. IL-1 is induced rapidly after various forms of CNS insult, such as stroke (Legos *et al.*, 2000) and TBI (Holmin & Hojeberg, 2004), but may remain elevated for some days (Holmin & Hojeberg, 2004) and is also associated with a number of chronic neurodegenerative conditions, including Alzheimer's disease (Griffin *et al.*, 1989), Parkinson's disease and multiple sclerosis (Basu *et al.*, 2004).

IL-1 exacerbates the acute damage observed after ischaemic and excitotoxic injury (Lawrence *et al.*, 1998; Stroemer & Rothwell, 1998; Hailer *et al.*, 2005) whilst IL-1ra reduces damage (Relton & Rothwell, 1992; Hailer *et al.*, 2005). Basu *et al.* (2005) have shown the IL1R1 receptor to be vital in this process. However IL-1 has been shown to have neuroprotective actions in some models (Carlson *et al.*, 1999; Pringle *et al.*, 2001), suggesting IL-1 receptor signalling is capable of initiating either

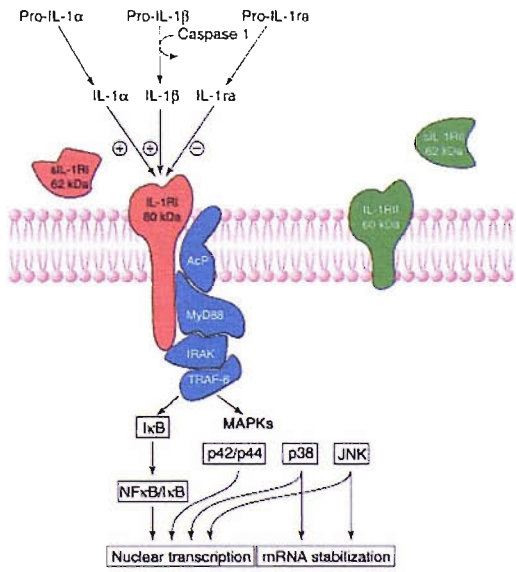


Figure 1.9 – Actions of IL-1. AcP=accessory protein, MAP=mitogen-activated protein, IRAK= interleukin-1 receptor associated protein, TRAF=tumour necrosis factor receptor associated factor, KF-κB=nuclear factor-κB, JNK= c-jun amino terminal kinase.

From Rothwell & Luheshi, 2000.

neuroprotective or neurodegenerative responses (Winter *et al.*, in press). After TBI IL-1 appears to promote neurodegeneration (Lu *et al.*, 2005), while IL-1ra attenuates damage (Tehranian *et al.*, 2002; Jones *et al.*, 2005). Inhibition of caspase-1 (preventing cleavage of pro-IL-1β reduces tissue damage and free radical production (Fink et al., 1999). In some clinical studies a rise in CSF IL-1 has been correlated with poor outcome (Chiaretti *et al.*, 2005; Hayakata *et al.*, 2004) although not in all (Winter *et al.*, 2004).

The genes coding for the IL-1 family exist in a cluster on chromosome 2. The IL-1α and IL-1β genes (IL1A and IL1B) contain a number of SNPs, the most significant being –889 (C/T) in IL1A and –31 (C/T), -511 (C/T) and +3953 (C/T) in IL1B. The -31 and -511 SNPs lie within the promoter region and have recently been confirmed as having transcriptional activity (Chen *et* al., 2006). They may also form part of a haplotype group (Chen *et al.*, 2006). The +3953 SNP (also identified as +3954 in some studies) lies within exon 5 of the IL1B gene. The SNP does not appear to cause a mutation within the protein, but does affect transcription (Pociot *et* al., 1992, Hulkkonen *et al.*, 2000).

Two studies of interleukin-1 polymorphisms and their association with outcome after TBI are available in the literature. Uzan *et al.* (2005) showed that patients who possessed the T allele at +3953 were significantly more likely to have a poor outcome. There was also an effect when the –511 SNP was considered together with the +3953. However the study was small and will require further validation. In a further small study the same group failed to demonstrate an association between the –889 polymorphism and outcome at 6-months. Studies have demonstrated an association between the IL1A –889, IL1B –511 and +3953 SNPs and increased risk of Alzheimer's disease (Grimaldi *et al.*, 2000; Nicoll *et al.*, 2000) and the IL1A –889 SNP with risk of cerebral infarction (Um *et al.*, 2003). The –889 SNP in IL-1A has been shown to influence the degree of microglial activation in Alzheimer's disease (Hayes *et al.*, 2004).

1.11.3 Interleukin-6

Interleukin-6 (IL-6) has a role in regulating cell numbers and neuronal function within the CNS (Allan & Rothwell, 2001). It also plays an important role in the acute phase response in the periphery (Fey & Gauldie, 1990), including macrophage activation, B cell differentiation and stem cell support. IL-6 production is stimulated by TNFα and IL-1 and, like these two cytokines, IL-6 levels have been shown to increase in a variety of models of CNS injury (Arvin *et al.*, 1996). IL-6 is produced primarily by microglia but also by astrocytes and neurons. It acts via a specific receptor (IL-6R) and the signal transducing glycoprotein gp130 (Allan & Rothwell, 2001).

In models of cerebral ischaemia IL-6 has been shown to be neuroprotective (Loddick *et al.*, 1998; Suzuki *et al.*, 1999; Herrmann *et al.*, 2003). However in clinical studies of stroke increasing IL-6 levels have been correlated with poorer long-term outcome (Vila *et al.*, 2000; Smith *et al.*, 2004). In studies of trauma higher IL-6 levels have been associated with better outcome (Singhal *et al.*, 2002; Winter *et al.*, 2004), although this is not consistent (Minambres *et al.*, 2003). In Alzheimer's disease there is some evidence demonstrating raised levels of IL-6 (Maes *et al.*, 1999; Licastro *et al.*, 2000), suggesting a possible role in chronic neurodegeneration.

The IL-6 gene on chromosome 7 has a number of significant SNPs within its promoter region including -174 (G/C), -572 (G/C) and –597 (G/A). These influence transcription through complex interaction (Terry *et al.*, 2000). The -174 SNP has been associated with a number of disease conditions, including Alzheimer's disease (Licastro *et al.*, 2003) and stroke (Greisenegger *et al.*, 2003). In the only study of IL-6 polymorphism and outcome after head injury Minambres *et al.* (2003) found no evidence that the –174 polymorphism was associated with outcome.

1.11.4 Transforming Growth Factor Beta (TGFβ)

The TGFβ family includes three isoforms TGFβ-1, 2 and 3. TGFβ-1 has been most extensively studied in the CNS, where it has a role in cell proliferation, differentiation and inflammation. TGFβ-1 is normally present in low levels in most

cell types in the CNS, although astrocytes and microglia are the main source after injury (Finch *et al.*, 1993). Two receptors (TβR1 and TβR2) have been identified in the brain (Allan & Rothwell, 2001).

TGFβ is believed to be a mostly anti-inflammatory cytokine, but under certain circumstances may promote neurodegeneration. Studies of acute neurodegeneration have confirmed the neuroprotective effects of TGFβ in both in-vivo and in-vitro models, although under certain conditions it may trigger cell death (see Flanders *et al.*, 1998, for review). TGFβ also plays a protective role in chronic neurodegeneration, possibly by limiting microglial activation (Basu *et al.*, 2002; Kim *et al.*, 2004; Stoll *et al.*, 2004).

Similar to the other cytokines discussed there are a number of polymorphisms within the TGFβ-1 gene on chromosome 19. Two SNPs in the promoter region, -509 (C/T) and -800 (G/A) are associated with higher concentrations of TGFβ-1 in plasma (Grainger *et al.*, 1999). If this holds true for concentrations within the CNS then these SNPs may enhance neuroprotection. No studies of the role of TGFβ-1 polymorphisms on outcome after head injury have been published. Luedecking *et al.* (2000) found a modest association between possession of the –509 T allele and risk of Alzheimer's disease, although this has not been confirmed in later studies (Araria-Goumidi *et al.*, 2002; Nishimura *et al.*, 2004). Sie *et al.* (2006) have recently shown the -509 T allele to be associated with an increased risk of stroke.

1.12 Non-cytokine genes

Although I shall be focussing on the potential effects of cytokine gene polymorphisms in the context of this project it is important to remember that these are only a handful amongst the many other genes which may be affecting outcome after TBI. Apolipoprotein E has been the focus of much attention but other genes that promote repair and regeneration (such as GAP43 or neurotrophic factors like NGF) need to be considered. Another alternative is to consider the genes which may promote or inhibit cell death after injury, such as p53. Due to the important role apolipoprotein E plays within this field I shall outline some of the other

polymorphisms, especially within the promoter region, that are certainly worthy of further investigation.

1.12.1 Apolipoprotein E

The importance of the ε4 allele of the APOE gene with respect to Alzheimer's disease risk is well documented. As already noted in section 1.6 it has been demonstrated that the ε4 genotype may similarly affect short-term outcome after head injury, although the long-term effects are less clear (Teasdale *et al.*, 1997; Friedman *et al.*, 1998; Millar *et al.*, 2003).

The APOE gene on chromosome 19 is complex, with a number of SNPs within the promoter region. If these SNPs were to result in increased *APOE* expression then possibly they could affect the response to apoE after TBI. SNPs at positions –219(T/G) and –491(A/T) alter the transcriptional activity of *APOE* (Artiga *et al.*, 1998). Other SNPs occur at positions –427(T/C) and +113(G/C).

The -219 SNP was associated with poor outcome after head injury in a small study (Lendon *et al.*, 2003). The -219 and -491 polymorphisms may influence Alzheimer's disease risk (Bullido & Valdivieso, 2000; Laws *et al.*, 2003). Further work is required to confirm the role of *APOE* in recovery after head injury, especially with regard to the promoter polymorphisms.

1.13 Summary

I have identified 11 cytokine gene polymorphisms that will be included in this study. The majority of these lie within the promoter region and are good candidates as SNPs that may affect cytokine gene transcription levels after TBI. The cytokine SNPs are summarised in table 1.3. The buccal DNA samples from the cohort of patients discussed in section 1.10 are currently stored in the Department of Neuropathology in Glasgow. An aliquot of each sample will be transferred to Southampton for genotyping of each SNP outlined above. When this genotyping

data is married with the clinical database it will be possible to detect any significant associations.

Gene	SNP	dbSNP ref	Effect
TNFα	-238 G/A	361525	Multiple positive association studies in various conditions, but functional effects now in question
	-308 G/A	1800629	
IL-1α	-889 C/T	1800587	Influences microglial activation
IL-1β	-31 C/T	1143627	Multiple positive association studies in various conditions, +3953 shown to increase expression
	-511 C/T	16944	
	+3953 C/T	1143634	
IL-6	-174 G/C	1800795	Complex interaction, which influences transcription
	-572 G/C	1800796	
	-597 G/A	1800797	
TGFβ	-509 C/T	1800469	Both SNPs associated with raised plasma concentrations
	-800 G/A	1800468	

Table 1.3 - Summary of cytokine polymorphisms included in the study.

Chapter 2

DNA quantitation and pre-amplification

2.1 Introduction

DNA samples were collected from patients in the form of mouth brush samples. The yield of DNA from such samples is usually considerably less than from a similar amount of blood, but subsequent successful polymerase chain reaction (PCR) is possible. APOE genotyping has been successfully carried out on all of the Glasgow patients (see below).

2.1.1 DNA collection

Obtaining high quality DNA is increasingly important for large-scale studies. It is often difficult to collect blood samples from such large populations, so alternative methods are required. Mouthwash and mouthbrush techniques are increasingly being used. Blood samples provide large amounts of genomic DNA but require a trained investigator to collect via an invasive technique (which may mean patients are unwilling to consent to the procedure) and can be difficult to transport. Epithelial cells derived from the buccal mucosa are an attractive alternative. They can be collected by self-administered, non-invasive, and relatively inexpensive techniques (Walsh *et al.*, 1992; Freeman *et al.*, 1997; Walker *et al.*, 1999). Buccal swabs and mouthwash protocols are the most commonly used methods for buccal cell collection. Buccal swabs are a 'dry' procedure, using a cytobrush to scrape the oral mucosa. Mouthwashes are 'wet' procedures involving swishing liquids in the mouth and spitting into a collection vessel. Both have advantages and disadvantages. Buccal swabs are efficient, cost effective and easy to process. Mouthwash sampling requires the handling of liquids, more complex processing and therefore higher costs. In a comparison of the two methods King *et al.* (2002) concluded that mouthbrush samples was cost-effective and provided yields of DNA sufficient for genotyping.

Even if the yield is insufficient for successful PCR-based genotyping it is possible to amplify the material to provide accurate results. DNA yields in paediatric populations are lower than those in adults. Zheng *et al.* (2001) demonstrated that they could use a method of whole genome amplification to improve PCR efficiency.

This technique is also useful to provide additional template material when a large number of PCR assays are to be performed (see 2.1.3).

2.1.2 DNA Quantitation

It is important to ensure sufficient DNA is available for both the planned SNP assays and for any future studies. In order to calculate the amount of DNA required as PCR template the concentration of DNA in the sample must be known. A number of techniques for DNA quantitation have been developed, most determining concentration by measuring absorbance at 260nm. As described by Singer *et al.* (1997) there are a number of disadvantages to this method, notably the large contribution of nucleotides and single-stranded DNA to the signal, the effect of contaminants and the insensitivity of the assay. The fluorescence enhancement of ethidium bromide has been used as an alternative, but ethidium bromide does not bind exclusively to double-stranded DNA and its high intrinsic fluorescence limits sensitivity.

Molecular Probes, Inc (Oregon, USA) have developed an unsymmetrical cyanine nucleic acid stain known as PicoGreen. This dye has high affinity for DNA, has negligible intrinsic fluorescence when unbound and large fluorescence enhancement when bound. The PicoGreen assay protocol can detect DNA amounts as low as 25pg/mL. The presence of single-stranded DNA and RNA has minimal effects on quantitation results (Singer *et al.*, 1997).

2.1.3 Whole genome amplification

Whole genome amplification is a valuable method for generating additional template from limited amounts of genomic DNA. This is important when the initial starting quantity of DNA is low but also when the number of assays to be run is high. This has become increasingly necessary as high-throughput techniques for SNP analysis have become available (Kwok, 2000). SNP analysis is increasingly performed on an industrial scale requiring milligram quantities of DNA in order to perform multiple assays.

When first conceptualised the PCR was intended to amplify a single locus in a target piece of DNA (Saiki *et al.*, 1985; Mullis *et al.*, 1986). As understanding and technology have developed the PCR has been modified to enable whole genome amplification.

The most efficient and widely used amplification method is the degenerate oligonucleotide primer (DOP) method, first described in the early Nineties (Telenius *et al.*, 1992). The technique is dependent upon initial low annealing temperature cycles, allowing the primers to initiate the PCR from short target sequences and degenerate primers. Degenerate primers have fixed 3' and 5' sequences flanking six degenerate positions (for example, Telenius *et al.* used the following primer in their original study 5'-CCGACTCGAGNNNNNNATGTGG-3', where N= A, T, G or C). The six degenerate positions create a pool of 4^6 primers to ensure priming at multiple positions throughout the genome. The annealing of the 3' end is stabilised by one or more of the degenerate positions at any one priming site. After several low temperature annealing cycles the annealing temperature is increased to allow more specific priming. Cheung & Nelson (1996) demonstrated the DOP method allowed unbiased, hundreds-fold whole genome amplification of human genomic DNA.

Genotyping of SNPs is possible from DOP amplified material. Barbaux *et al.* (2001) found DOP-PCR products could be successfully used for accurate genotyping, although a proof-reading *Taq* polymerase was required. Their DOP protocol enabled 5000ng of material to be generated from 10ng of starting DNA. The genotyping results from DOP template were identical to results previously obtained from genomic DNA. Grant *et al.* (2002) looked at a larger panel of SNPs (10 SNPs from different genes) and found there was a slight drop in data quality. The number of no-calls remained about the same (1.3% in the DOP template group vs. 1.6% in the genomic) but the number of ambiguous calls rose (5.8% vs. 2%). However they still concluded that DOP amplification provided a valuable technique for increasing the amount of template available for SNP genotyping.

The technique of DOP-PCR amplification has been successfully applied to a number of studies from the human genetics group in Southampton (Lawlor *et al.*, 2004). Although the buccal swabs from the Glasgow cohort provided sufficient DNA for

successful APOE genotyping (Teasdale *et al.*, 1997; Millar *et al.*, 2003) it is expected that a pre-amplification step will be required to ensure adequate template for multiple SNP assays.

An alternative method of pre-amplification has recently become available, utilising a novel proofreading DNA polymerase that does not require thermal cycling. Bacteriophage Phi29 polymerase can exponentially amplify linear DNA template by strand displacement (Dean *et al.*, 2001). The GenomiPhi DNA amplification kit (Amersham Biosciences, Piscataway, NJ) enables representative amplification in a different manner to the DOP protocol. Genomic DNA is added to a buffer containing random hexamer primers. The mixture is heated to denature the DNA and then cooled to allow random priming of the hexamers. The Phi29 enzyme is then added and incubated at 30°C. Thermal cycling is not required as the enzyme displaces downstream complementary strands to enable further priming by available hexamer primer (see Figure 2.1). The amplified DNA has high fidelity to the template as Phi29 polymerase has an error rate reportedly 100-fold lower than *Taq* polymerase (Estaban *et al.*, 1993).

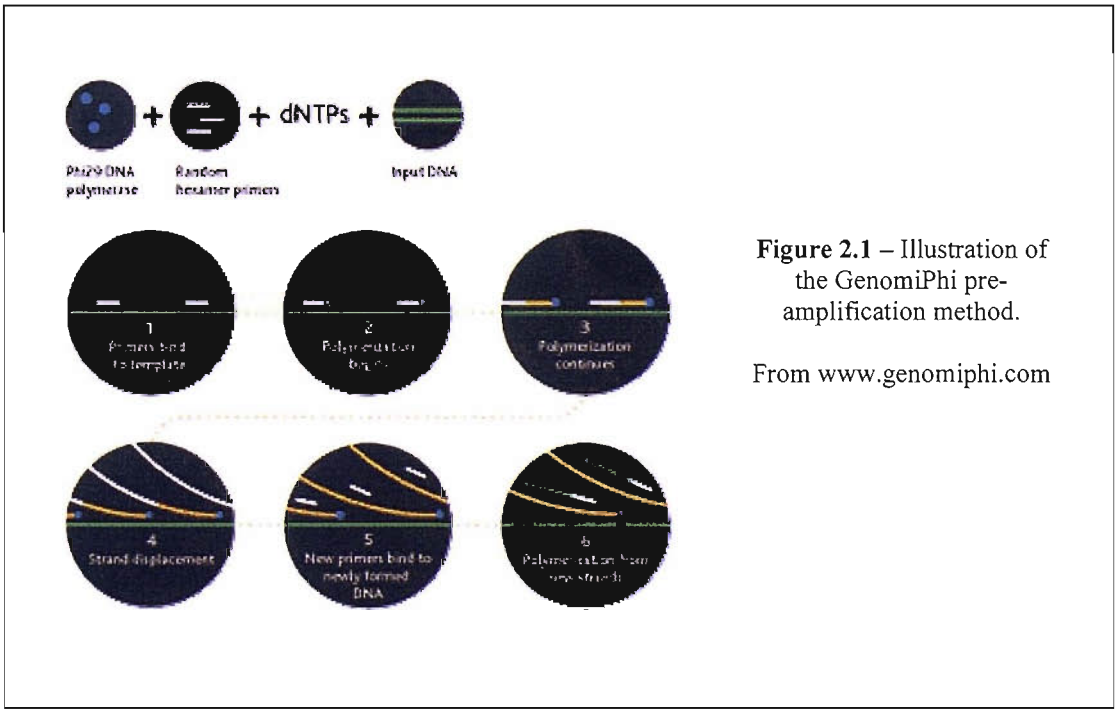


Figure 2.1 – Illustration of the GenomiPhi pre-amplification method.

From www.genomiphi.com

SNP genotyping can safely be performed on GenomiPhi amplified material (Mamone, 2003). No loss of heterozygosity has been shown and amplification from 1ng DNA gives equivalent results to genomic DNA. A much larger examination of the fidelity of Phi29 amplification has recently been completed (Paez *et al.*, 2004) that found near-complete genome representation and no degradation of SNP genotyping accuracy.

I shall compare the DOP-PCR and GenomiPhi pre-amplification protocols in order to establish which will be more suitable for my cohort of buccal swab samples.

2.2 Methods

2.2.1 Ethical Approval

At the time of DNA collection consent was obtained to use samples for future studies of genetic influence on outcome after TBI. Ethical approval for this was sought and obtained from the Southern General Hospital ethics committee at the time. At the outset of this study the ethics committees in both Southampton and Glasgow were contacted and submissions presented. Approval to proceed was obtained from the Southampton and South West Hampshire committee in February 2004 (Ref. No. 004/04/t), while an application to the Southern General Hospital committee in Glasgow was approved in March 2004 (Ref. no. EC/04/S/07). The relevant NHS Trust Research & Development officers were also informed.

2.2.2 DNA Preparation

Buccal swabs were obtained by rubbing a cytology brush on the inner surface of the patient's cheek for approximately 15 seconds. The brush was then inserted into an Eppendorf tube with 400µl ethanol and agitated. The samples were then stored at 4°C until return to the laboratory. In the laboratory the samples were centrifuged at 13000 rpm for 30 seconds to pellet the cells. The ethanol was decanted. The pelleted samples were dried on a heat block at 55°C for 30 minutes and then digested with proteinase K (100µl/ml) at 56°C for 1 hour. The proteinase K was then

inactivated by heating to 95°C for 10 minutes. DNA preparation was carried out at the time of the earlier studies (Millar *et al.*, 2003; Teasdale *et al.*, 2005) by Janice Stewart at the Institute of Neurological Sciences, Glasgow. 20μl aliquots of each sample were transferred to Southampton for cytokine SNP genotying.

2.2.3 APOE Genotyping

Janice Stewart also performed the initial APOE genotyping in Glasgow. 0.8μl of the proteinase digest was used as template. Hot start PCR amplification was performed as previously described by Wenham *et al.* (1991). The standard primers were used in a final volume of 15μl per sample. Thermal cycling consisted of 40 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes. The PCR product was digested with *Hha 1* for at least 3 hours. The digestion products were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide and displayed by ultra-violet transillumination.

2.2.4 PicoGreen Quantitation

PicoGreen quantitation reagent (Molecular Probes) was diluted 1 in 200 in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). In order to create a calibration curve a stock standard solution of DNA was diluted to provide samples with known concentrations of 40, 20, 10 and 5ng/μl. 6 patient samples were selected at random to test on the initial run.

For each sample 2μl was added to 50μl TE buffer in the well of a 96-well plate. 50μl of diluted PicoGreen reagent was added to all wells and incubated in the dark for 15 minutes. The plate was then scanned on a Fluorimager 595 (Molecular Dynamics, Sunnyvale, CA). ImageQuant software (Amersham Biosciences) was used to analyse the data.

2.2.5 DOP-PCR

The DOP protocol was a modified version of that used by Cheung and Nelson (1996), in order to minimise the loss of representation of GC-rich regions. DOP-PCR was performed in a final volume of 50µl per reaction. All reactions were performed in 96-well plates (Abgene®, Epsom, Surrey, UK). The reaction mix contained 10x DOP Buffer (500mM Tris-HCl pH 8.9, 140mM Ammonium Sulphate), 250µM dNTPs, 1µM DOP primer (5'-CCGACTCGAGNNNNNNATGTGG-3'), 2.5mM MgCl, 1.3M Betaine, 2.5 units *Taq* DNA polymerase (Gibco, Paisley, UK) and 0.02 units Pwo polymerase (Roche, Basel, Switzerland). PCR cycling conditions were as follows: 94°C for 5 minutes followed by 8 cycles of 94°C for 1 minute, 30°C for 1 minute and 72°C for 3 minutes. This was followed by 28 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 3 minutes. Heat cycling was performed on an MJ Research PTC-225 DNA Engine Tetrad® (Genetic Research Instrumentation Ltd, Braintree, UK).

2.2.6 GenomiPhi Amplification

The GenomiPhi amplification kit (Amersham Biosciences) consists of three components; sample buffer (containing hexamer primers), reaction buffer (salts and deoxynucleotides at optimum pH) and enzyme mix (Phi29 polymerase with random primers).

Pre-amplification was carried out in 96-well plates (Abgene). 9µl of sample buffer is added to the template DNA (1µl and 3µl of genomic DNA were trialled as template) and then heated to 95°C for 3 minutes. The sample was then cooled on ice prior to adding 9µl reaction buffer and 1µl enzyme mix to each sample. The plate was then sealed with a rubber mat and left to incubate at 30°C for 18 hours. The Phi29 polymerase was then heat inactivated during a 10 minute incubation at 65°C. Heating and incubation were carried out using the same PCR block as for the DOP-PCR amplification.

2.2.7 Post amplification PCR

The amplification products were tested using two reliable PCR protocols available in the laboratory. R52 and Q90 are polymorphisms within the GHRelin gene.

PCR products were displayed on microplate array diagonal gel electrophoresis (MADGE) plates. The MADGE system (see Day *et al.*, 1995; Gaunt *et al.*, 2003) uses 96 track origins at the same pitch as the wells of a 96-well plate. The long axis of the array is angled at 18.4° to the direction of electrophoresis allowing samples to pass between wells on adjacent rows, giving a track length of 26mm. 5% polyacrylamide gels were formed by mixing 8.3ml 30% acrylamide: bis solution with 5ml 10xTBE buffer (108g Tris base, 55g Boric acid, 9.3g EDTA disodium salt in 1litre, pH 8.3) made up to 50ml with water. To polymerise the gel 150µl 25% ammonium persulphate and 150µl tetramethylethylenediamine (TEMED) are added and the mix poured into a moulded plate former. The former is covered with a glass plate coated with silane and left to set. After five minutes the gel, now adherent to the glass plate, can be removed from the former.

Prior to electrophoresis the gel is pre-stained with ethidium bromide by submerging it in 100ml TBE with 10µl stock ethidium bromide and agitating for 15 minutes on a shaker. 5µl of PCR product together with 2µl formamide (98% deionised formamide, 10mM EDTA, xylene cyanol) loading dye are loaded into each well. Electrophoresis is then performed at 150V for 8 minutes. The gels were then visualised on a Fluorimager 595 (Molecular Dynamics) and analysed with ImageQuant software (Amersham Bioscience).

2.3 Results

2.3.1 DNA Quantitation

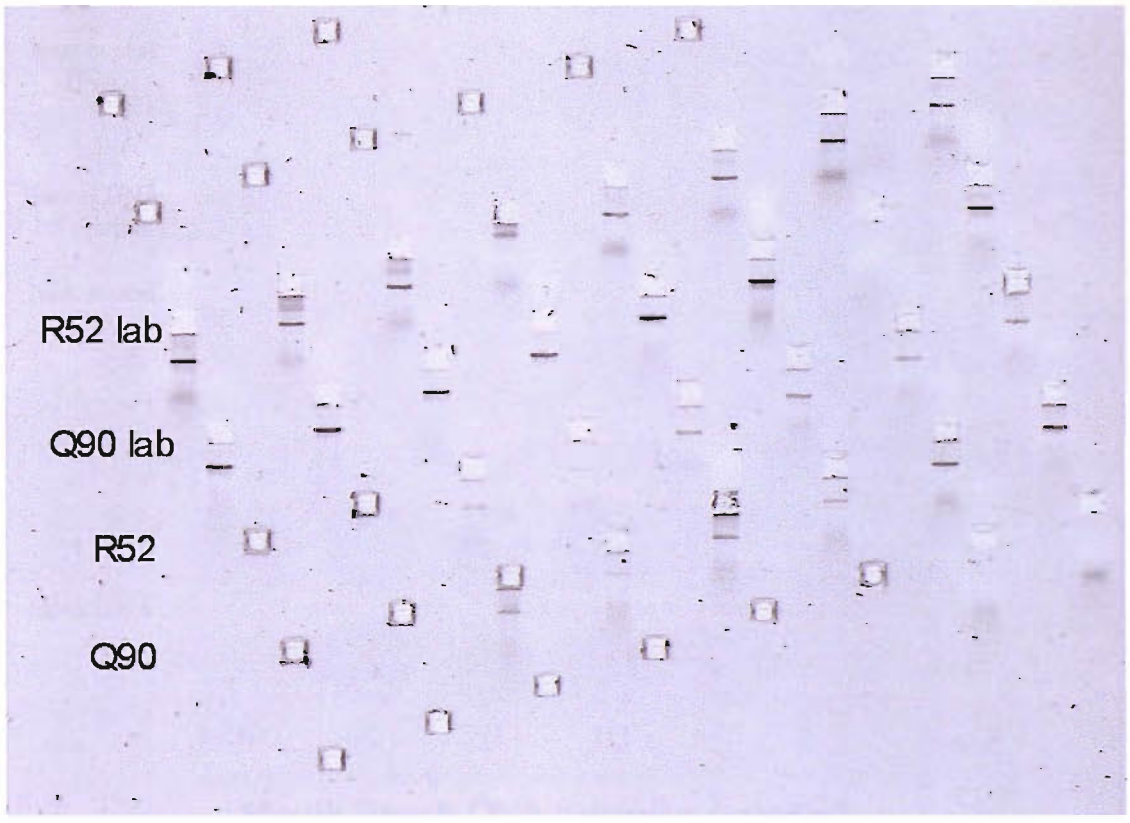


Figure 2.2 – Q90 and R52 PCRs run on blood-derived 'control' DNA ('lab' samples) and on the buccal swab DNA (labelled only with the assay type). 2µl of sample was used as template for each reaction.

The Q90 and R52 PCRs were successfully performed on a template of genomic DNA from the buccal swabs (Figure 2.2), although not for all samples (six out of eight samples were successfully typed for both of the assays). The bands observed after electrophoresis were of considerably lower intensity than those from blood derived 'control' DNAs, confirming the concentration of DNA in the buccal swab samples was lower than in blood derived samples.

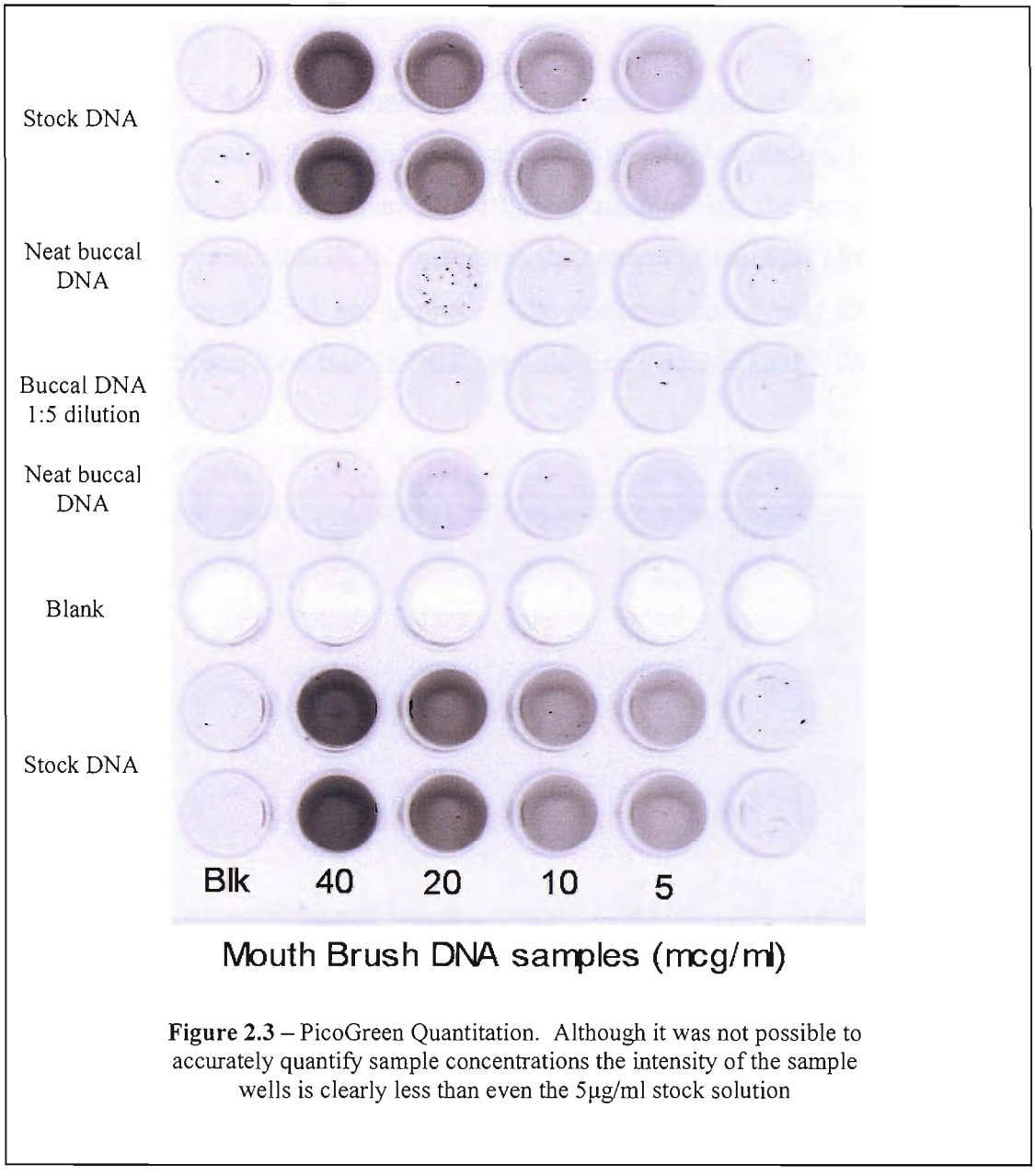


Figure 2.3 – PicoGreen Quantitation. Although it was not possible to accurately quantify sample concentrations the intensity of the sample wells is clearly less than even the 5μg/ml stock solution

PicoGreen quantitation of six random samples demonstrated DNA concentrations <5μg/ml for all six samples (Figure 2.3). The samples were quantified twice (on separate runs) to ensure against error (the two 'neat buccal DNA' rows). A dilution had also be performed in case the sample concentration was higher than the stock DNA concentrations. The exact concentrations of the samples could not be calculated accurately as they lay below the lower points of the calibration curve.

2.3.2 PCR on DOP product

Figure 2.4 shows the results when the PCR protocols were run on varying amounts of DOP amplified material. Some improvement in electrophoresis band intensity was seen in comparison to the unamplified buccal samples, but the template was still insufficient to run reliable PCR. Increasing the amount of template DNA used in the DOP reaction to 5µl did not improve PCR performance. Using DOP amplified material as template for a further DOP amplification ('double DOP') did not improve PCR results.

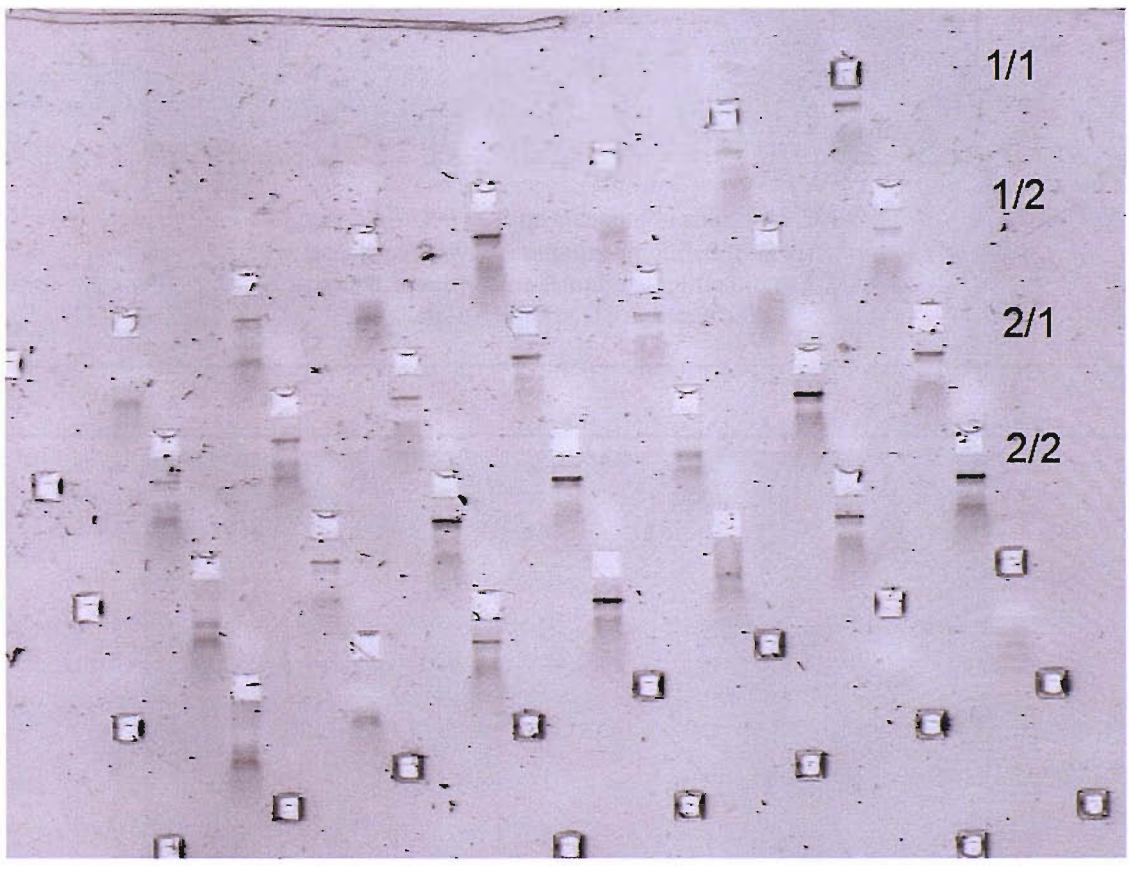


Figure 2.4 – Q90 PCR run on DOP product. 1/1 indicates 1µl of genomic DNA used as template for DOP amplification then 1µl of the DOP product used as template for Q90 PCR, 1/2 indicates 1µl of genomic DNA used as template for DOP amplification then 2µl of the DOP product used as template for Q90 PCR etc.

2.3.3 PCR on GenomiPhi product

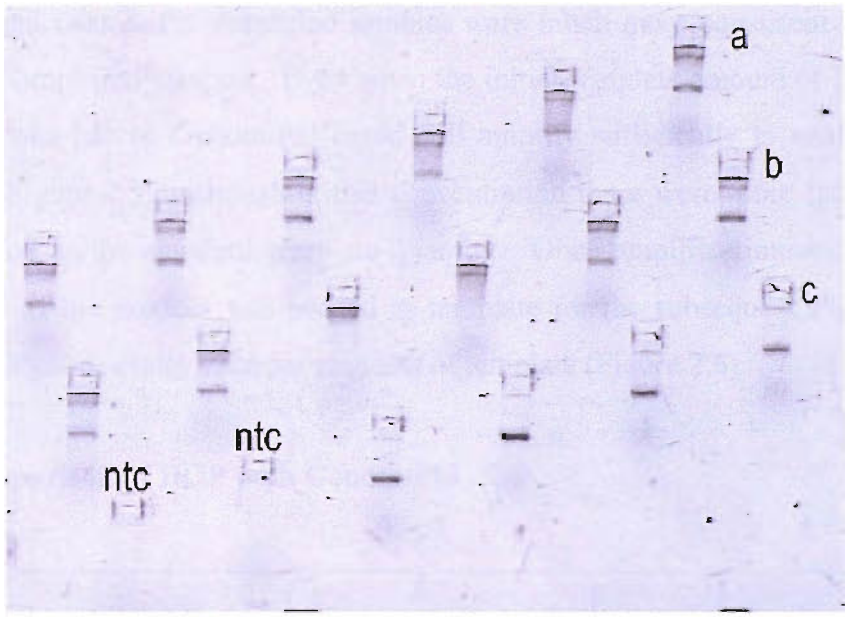


Figure 2.5 – Q90 PCR on GenomiPhi amplified DNA template. Row a=6 samples amplified from 1µl genomic, row b= same samples amplified from 1:2 genomic, c = control DNA, ntc=blanks

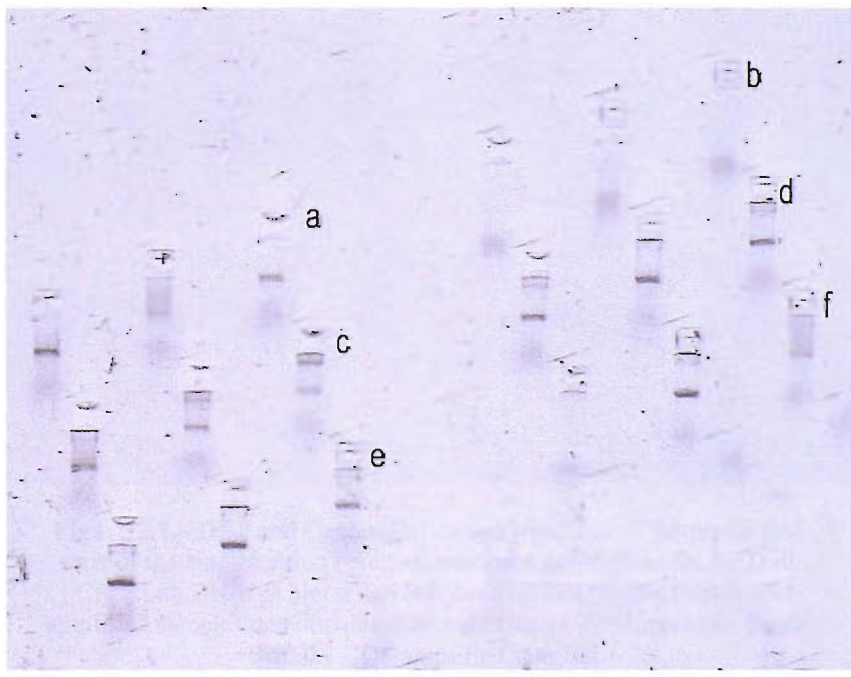


Figure 2.6 – three samples amplified from 1µl genomic. Varying amounts of this material then used as template for subsequent PCR. a=control DNA, b=blanks, c=2µl, d=1µl, e=0.5µl, f=0.25µl

PCR on GenomiPhi amplified material was more successful. Although not approaching the intensity of blood derived samples, the electrophoresis bands derived from GenomiPhi amplified samples were much more consistent than those from DOP amplified samples. Even when the initial template amount of DNA to be amplified was halved GenomiPhi could still amplify sufficiently to enable further PCR (see Figure 2.5), although at this concentration there were more failures. 1µl was adopted as the standard template quantity. Once amplification was achieved only 0.5µl of the product was needed as template for the subsequent PCR, as this achieved as good results as larger amounts of template (Figure 2.6).

2.3.4 Comparison of DOP with GenomiPhi

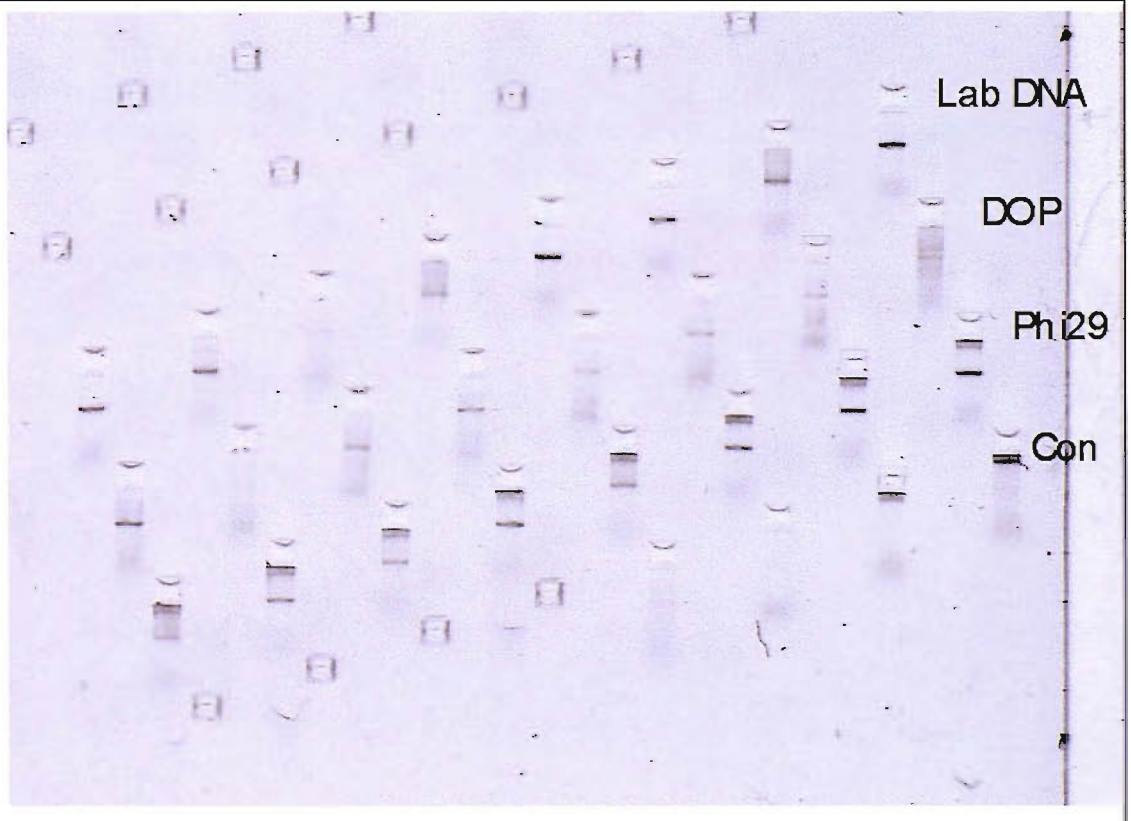


Figure 2.7 – DOP and GenomiPhi comparison. 2µl of the product of each of the amplification reactions was used as template for the Q90 PCR. 'Lab DNA' is blood derived 'control' DNA. The GenomiPhi amplified samples demonstrate markedly clearer electrophoresis bands than the DOP amplified samples.

When the DOP and GenomiPhi methods were directly compared a difference could be observed. GenomiPhi provided superior amplification (Figure 2.7). 8 samples were used, pre-amplified by both the DOP and Phi29 methods. The Q90 PCR was then used on all of the samples. As can be seen in Figure 2.7 six out of eight samples were genotyped after DOP amplification. All eight were typed using the Phi29 amplification. Clearer electrophoresis bands are obtained using Phi29, as compared with DOP, in seven out of eight of the samples.

3.3.5 GenomiPhi on larger sample numbers

When applied to a larger sample size the GenomiPhi amplification remained consistent, although it was not possible to obtain a PCR product in 6 samples (Figure 2.8).

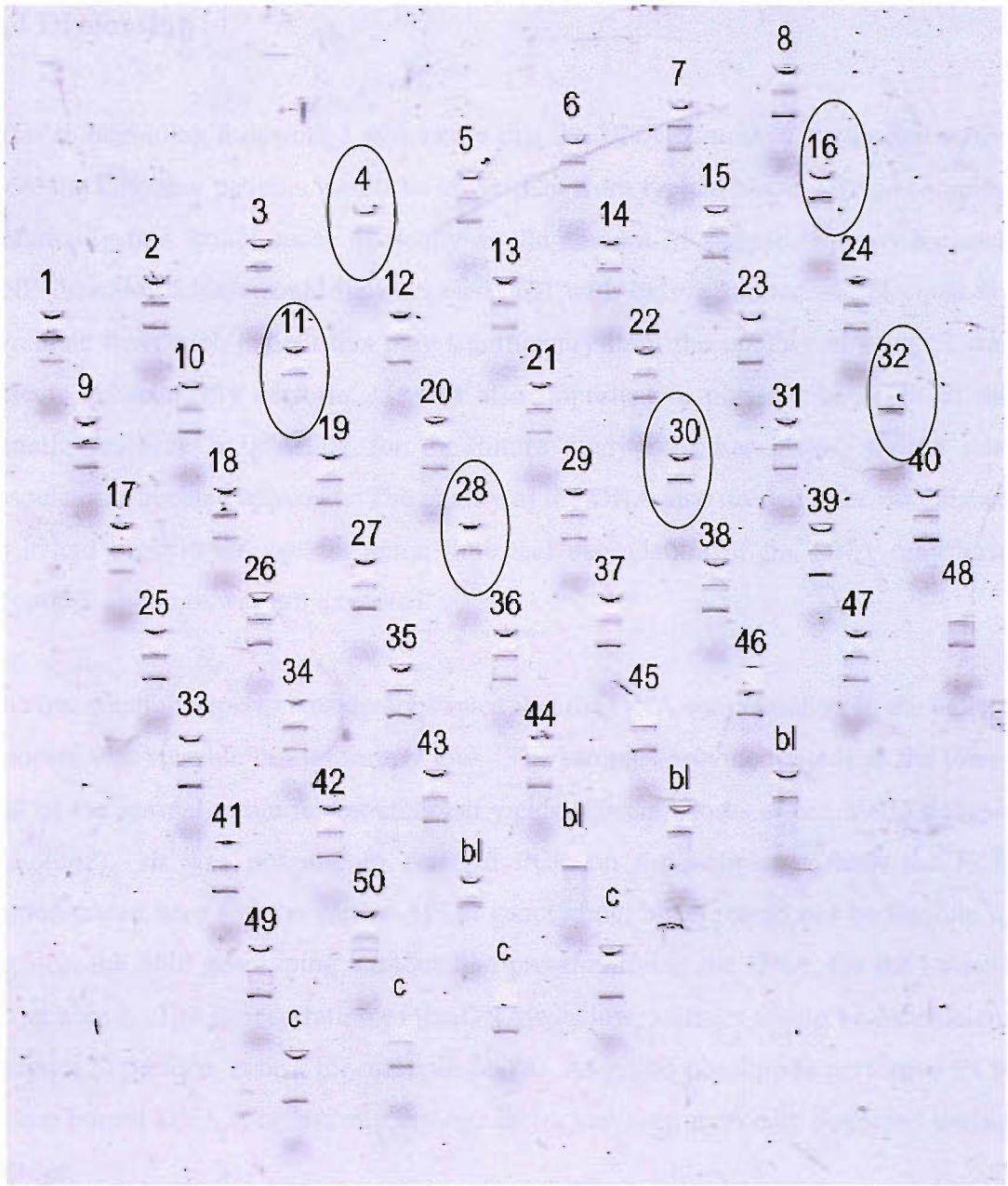


Figure 2.8 – Q90 PCR on GenomiPhi amplified samples. Circled samples show non-results bl=blanks, c=control DNA

2.4 Discussion

Prior to beginning this work I was aware that the DNA content of the buccal swabs from the Glasgow patients would be lower than from typical blood-derived samples. Potentially this would cause difficulty as the amount of sample required for each SNP detection assay would be increased, and with only a limited sample quantity available from each patient this may significantly limit the number of assays I was able to subsequently perform. It was also important to preserve as much of the genetic resource as possible for the future study of other genes, should new associations become apparent. The quality of the DNA also needed to be established as it had been in storage for some time and degradation of the DNA may have occurred, although was not expected.

The quantitation experiments demonstrated that the DNA concentration in the patient samples was variable but uniformly low. The samples provided yields at the lower end of the normal range for mouthbrush yields (Garcia-Closas *et al.*, 2001; King *et al.*, 2002). It was possible to perform PCR on the samples – both the PCR demonstrated here and the earlier APOE genotyping, but it would not be feasible to perform the SNP genotyping without first pre-amplifying the DNA, for the reasons given above. The concentration of the DNA was low, so there would be insufficient material to perform assays for multiple SNPs. As it was possible to perform a PCR on the buccal DNA it is unlikely that the DNA had been markedly degraded during storage.

The DOP-PCR method of pre-amplification is well established in the Human Genetics laboratory in Southampton. It is used for preservation of large DNA banks, derived from blood samples. This method had not previously been used to pre-amplify buccal derived material in our lab. The GenomiPhi method is entirely new, having only recently come onto the market.

When using the DOP method amplification was achieved but the performance of the template in subsequent PCR was poor. In order to obtain good haplotype data it will be important to be able to accurately genotype each SNP of interest in the majority of

samples. The evidence from running the Q90 PCR was that this would not be possible in the DOP amplified samples. Running a 'double DOP' protocol (as used by Barbaux *et al.* (2001)) did not improve results.

GenomiPhi provided good amplification of genomic DNA and this could be used as a template for further PCR. When directly compared with the DOP method GenomiPhi provided superior results. GenomiPhi amplification is also easier to perform as it is available in kit form and does not require thermal cycling. It is however more expensive. Only 1μl of genomic DNA is required as template for pre-amplification. The reaction takes place in a final volume of 20μl. As demonstrated in my results subsequent PCR can be performed on 0.5μl of this pre-amplified material, suggesting that nearly 40 SNP assays could be performed from just 1μl of the original sample. This is considerably more than could have been achieved from the whole sample without the pre-amplification step.

When pre-amplification and subsequent PCR was applied to 50 samples a 12% failure rate was observed. It is likely that these failed samples have the lowest genomic DNA concentrations and even after pre-amplification DNA concentrations were too low to perform PCR under conditions available in the laboratory. This problem may be solved by designing more specific assays (see Chapter 3), but I may need to assume that there will be approximately a 10% failure rate across my cohort. There is sufficient power in the study that this will not adversely affect my results.

The GenomiPhi protocol will be adopted for the pre-amplification phase of my study, using 1μl of genomic DNA as template. 0.5μl of this material was sufficient for subsequent PCR, so this amount is likely to provide sufficient template for future assays.

Chapter 3

SNP assay design

3.1 Introduction

As we saw in Chapter 1 genetic association studies are designed in order to establish whether particular alleles or genotypes are risk factors for a given disease or condition. The expectation is that any 'risk' allele will be seen in a much higher proportion of disease sufferers. Population studies designed to detect these variables need sufficient statistical power – and this is achieved by using large subject numbers. In order to perform these studies a high-throughput method of genotyping is therefore required. Ideally any technique should also be fast, accurate and inexpensive, especially when a large panel of SNP genotypes need to be determined. Since the introduction of PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986) many techniques have been developed. Traditionally these have involved variations of the PCR technique followed by gel-based analysis of products. Examples include RFLP (restriction fragment length polymorphism) analysis (Lander & Botstein, 1986) and ARMS-PCR (amplification refractory mutation system) (Newton *et al.*, 1989). When multiple SNPs are being investigated it becomes increasingly cost and time prohibitive to use these techniques. Due to developments in molecular biology over recent years an increasing number of alternative methods of high throughput genotyping have become available. These include the 5' nuclease assay (TaqMan) (Johnson *et al.*, 2004), multigene array methods and gene chips (Yauk *et al.*, 2004). Choosing the most useful and productive assay method for SNP genotyping is becoming increasingly important.

At the outset of this study a number of techniques for SNP detection were available to me. As well as techniques already available in the Human Genetic laboratory it was also possible to use techniques elsewhere in the University. When deciding which technique or techniques to use it was necessary to establish which would provide the most accurate results, which would be most practical in terms of equipment availability and time consumption and also what costs would be involved. In the next section I will describe the techniques considered at the beginning of the project. The following work demonstrates how the final assay method was decided upon after comparison of a number of methods. In the second part of the chapter the actual SNP assay design and optimisation will be described.

3.2 Assay Choice

3.2.1 RFLP analysis

This technique is often considered the 'gold standard' for SNP genotyping. It relies upon the presence of an enzymatic restriction site at or near the site of a SNP. After PCR is used to amplify the region containing the SNP/restriction site the amplicon is then 'digested' with the enzyme to produce DNA fragments of differing sizes depending upon the allele/s present in the sample. The digested products can be visualised by gel electrophoresis. Genotypes can then be called manually or by image analysis software. The MADGE system (Day *et al.*, 1995) enables SNP typing of large numbers of samples simultaneously (Gaunt *et al.*, 2003), although it is still quite labour intensive to genotype many SNPs, as the gels needed to be formed and loaded. Not all SNPs can be detected in this manner if the SNP does not form a restriction site.

It was felt that this method was too labour intensive in comparison to the other methods available to us so was not tested as a possible assay method. Should it prove difficult to accurately genotype my samples with any of the other techniques this method will be used as a reliable 'fall back' method.

3.2.2 ARMS-PCR

This modification of the PCR method relies on the principle that a single base mismatch at the 3' end of a primer will inhibit non-specific amplification. DNA amplification will therefore only occur if the precise primer target sequence occurs in the target strand of DNA (Newton *et al.*, 1989). This is often termed allele specific PCR. In order to detect both alleles of a SNP two reactions will be required, one for each allele.

This technique avoids the need for any post-PCR manipulation (such as the digestion of PCR products in the RFLP method) but two separate reactions for each sample are

necessary. Relatively high-throughput can be achieved by using the MADGE technique for resolution of the products.

3.2.3 5' Nuclease Assay (Taqman®)

The 5' nuclease polymerase chain reaction assay is a non-gel based technique first described in 1993 by Holland *et al.* (1991). Using fluorogenic probes Lee *et al.* (1993) modified the assay to enable amplification and detection to be performed in a single step. Because of this the technique is often referred to as 'real-time' PCR. As in a standard PCR reaction primers are used to replicate the region containing the SNP. Fluorescently labelled probes specific for each allele of the SNP are added. As the primer is extended by *Taq* polymerase and reaches the bound probe the fluorogenic probe is cleaved, causing an increase in fluorescent intensity. By labelling each probe with a different fluorescent dye each allele can be detected separately.

The Taqman system is very economical in terms of time required to genotype SNPs, although this comes at an increased financial cost due to the need for fluorescently labelled probes and the initial equipment outlay (Holloway *et al.*, 1999).

3.2.4 Odyssey LightTyper

This recently available technology also utilises fluorescently labelled probes, but an entirely different detection method (see Figure 3.1). The Odyssey utilises the principle of 'melt curves'. A fluorescently labelled probe, designed to anneal precisely with one of the SNP alleles, is included during PCR setup. If the sample is subsequently heated any probe that is imprecisely bound (i.e. bound to the alternate allele of the SNP) will 'melt off' the target DNA at a lower temperature than the precisely bound probe. As the probe melts off its target a change in fluorescence can be detected, and this change will occur at different temperatures for each SNP allele, enabling each SNP to be differentiated. The LightTyper detector and software had recently become available for use in the Department of Human Genetics.

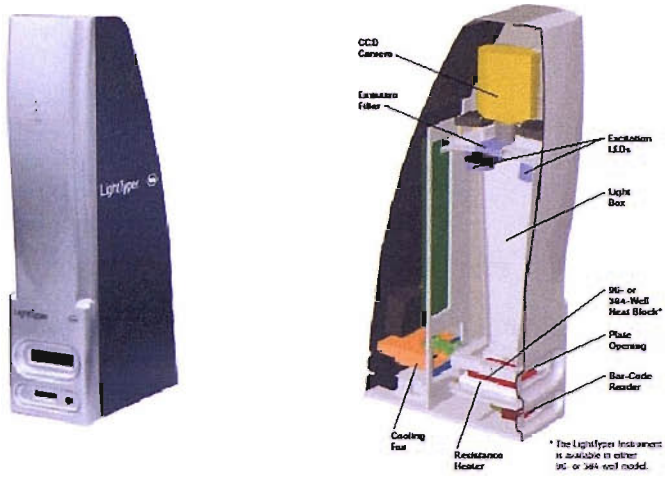


Figure 3.1 – Image of the Odyssey LightTyper and schematic. Images from www.roche.com

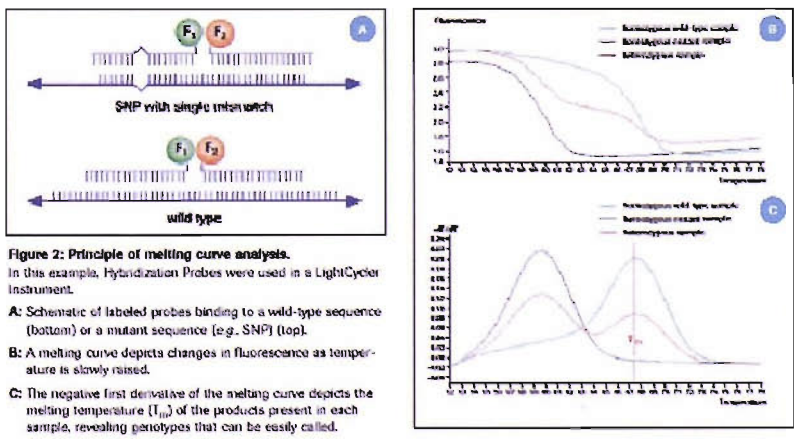


Figure 2: Principle of melting curve analysis.
In this example, Hybridization Probes were used in a LightCycler Instrument.

A: Schematic of labeled probes binding to a wild-type sequence (bottom) or a mutant sequence (e.g. SNP) (top).

B: A melting curve depicts changes in fluorescence as temperature is slowly raised.

C: The negative first derivative of the melting curve depicts the melting temperature (T_m) of the products present in each sample, revealing genotypes that can be easily called.

Figure 3.2 – Illustration of melt curve analysis. Figure from www.roche .com

3.3 Methods

3.3.1 Subjects

The first fifty samples from the Glasgow cohort (samples P0001-P0050) were used to examine the efficiency of the assays tested in this segment of work. 1µl of each sample was pre-amplified using the GenomiPhi method. 0.5µl of the pre-amplified sample was then used as template for the assay reactions.

3.3.2 ARMS-PCR typing of TNFA -308

An optimised ARMS-PCR assay was available for the TNFA -308 promoter polymorphism, courtesy of Dr Martin Howell (see Perrey *et al.*, 1999). This was modified to test the ARMS-PCR method on the Glasgow samples. 2 separate PCR reactions were necessary for each sample in order to type each allele. Each reaction was carried out in a final volume of 10 µl consisting of 1µM common primer (5'-TCTCGGTTTCTTCTCCATCG-3', Oswel, Southampton, UK), 1µM specific primer (either 5'-ATAGGTTTTGAGGGGCATGG-3', Oswel, for the G allele, or 5'-ATAGGTTTTGAGGGGCATGA-3', Oswel, for the A allele), 0.3µM control primers (5'-TGCCAAGTGGAGCACCCAA-3' and 5'-GCATCTTGCTCTGTGCAGAT-3', Oswel), 1x reaction buffer (Promega, Southampton, UK), 200 µM dNTPs (Invitrogen, Paisley, UK), 1.75mM MgCl, 0.25 units *Taq* polymerase (Promega), 12% sucrose and 200µM cresol red. Thermal cycling was carried out on an MJ Research PTC-225 DNA Engine Tetrad® (Genetic Research Instrumentation Ltd) using the following cycles; 96°C for 1 minute, 10 cycles of 96°C for 15 seconds, 65°C for 50 seconds and 72°C for 40 seconds then 20 cycles of 96°C for 10 seconds, 60°C for 50 seconds and 72°C for 40 seconds. The PCR products were loaded onto a MADGE gel (see Chapter 2) and electrophoresis carried out at 150V for 10 minutes. Image analysis was performed using ImageQuant software (Amersham Bioscience).

3.3.3 TaqMan assay for TNFA -308

An optimised 5' nuclease assay for TNFA –308 was available from the Department of Molecular Pathology, Southampton General Hospital. 5µl reaction mixes contained 0.2µM probes, 0.9µM primers and QPCR mix (Oswel). Reactions were performed in 384-well plates on the Applied Biosystems 7900HT Sequence Detection System using the allelic discrimination protocol. SDS software (Applied Biosystems) was used to analyse fluorescence results. This method was not tested on the full fifty samples but on 10 samples at various template concentrations.

3.3.4 Odyssey assay for TNFA -308

The Odyssey assay for TNFA -308 entails an asymmetrical PCR with the addition of FITC- and DABCYL-labelled probes. PCR mixes consist of 1x PCR buffer (Promega), 200 µM dNTPs (Promega), 100nM forward primer (5'-CCTCACACTCCCCATCCTC-3'), 500nM reverse primer (5'-CCTGCATCCTGTCTGGAAGT-3'), 200nM FITC-labelled probe (5'-CCGTCCCCATGCCCC-3'), 200nM DABCYL-labelled probe (5'-TGTGTGTAGGACCCTGGAGGCTGAA-3'), 1.5mM MgCl and 0.2 units of *Taq* polymerase (Promega) per reaction. Heat cycling was performed on an MJ Research PTC-225 DNA Engine Tetrad® (Genetic Research Instrumentation) using a protocol of 94°C for 2 minutes, then 50 cycles of 94°C for 20 seconds, 59°C for 30 seconds and 72°C for 30 seconds, followed by a final 2 minutes at 72°C. After thermal cycling the samples were overlaid with 5µl Chill-Out™ wax (Genetic Research Instrumentation) to prevent evaporation during analysis. Analysis was carried out in a 384-well Odyssey (Idaho Technology, Salt Lake City, Utah, USA). Samples were melted from 35°C to 70°C. LightTyper software (Roche Diagnostics Ltd) was used to analyse the fluorescence change during melting. Results were then manually checked.

3.4 Results

3.4.1 ARMS-PCR typing of TNFA -308

Although I was able to generate control bands and some allele bands when the assay was run on a small number of samples these were not visible when the assay was run on the full fifty samples (see Figure 3.3)

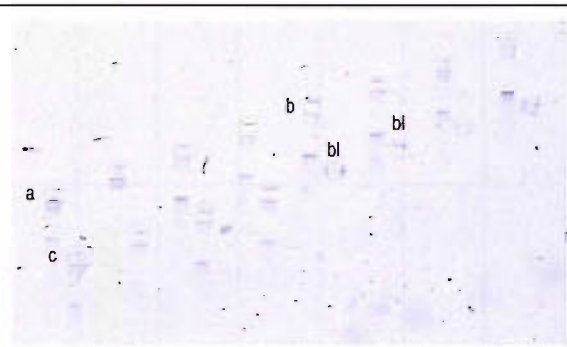


Figure 3.3a - TNFα ARMS assay (single allele) on various template amounts. a=0.75µl, b=0.5µl, c=0.25µl, bl=blanks

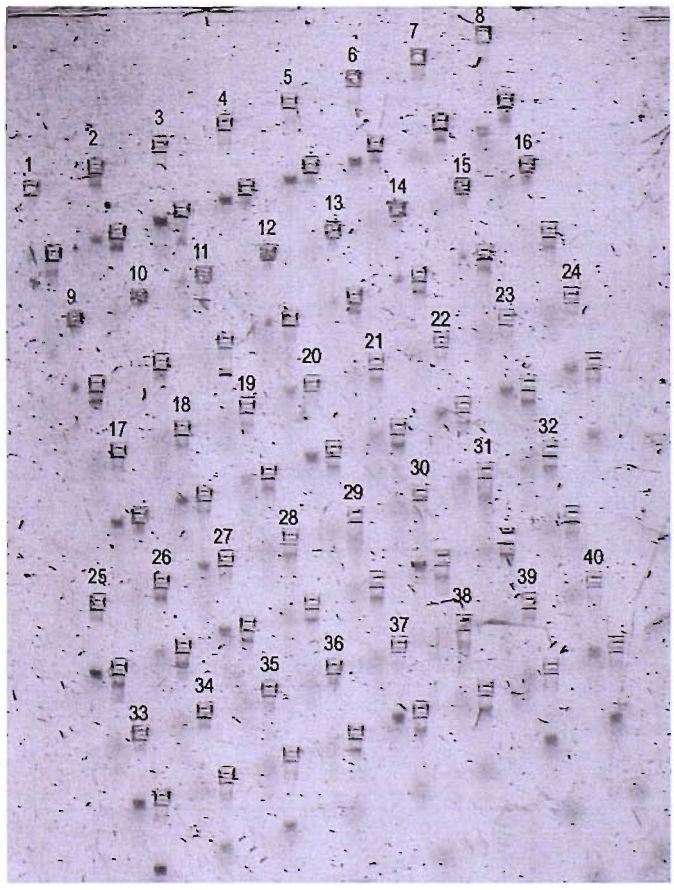


Figure 3.3b - TNFA ARMS assay on 40 samples (0.5µl template). Upper row G allele, lower row A allele

3.4.2 TaqMan assay for TNFA –308

Figure 3.4 shows the dot plot generated. There was poor clustering of the samples and a large number of samples remained undetermined.

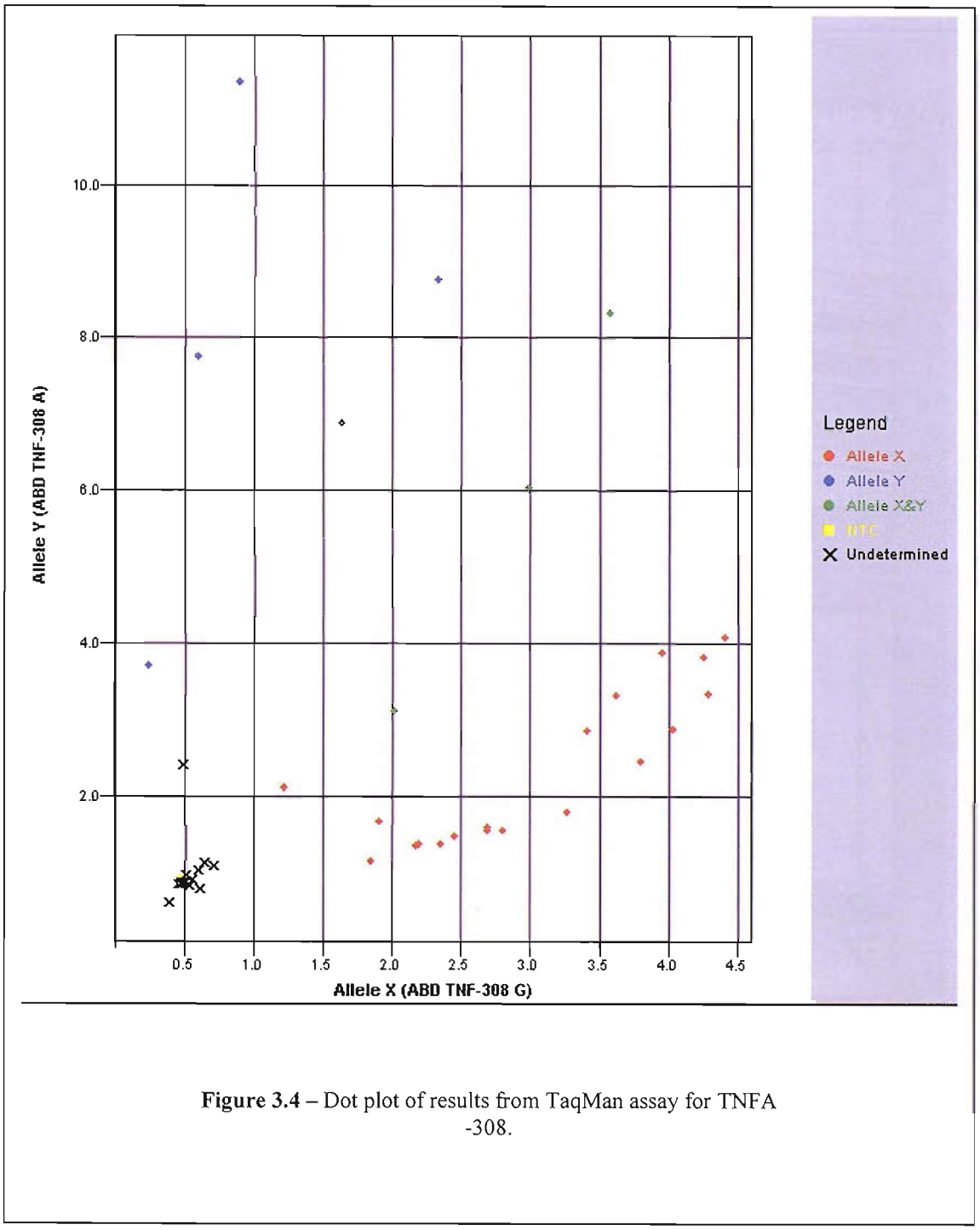


Figure 3.4 – Dot plot of results from TaqMan assay for TNFA -308.

3.4.3 Odyssey assay for TNFA -308

50 GenomiPhi amplified patient samples were tested using the Odyssey (see Figure 3.5). 43/50 (86%) were successfully genotyped, giving allele frequencies of GG 0.62, GA 0.16, AA 0.08 and fails of 0.14.

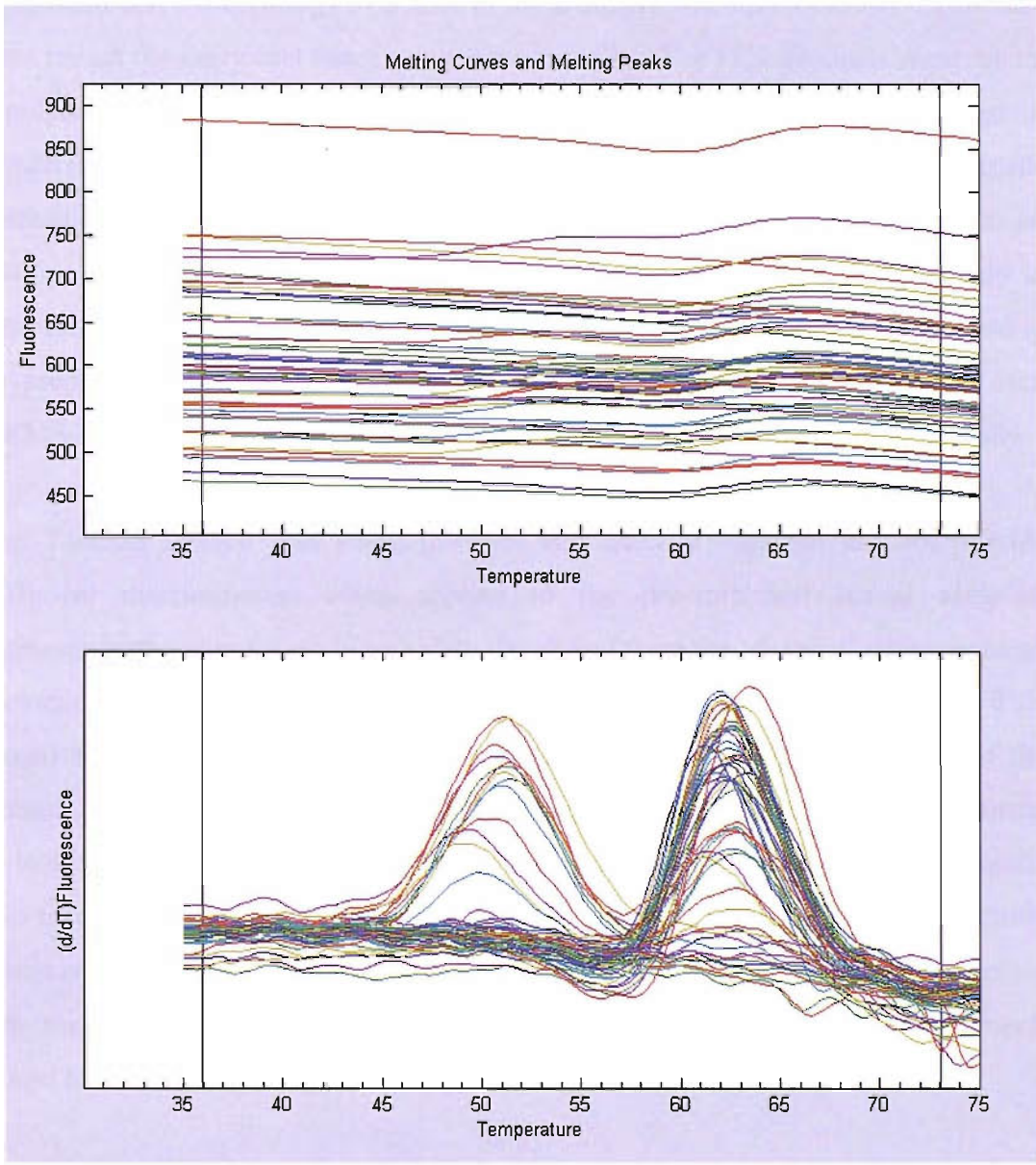


Figure 3.5 – Melt curves and peaks achieved when running the TNFA -308 assay on GenomiPhi amplified samples.

3.5 Summary

Three methods for determining SNP genotypes were compared. The ARMS-PCR method failed to genotype any of the pre-amplified Glasgow samples. The reason for the failure was not clear but the assay was modified slightly in order to use equipment and methodology available in the Human Genetics laboratory. This may have meant the assay was functioning sub-optimally. The PCR products were run on a polyacrylamide gel, as opposed to an agarose gel, in order to take advantage of MADGE to enable multiple assays to be run simultaneously. This may have made distinction of the bands more difficult, as the assay was designed to be run on an agarose gel. Even if the results had been better the assay was quite unwieldy to perform. The need to run two separate reactions means two reaction 'mixes' need to be prepared each time the assay is run, and twice as many gels need to be run. ARMS-PCR was discounted as a possible method for SNP genotyping in this study.

The Taqman method was straightforward and quick to use but did not provide sufficient discrimination when applied to the pre-amplified buccal samples. Although some samples could be safely genotyped from the 'dot plot' many required crosschecking with the real-time data to confirm the accuracy of the result. This turned a process that should be relatively swift into a far longer one. One of the benefits of the Taqman system is the efficiency of the data collection, which would be lost if the real time data needed to be examined for many of the samples. It would also mean that all of the PCRs would need to be performed in the Taqman machine, whereas performing the PCR on a heat block and then transferring the sample plate into the Taqman machine for analysis could enhance throughput. This approach would mean the real-time data was not available.

The LightTyper system provided the most promising results. The assay set up was quick and the post PCR analysis could be rapidly performed with the aid of the LightTyper software. Although it was not possible to genotype all of the 50 samples for each SNP this was likely to be more a problem with the sample quantity and quality rather than a problem with the assay. The technology is new, so if it was adopted assays would need to be designed from scratch for each of the SNPs, apart

from the two in the TNFα gene as these were already available following other studies performed in the laboratory. The need for FITC- and DABCYL-labelled probes within the assays is expensive, although the increased cost per sample is not significant.

In comparison to the other two methods the Odyssey LightTyper was the only method that provided results good enough to use for the genotyping of the whole Glasgow cohort. In addition, the ease of use and high-throughput methodology confirmed this as the technique we would adopt for SNP genotyping during this project.

3.6 Odyssey assay design

SNP genotyping will be carried out on the Odyssey LightTyper, as described in the previous section. Assays for the two TNFA SNPs (-238 & -308) were already available in the laboratory but assays for all of the remaining polymorphisms required development from scratch. Each SNP will require its own assay. Each assay will require the design of a primer pair to amplify the region containing the SNP, a fluorescently labelled probe to detect the SNP and a second probe with an attached fluorescence quencher-dye to enhance the change in fluorescence as the detector probe 'melts off'.

All of the PCR-based assays conform to a basic design. Primers and probes should be in the ratio 5:2:1 (opposite sense primer: probe(s): same sense primer). FITC (fluorescein-5-isothiocyanate) is used to label the probe that sits over the SNP, whilst DABCYL (4-dimethylaminoazobenzene-4''-carboxylic acid) is attached to the 'quencher' probe. When bound the quencher probe should ideally be separated from the fluorogenic probe by 2 base pairs. The SNP should occur near the centre of the FITC-labelled probe, which should be 16-20 nucleotides long. The DABCYL probe should be longer; ensuring its melt temperature (T_m) is at least 10°C higher than the FITC probe. The PCR product ideally should be the minimum size possible without the primers and probes overlapping.

3.7 Methods

3.7.1 Sequence Identification

In order to design sequence specific primers and probes the correct DNA sequence containing the SNP needed to be identified. SNPs were identified in Entrez SNP/dbSNP, the SNP database available from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Within dbSNP each SNP is identified by an individual reference number, and not necessarily by its position within the gene sequence. Where it was unclear the SNP identified in dbSNP was the correct one then the literature referenced by dbSNP was examined and cross-referencing with other SNP databases (SNP500Cancer (snp500cancer.nci.nih.gov), the Human Genome Variation database (hgvbase.cgb.ki.se) & the Bristol Cytokine Gene Polymorphism database (www.bris.ac.uk/pathandmicro/services/GAI/cytokine4.htm) was performed to ensure correct identification.

3.7.2 Primer design

Forward and reverse primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, www-genome.wit.mit.edu/ genome_software/other/primer3.html) (see Rozen *et al.*, 2000). Where the software suggested multiple primer pairs the pair that would produce the shortest PCR product were selected. Primers were synthesised by MWG Biotech (Milton Keynes, UK) and TAGN (Gateshead, UK).

3.7.3 Probe design

Probes were designed manually, according to the requirements set out in section 4.6. All fluorogenic probes were labelled with a 5'-FITC and a 3'-phosphate group to prevent extension during PCR. All quencher probes were labelled with a 3'-DABCYL. Probes were synthesised by MWG Biotech and TAGN.

3.7.4 Assay optimisation

A standard PCR set-up was used for each SNP assay. Each 10µl reaction contained 1x PCR Buffer (Promega), 200µM dNTPs (Promega), 100nM primer, 500nM primer (see below), 200nM FITC probe, 200nM DABCYL probe, 1.5 or 2mM MgCl and 0.2 units of *Taq* polymerase (Promega). If the FITC probe was designed as a sense probe then 100nM forward primer and 500nM reverse primer were used. If the FITC probe was designed as an antisense probe then 500nM forward primer and 100nM reverse primer were used.

Thermal cycling was performed on a Tetrad heat block as detailed in section 3.3.3. Heat cycling entailed 2 minutes at 94°C, then 50 cycles of 94°C for 20 seconds, 30 seconds at an optimal annealing temperature for the SNP and 30 seconds at 72°C, followed by a final 2 minutes at 72°C. PCR was performed in 96-well plates (Abgene, Epsom, Surrey). The samples were then transferred to a 384-well plate (Abgene), covered with 5µl Chill-Out™ wax and analysed using the Odyssey LightTyper.

An annealing gradient was performed for each SNP assay in order to establish the optimum annealing temperature and MgCl concentration. Each assay was performed on 2µl of pooled genomic DNA (100ng/µl) across a range of annealing temperatures between 50°C and 65°C. MgCl concentrations of 1.5mM and 2mM were used.

Once the optimum annealing temperature and MgCl concentration was determined then the assay was run on 40 of the pre-amplified Glasgow samples. PCR was performed in 96-well plates (Abgene) and analysis in 384-well plates (Abgene) for all assays except the three IL-1β assays, which were performed in 384-well plates only.

3.8 Results

3.8.1 Assay sequences and optimisation

SNP IDs and primer and probe sequences are shown in Table 3.1. The initial primers designed for all of the IL-6 assays did not provide suitable amplification. These were re-designed using Primer3 and are included in the table. Table 3.2 shows the

Gene	SNP	Anealing Temperature (°C)	MgCl concentration (mM per reaction)
IL-6	-174 (G/C)	59	1.5
	-572 (G/C)	62	1.5
	-597 (G/A)	62	1.5
TGFβ	-509 (C/T)	53	2.0
	-800 (G/A)	54	2.0
IL-1α	-889 (C/T)	56	2.0
IL-1β	-31 (C/T)	53	1.5
	-511 (G/T)	53	1.5
	+3953 (C/T)	57	1.5

Table 3.2 – Optimum annealing temperature (in 96-well plates) and magnesium concentration for each cytokine SNP assay

Gene	SNP	dbSNP ID	Primers Forward then Reverse	Probe Orientation	FITC Probe	DABCYL probe
IL-6	-174 (G/C)	1800795	5'- GCCTCAATGACGACCTAAGC 5'- TCATGGGAAAATCCCACATT	antisense	5'-CTTTAGCATcGCAAGACAC	5'-AACCTTATTAAGATTGTGCAATGTGACG
	-572 (G/C)	1800796	5'- TGGCAAAAAGGAGTCACACA 5'- CCAAGCCTGGGATTATGAAG	antisense	5'- CTGTGAGcGGCTGTTG	5'- CATCTGAGTTCTTCTGTGTTCTGGCTCT
	-597 (G/A)	1800797	5'- TGGCAAAAAGGAGTCACACA 5'- CCAAGCCTGGGATTATGAAG	antisense	5'- CAcCCTCAAATTTCGTG	5'- GGCTGTTGTAGAACTGCCTG
TGFβ	-509 (C/T)	1800469	5'-TGGGAGGTGCTCAGTAAAGG 5'-ACCCAGAACGGAAGGAGAGT	sense	5'-CTTCCATCCcTCAGGTGT	5'-CTTACAGGTGTCTGCCTCCTGA
	-800 (G/A)	1800468	5'-CTGCTCCGCAACTTCGAC 5'-CTGGGGTCAGCTCTGACAGT	sense	5'-CTCCAACgTCACCACCAT	5'-CGTGGAGTGCTGAGGGACTCT
IL-1α	-889 (C/T)	1800587	5'-TAGGCTGGCCACAGGAATTA 5'-CGTAAGGCCTCAGCCAGAAG	sense	5'-CAGGCAACAcCATTGAAG	5'-AGCTGAGAAATTCTTTAATAATAGTA
IL-1β	-31 (C/T)	1143627	5'-CCCTTCCATGAACCAGAGAA 5'-GCTGAAGAGAATCCCAGAGC	antisense	5'-CTCGCTGTTTTTATgGCTTT	5'-GAAGAGGTTTGGTATCTGCCAGTTTCT
	-511 (G/T)	16944	5'-AAACCTCTTCGAGGCACAAG 5'-CAGAGAGACTCCCTTAGCACCT	antisense	5'-CaGAGCAGCCTGTTGTG	5'-GCAATGAAGATTGGCTGAAGAGAAT
	+3953 (C/T)	1143634	5'-ATGCTCAGGTGTCCTCCAAG 5'-TGGAGGTGGAGAGCTTTCAG	antisense	5'-CATGTGTCgAAGAAGATAG	5'-TCGTGCACATAAGCCTCGTTAT

Table 3.1 – Primer and probe sequences for in-house Odyssey assays.
Bases indicated in lower case in the FITC sequences denote the SNP position.

optimum annealing temperatures and magnesium concentrations derived from the annealing gradient experiments.

3.8.2 Sample results

These are shown in Table 3.3. Across all eleven assays the genotyping was not successful in 23.9% of cases. The IL-1B PCRs were performed in 384-well plates and two out of three of the assays showed markedly higher failure rates (25/40 for IL-1B +3953). If the IL-1B assays are disregarded the failure rate drops to 18.1%. The IL-1B +3953 assay was repeated, running the PCR in 96-well plates, although on a different selection of samples. This dropped the failure rate to 20%.

There was good concordance of results when each of the assays was repeated. Using the TGFB -509 SNP assay as an example; after performing the assay twice 22.5% (9/40) of cases had not been typed. In 4 cases (10%) a definite SNP type was obtained in only one of the runs. In the remaining 27 cases there was 100% concordance of SNP types. Similar findings were obtained for the other SNP assays.

SAMPLE	238(G/A)	308(G/A)	174(G/C)	572(G/C)	597(G/A)	509(C/T)	800(G/A)	889(C/T)
P001	GG	GG		GG		TT		CC
P002	GG	GG	GG	GG	GG	CC	AA	CC
P003	GA	GG	GC	GG	GA	TT	GG	CC
P004	GG	GG	GC	GG	GA	CC	GG	CC
P005	GG	GG	GG	GG	GG	CC	GG	CC
P006	GG	GG	GG	GG	GG	CC	GG	TT
P007	GG	GG	GG	GG	GG	TT	GG	TT
P008	GG	GG		GG	GA	CC	GG	CC
P009	GG	GG		GG				CC
P010	GG		GC	GG	GA	TT	GG	CC
P011				GG		CC	GG	CC
P012	AA	AA		GG	GA	TT	GG	CC
P013	GA	GG	GG	GG	GG	CT	GG	CT
P014	GG	GG		GG	GA	CC	GG	TT
P015	GG	GG	CC	GG	AA		GG	CC
P016					GG			CC
P017	GG	GG	GG	GG	GG	CC	GG	CC
P018	GG	GA	GG	GC	GG	TT	GG	CT
P019	AA	GG		GG		CC	GG	TT
P020	GG	GG	CC	GG	AA	CC	GG	CT
P021	GG	GG	GG	GG	GG			CT
P022	GG	GA	GC	GG	GA		GG	CC
P023	GG	GG	GC	GG	GA	CC	GG	CC
P024	GG	GA	GG	GG	GG	CC	GG	CC
P025	GG	GG	GC	GG	GA	TT	GG	CC
P026	GG	GG	GG	GG	GG	TT	GG	CC
P027	GG	GG	CC	GG	AA	TT	GG	TT
P028			CC	GG	AA	CC	GG	
P029	GG	GG		GG	GG	CC	GG	CC
P030	GG	GG		GG		CC	GG	CC
P031			GG	CC	GG			
P032								CC
P033	GG	GA	GG	GG	GG	TT	GG	TT
P034	GG	AA	CC	GG	AA			CC
P035	GG	AA	CC	GG	AA	CC	GG	CT
P036	GG	GG	GG	GG	GG			
P037	GG	GG	GG	GG	GG	CC	GG	CC
P038								
P039	GG	GA	GG	CC	GG	CC	GG	CC
P040	GG	GA	CC	GG	AA	CC	GG	CC

Table 3.3 – Genotype results for the 40 samples used to assess the cytokine Odyssey assays

3.8.3 Transfer to 384-well plates

All of the assay optimisation, and initial genotyping (see Table 3.3) was performed in 96-well plates. This was done as the temperature gradient function was only available on the 96-well Tetrad heat blocks.

After initial testing of some of the assays in 384-well plates (which I was planning to use for the whole cohort genotyping, for reasons of efficiency) it became apparent that the efficiency of the assays was much reduced. There was a much higher failure rate on the plates, although good results from some samples, indicating that the pre-amplification process and the Odyssey assay had worked (as we were able to obtain clear, well-defined 'melt' peaks from some samples on the plate) suggesting this was more a problem of pre-amplification i.e. there was still insufficient DNA in some samples for the assay to be successfully performed despite preamplification. However in some assays, on plates derived from the same pre-amplified material, there was a loss of peak definition, suggesting a problem with the assay itself.

The GenomiPhi preamplification process requires a minimum quantity of template DNA to be available in order for the reaction to proceed satisfactorily (between 5 and 10ng). We know that the concentration of DNA in the buccal samples is at the lower end of the range of expected yields and that the concentration across samples was varied. We therefore hypothesised that we were not adding a sufficient quantity of starting DNA to the pre-amplification reaction leading to a failure of the pre-amplification. Therefore the amount of template added to the reaction was increased from 1μl to 3 μl and then 5 μl. 5 μl provided better results than either 1μl or 3μl in terms of the number of samples successfully genotyped, and therefore this was adopted as the new standard for the pre-amplification of the whole cohort.

The loss of peak definition experienced within some assays was likely due to inefficiency of the PCR, probably caused by the temperature during the annealing phase being sub-optimal. This may occur due to the change from 96-well plates to 384-well, as they will have different thermodynamics. The assays were re-tested in 384-well plates at a range of temperatures above and below the previously derived

annealing temperatures and it was determined that a drop of 2°C in the annealing temperature was required to compensate for the change in plates.

3.9 Discussion

Assays for the cytokine SNPs outlined in Chapter 2 have been successfully designed for the Odyssey LightTyper system. These will now be used to genotype the complete Glasgow cohort.

Assay design proved to be relatively straightforward. Because the primer and probe requirements were strict it was not possible to utilise primer sequences previously published in the literature. For any given SNP identifying the correct DNA sequence was not always easy. As in this work, most papers identify SNPs by their position in relation to the gene transcription start site. This is not the case in the NCBI SNP database, where ID numbers identify SNPs. It was not always easy to clearly correlate the two together, necessitating careful examination of the supporting literature to ensure the correct sequence was identified.

Primer3 software successfully identified suitable primers for all of the assays except for the three SNPs in IL-6. The first sets of primer pairs identified for the IL-6 assays did not enable the asymmetric amplification of the required target region. The sequence was reanalysed using Primer3 and alternative primer pairs were identified. These primer pairs were successfully incorporated into the IL-6 assays.

None of the assays enabled SNP genotyping of all of the test samples, in keeping with the findings of the initial comparison results using the TNFα –308 assay. If the mean failure rate of 18% is replicated for the whole cohort there would still be sufficient power in the study to detect any significant effect. By repeating the assays I expect the failure rate to drop even further.

In order to achieve high–throughput of the assays the PCRs will need to be performed in 384-well plates. The finding that the failure rate of the assays increased dramatically when work was transferred from 96-well plates to 384-well plates was

disappointing. The change in the required annealing temperature depending upon the plate design being used was possibly predictable. It was unfortunate that optimisation had to be performed in 96-well plates, and that this did not generalise to the 384-well plates (as is often the case). Only a small reduction in the temperature was needed to rectify the problem. However what this did indicate was that some of the assays were more robust than others and that it was likely that I would get a varied rate of successful genotyping across the assays.

Using the GenomiPhi kit pre-amplification from only 1μl of template appeared to be relatively consistent on the first 50 samples tested. However when applied to a larger number of samples the subsequent PCR failure rate increased from the level initially observed. This observation was discussed with the representatives of GE Healthcare (previously Amersham) who agreed the likely cause of failure was an inadequate amount of template DNA. Increasing the amount of template DNA used in the pre-amplification reaction did indeed improve the failure rate. This leads us to conclude that the concentration of DNA in the buccal samples is more variable than anticipated, and in most cases this variability is in the direction of reduced concentrations.

When running the assays on the whole cohort it may become necessary to use 96-well plates rather than 384-wells for PCR if the failure rate climbs too high. As analysis by the LightTyper can only be carried out on 384-well plates this will mean transferring samples from 96-well plates to 384-well plates after the PCR stage. This will increase the chance of sample contamination, but the process could be automated to minimise this. Many of the assays worked well in 384-well plates so the use of 96-well plates will be kept to a minimum.

The assays will now be used to genotype the cytokine SNPs. 5 μl of each sample will be used for each pre-amplification reaction. When running the SNP assays on 384-well plates the annealing temperatures (shown in Table 3.2) will be reduced by 2 degrees.

Chapter 4

Genotyping Results

4.1 Introduction

In previous chapters I have demonstrated the methods whereby I have developed a reliable method for pre-amplifying the buccal-derived patient DNA and then performing assays in order to genotype each sample for each of 11 SNPs across 5 genes.

In this chapter I shall demonstrate how all of the samples were assayed at each SNP position, the results of those assays and demonstrate the genotypic data collected in order to proceed to association analysis with the clinical data.

4.2 Methods

4.2.1 Sample identification and array assignment

The buccal DNA samples had been prepared previously in Glasgow (see Section 2.2.2). 20μl aliquots of each sample were transferred to Southampton for use in this study. A combined total of 1510 samples were received. Each sample was identified by a unique 'P' study number, enabling linkage with the clinical data for the given patient.

Samples were then arranged into 96-well arrays. Each array consisted of 90 consecutive samples together with 6 blank controls (sterile distilled water was used in place of sample). 17 of these arrays were required to accommodate all samples. The position of the control blanks was systematically altered on each array.

4 arrays were then combined onto a single 384-well plate for genotyping. Each 384-well plate therefore contained 360 samples and 24 control blanks.

4.2.2 Pre-amplification

Pre-amplification was performed using GenomiPhi (GE Healthcare) pre-amplification kits (see Chapter 2) in 96-well plates. As discussed in the previous chapter 5μl of each buccal DNA sample was used as template. The standard GenomiPhi protocol was used, with a maximum 18-hour incubation period at 30°C.

4.2.3 Transfer to 384-well plates

After pre-amplification it was necessary to transfer the samples to 384-well plates, ready to perform the Odyssey SNP assays. 0.5μl aliquots of the pre-amplified material were required for each SNP assay. In order to perform all SNP assays at least 11 replicates (plates) would be required.

Samples were transferred between 96-well plates and 384-well plates using a TomTec robot that could accurately dispense a minimum volume of 2μl. The pre-amplified material was therefore diluted 1:4 in sterile distilled water prior to the 384-plating.

Two batches of 15 384-well plate replicates were produced from each set of 4 96-well plates containing pre-amplified samples, allowing enough plates to run each assay twice (once on each batch) on every sample, as well as additional plates to use in the event of assay failure or if further repetition was needed.

4.2.4 SNP assay protocol

Each assay was performed on at least one of every 15 384-well plates in a batch. This meant that each assay was performed at least twice (and for some assays three or four times) on any given sample.

By repeating the assays I was able to ensure the consistency of the results I obtained, and also increase the number of samples successfully genotyped as some samples were more difficult to genotype than others (i.e. a result was not obtained every time the assay was performed).

In the ideal situation I would obtain 11 SNP genotypes for each sample. Unsurprisingly this was not the case for all samples. At this stage any sample that had at least one missing genotype was identified. Each of these samples was then pre-amplified from the original sample for a second time and the process of running the assays repeated. Again each assay was performed at least twice on each sample. This was done to ensure that a failure during the original pre-amplification stage was not to blame for subsequent PCR failure. Also due to the random nature of the pre-

amplification process it is possible that there was poor amplification of the region to be targeted in subsequent PCRs. A second attempt at pre-amplification in the affected samples would hopefully reduce this occurrence.

This process is summarised in the accompanying flow diagram (see Figure 4.1).

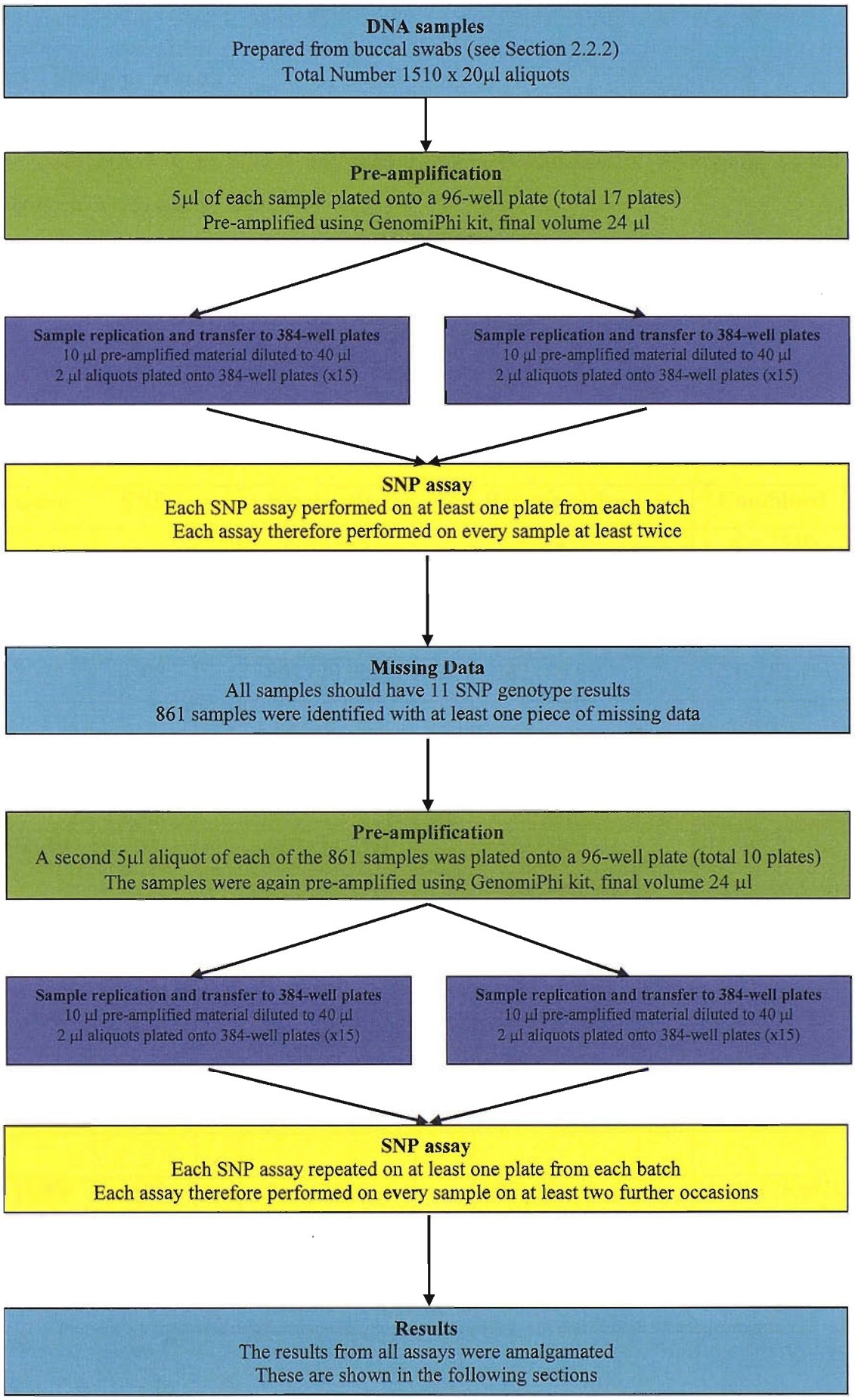
4.2.5 Assigning SNPtypes

After each 384-well plate had been run through the LightTyper the raw data was assessed using the LightTyper software (Roche). This assigns each sample on the plate into a group of similar melt curves, enabling easy and rapid genotyping. The number of groups (and curve characteristics) for each group can be defined by the operator and adjusted according to each assay and plate. For each assay five groups were defined: 1) homozygous (common allele) 2) heterozygous 3) homozygous (rare allele) 4) no result 5) result unclear. Unclear results are those that the software was unable to categorise into any of the other groups, and thus required assigning manually.

The data from the LightTyper software was then also entered into an Excel (Microsoft Corp) macro, designed by Tom Gaunt in the Department of Human Genetics. This enables rapid manual checking of the genotypes assigned by the LightTyper software.

After each running of the assay the results were compared with any results for the same samples from previous running of the assay, and amalgamated to give a final genotype.

Figure 4.1 – Schematic illustrating the protocol utilised to complete the SNP genotyping of all DNA samples



4.3 Initial Results

4.3.1 SNPtype results

The genotyping results after completion of our protocol are shown in Table 4.1, divided according to cohort and also combined. Successful SNPtyping varied between 60.7% and 97.4%.

4.3.2 SNPtyping success by assay

It can be observed from the range of n numbers in table 4.1 that the success of each assay, when applied to the DNA samples, was variable. A summary of the genotyping success rate per assay is shown in table 4.2

Gene	SNP		Prospective		Retrospective		Combined
			n = 1085		n = 425		n = 1510
TNFA	-238		1014 (93.5%)		414 (97.4%)		1428 (94.6%)
	-308		867 (79.9%)		382 (89.9%)		1249 (82.7%)
IL-1A	-889		980 (90.3%)		406 (95.5%)		1386 (91.8%)
IL-1B	-31		810 (74.7%)		379 (89.2%)		1189 (78.7%)
	-511		912 (84.1%)		400 (94.1%)		1312 (86.9%)
	+3953		1004 (92.5%)		404 (95.1%)		1408 (93.2%)
IL-6	-174		659 (60.7%)		324 (76.2%)		983 (65.1%)
	-572		845 (77.9%)		389 (91.5%)		1234 (81.7%)
	-597		961 (88.6%)		405 (95.3%)		1366 (90.5%)
TGFB	-509		805 (74.2%)		351 (82.6%)		1156 (76.6%)
	-800		896 (82.6%)		388 (91.3%)		1284 (85.0%)

Table 4.1 – SNPtyping results by cohort.
Percentage figures are percentage of genotyped samples, not percentage of whole cohort.

Gene	SNP		Prospective		Retrospective		Combined
			n / (%)		n / (%)		n / (%)
TNFA							
	-238	GG	904 (89.2)		367 (88.6)		1271 (89.0)
		GA	99 (9.8)		41 (9.9)		140 (9.8)
		AA	11 (1.0)		6 (1.4)		17 (1.2)
	-308	GG	599 (69.1)		261 (68.3)		860 (68.9)
		GA	204 (23.5)		99 (25.9)		303 (24.3)
		AA	64 (7.4)		22 (5.8)		86 (6.9)
IL-1A							
	-889	CC	555 (56.6)		204 (50.2)		759 (54.8)
		CT	313 (31.9)		163 (40.1)		476 (34.3)
		TT	112 (11.4)		39 (9.6)		151 (10.9)
IL-1B							
	-31	CC	152 (18.8)		67 (17.7)		219 (18.4)
		CT	227 (28.0)		141 (37.2)		368 (31.0)
		TT	431 (53.2)		171 (45.1)		602 (50.6)
	-511	TT	172 (18.9)		78 (19.5)		250 (19.1)
		TC	289 (31.7)		143 (35.8)		432 (32.9)
		CC	451 (49.5)		179 (44.8)		630 (48.0)
	+3953	CC	623 (62.1)		249 (61.6)		872 (61.9)
		CT	308 (30.7)		132 (32.7)		440 (31.3)
		TT	73 (7.3)		23 (5.7)		96 (6.8)
IL-6							
	-174	GG	284 (43.1)		132 (40.7)		416 (42.3)
		GC	213 (32.3)		120 (37.0)		333 (33.9)
		CC	162 (24.6)		72 (22.2)		234 (23.8)
	-572	GG	664 (78.6)		292 (75.1)		956 (77.5)
		GC	165 (19.5)		86 (22.1)		251 (20.3)
		CC	16 (1.9)		11 (2.8)		27 (2.2)
	-597	GG	437 (45.5)		185 (45.7)		622 (45.5)
		GA	358 (37.3)		163 (40.2)		521 (38.1)
		AA	166 (17.3)		57 (14.1)		223 (16.3)
TGFB							
	-509	CC	490 (60.9)		184 (52.4)		674 (58.3)
		CT	199 (24.7)		123 (35.0)		322 (27.9)
		TT	116 (14.4)		44 (12.5)		160 (13.8)
	-800	GG	773 (86.3)		343 (88.4)		1116 (86.9)
		GA	104 (11.6)		40 (10.3)		144 (11.2)
		AA	19 (2.1)		5 (1.3)		24 (1.9)

Table 4.2 – Quantification of SNPtyping success by individual SNP assay. Figures show the number of samples successfully genotyped for the given SNP, broken down by cohort and combined. Highest combined success rate was 94.6% (TNFα -238). Lowest rate was 65.1% (IL6 -174).

4.3.3 Quantity of samples with missing data points

When the results of each individual assay are combined to look at the profile of results per sample we observe that some samples provide a more reliable template than others (Table 4.3). This presumably reflects the range of DNA concentrations within the samples, with samples with the most missing data being those with the lowest concentration of 'viable' DNA.

Missing Data / Sample	Prospective Cohort	Retrospective Cohort	Combined
	n = 1085	n = 425	n = 1510
0	548 (50.5%)	287 (67.5%)	835 (55.3%)
1	160 (14.7%)	67 (15.8%)	227 (15.0%)
2	86 (7.9%)	20 (4.7%)	106 (7.0%)
3	41 (3.8%)	9 (2.1%)	50 (3.3%)
4	40 (3.7%)	10 (2.4%)	50 (3.3%)
5	43 (4.0%)	2 (0.5%)	45 (3.0%)
6	38 (3.5%)	5 (1.2%)	43 (2.8%)
7	30 (2.8%)	6 (1.4%)	36 (2.4%)
8	37 (3.4%)	5 (1.2%)	42 (2.8%)
9	21 (1.9%)	5 (1.2%)	26 (1.7%)
10	23 (2.1%)	7 (1.6%)	30 (2.0%)
11	18 (1.7%)	2 (0.5%)	20 (1.3%)

Table 4.3 – Completeness of data, illustrated by categorization of samples according to the amount of data points missing from the maximum of 11.

4.3.4 Hardy-Weinberg Equilibrium

Conformation to the Hardy-Weinberg Equilibrium (see Discussion) was assessed for each SNP assay – see Tables 4.4 (prospective cohort) & 4.5 (retrospective cohort). The general trend is for the degree of deviation from the equilibrium to increase as samples with increasing amounts of missing data are included in the test. This suggests that the data obtained from samples with a high amount of missing data was possibly not reliable (although see Discussion).

SNP Assay Missing Data	IL-1 -889	IL1 -31	IL1 -511	IL1 +3953	TGF -509	TGF -800	IL6 -174	IL6 -572	IL6 -597	TNF -238	TNF-308
0	3.32	30.48	20.10	1.95	66.97	11.00	46.20	1.60	16.00	5.53	9.48
1	9.01	59.03	38.99	3.22	81.12	23.02	57.98	1.34	18.07	7.31	19.39
2	12.03	75.51	44.85	2.73	94.03	33.01	61.43	2.03	16.83	19.54	27.47
3	14.37	84.96	54.05	4.86	103.58	30.76	64.06	2.25	23.98	18.28	32.64
4	18.37	91.92	60.53	4.71	100.29	31.98	68.22	2.90	25.42	14.83	34.06
5	23.30	99.89	69.82	6.99	106.10	32.38	68.62	2.83	28.59	14.39	36.39
6	27.05	103.07	74.92	9.21	108.40	33.62	70.53	2.35	32.54	17.85	43.35
7	31.27	104.49	77.91	10.40	106.97	36.66	71.28	2.39	33.75	18.23	48.89
8	34.97	105.20	81.63	12.51	109.29	37.22	71.28	2.28	33.66	17.38	49.95
9	37.78	106.27	81.63	13.58	109.60	37.36	71.28	2.28	34.92	16.77	49.95
10	37.94	106.27	81.63	15.21	109.60	37.43	72.04	2.28	35.33	17.08	49.95

Table 4.4 - χ^2 values assessing conformation to Hardy-Weinberg equilibrium for the prospective cohort (n=1085). Missing data groups contain samples with up to that amount of missing data (e.g. the analysis of the '2' group contains samples with 2 or less pieces of missing data). Highlighting indicates significant deviation ($p<0.05$).

SNP Assay Missing Data	IL-1 -889	IL1 -31	IL1 -511	IL1 +3953	TGF -509	TGF -800	IL6 -174	IL6 -572	IL6 -597	TNF -238	TNF-308
0	0.02	2.49	5.03	0.17	3.12	9.90	10.56	0.87	2.13	5.63	0.51
1	0.00	9.79	13.52	0.24	8.38	8.62	14.96	2.00	3.90	5.76	5.75
2	0.01	11.32	15.78	0.22	8.86	8.71	16.72	2.80	3.00	4.91	5.95
3	0.01	11.72	17.23	0.38	9.15	8.96	17.28	2.35	3.43	4.72	7.41
4	0.15	12.12	18.47	0.60	9.59	8.54	17.58	2.15	4.30	8.34	8.45
5	0.16	12.12	18.97	0.62	9.74	7.96	17.58	2.18	4.08	8.41	8.16
6	0.12	12.68	21.15	0.74	9.74	8.11	17.58	2.18	4.07	8.57	8.16
7	0.28	13.88	21.41	0.70	9.74	8.15	17.58	2.18	3.75	8.80	8.32
8	0.35	14.46	22.34	0.61	9.74	8.15	17.58	2.18	3.75	8.36	8.40
9	0.40	14.46	22.34	0.66	9.74	8.15	17.58	2.21	4.31	12.36	8.40
10	0.59	14.46	22.34	0.97	9.74	8.15	17.58	2.21	4.53	12.46	8.40

Table 4.5 - χ^2 values assessing conformation to Hardy-Weinberg equilibrium for the retrospective cohort (n=425). Missing data groups contain samples with up to that amount of missing data (e.g. the analysis of the '2' group contains samples with 2 or less pieces of missing data). Highlighting indicates significant deviation ($p<0.05$).

4.4 Discussion of Initial Results

The genotyping results from the initial analysis of my SNP assays, performed on the complete set of prospective and retrospective cohorts, are illustrated in the section above. Based upon my preliminary data I had expected a success rate (proportion of samples successfully genotyped) of around 82% (see section 3.9). This was matched or exceeded in eight of eleven assays. The remaining three assays (IL6 –174, IL1B -31 and TGFB -509) performed consistently worse than the other assays, despite multiple repetitions. Although careful optimisation of these assays was undertaken with the given primers (see Section 3.8.1) I was unable to improve their efficiency. The reasons for this are unclear as the other SNP assays from similar genomic regions performed adequately. If the pre-amplification process was not efficiently amplifying the required region then all of the SNPs from that gene would be affected, as the SNPs are relatively close together. The amplicon for the TGFB -509 SNP is relatively GC-rich (approaching 60%), which may explain the difficulties with this assay. The IL6 –174 and IL1B -31 assays have lower amplicon GC-contents (under 50%) so this would not explain the relative failure in these assays. It may simply be that the primer sets chosen for these assays are sub-optimal and a redesign of the affected assays with a different primer pair may yield better results. However the assays worked well when applied to 'control' blood derived DNA and in the original testing on the pre-amplified buccal DNA.

Of more concern was the large deviation from the Hardy-Weinberg equilibrium (HWE) observed in both cohorts. The HWE was developed from initial work by Castle (1903), and independently developed further by Hardy (1908) and Weinberg (1908) (see Wittke-Thompson *et al.*, 2005 for brief summary) and states that gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. Where the HWE applies then allele frequency data can be used to derive population expected genotypic proportions. In this circumstance, if a SNP allele (*A*) has a frequency of p then the alternate allele (*a*) will have a frequency q (where $p+q=1$) and the expected genotype proportions will be p^2 for the AA genotype, $2pq$ for the Aa genotype and q^2 for the aa genotype. The HWE does not apply when the frequencies of alleles are not constant (if there is ongoing

mutation or natural selection is occurring, for instance) or if random mating is not occurring within the population. Provided the HWE is applicable it can be used to assess the possibility of genotyping error in control populations (in case-control studies, for example) (Leal, 2005) as this is the most likely explanation for deviation from HWE in these populations. The simplest way of assessing conformation with the HWE is to perform a χ^2-test, comparing observed with expected genotypes (the procedure undertaken in section 4.3.4).

Although our cohorts are 'case' data, as opposed to 'control', and therefore may not be expected to conform to the HWE, it is important to assess the reason for the large observed deviation from the HWE, mainly to exclude the possibility of genotyping error. In order to do this, each step of the process leading to our final genotyping results must be examined in order to assess the potential impact on the deviation from HWE that has been observed for all bar one of the SNP assays in the prospective cohort (as well as eight out of eleven SNPs in the retrospective cohort).

The two cohorts have been selected (all of the patients included have suffered a head injury), and therefore may not resemble the general population (which may be assumed to fulfil the criteria of a 'randomly mating population'). Results from previous studies have found the SNPs under study to fulfil HWE in control populations (see snp500.nci.nih.gov; also McCulley *et al.*, 2004; Zienolddiny *et al.*, 2004; Hefler *et al.*, 2005; Shin *et al.*, 2005; Seifart *et al.*, 2005 for examples). The results from our cohorts may therefore not be expected to fulfil HWE, simply due to population stratification. However we must consider whether the fact of suffering a head injury would select a population whose cytokine SNP-types were no longer in HWE, assuming that HWE applies in the general population. This may occur if the SNPs I am typing are risk factors for suffering a head injury, or are in linkage disequilibrium with a risk locus. There is no evidence to suggest an association between cytokines, or cytokine SNPs, and risk of sustaining a TBI, and no biological reason to suggest this would be the case. However the prospective cohort was identified at a tertiary referral centre, so any patients who died either at scene or prior to transfer could not be included. Should any of the cytokine SNP alleles be involved in predisposing to very early death after TBI then they will be

underrepresented in the prospective cohort. It is therefore possible that the cytokine SNP alleles I have examined could deviate from the HWE in the prospective cohort. The retrospective cohort is highly selected, as only patients who had a GOS of 3 or greater at 6-months and who survived to long-term follow-up were considered for inclusion in the cohort. Any deviation from HWE in this cohort may thus be due to population selection 'bias'.

In summary, population selection effects would be unlikely to explain a large deviation from HWE observed in the prospective cohort. There may be some influence in the both cohorts, but this would not explain the large deviations observed in many of the assays.

We must next consider the possibility of error introduced during the pre-amplification process, which would lead to subsequent genotyping errors and an apparent deviation from HWE. The GenomiPhi pre-amplification kit should achieve whole genome amplification by the binding of random hexamers throughout the genome. Errors may be introduced if the DNA strands containing the alleles of interest are not amplified equally (leading to the possibility of heterozygous samples appearing to be homozygous due to preferential amplification of one strand) or if errors occur during primer extension by the DNA polymerase. Evidence suggests that the Phi29 DNA polymerase has a far lower error rate than *Taq* DNA polymerase (Estaban *et al.*, 1993), effectively excluding polymerase errors in the pre-amplification step as a cause of apparent deviation from HWE.

Leviel *et al.* (2004) demonstrated that genotyping from Phi29 amplified DNA from buccal cells was as accurate as genotyping from lymphocytes. Holbrook *et al.* (2005) found that in a small series of samples, of varying DNA concentration and quality, pre-amplification with GenomiPhi produced material with no detectable differences from the starting material. Bergen *et al.* (2005) found a reduction in concordance when comparing GenomiPhi amplified material with genomic. The majority of the discordance was due to a genomic heterozygote being scored as an amplified homozygote. When examining the genotype results in our samples it is apparent that this may also be occurring in our samples. When comparing observed to expected genotypes there is an under-representation of heterozygotes, with a

consequent over-representation of homozygotes. The pre-amplification step may be contributing to genotyping error, and consequently to a deviation from the HWE, but the degree of error is difficult to gauge (as the pre-amplified samples cannot be compared to the original buccal samples due to insufficient material). If the discordance is similar to that observed by Bergen *et al.* (2005) then it would affect less than 1% of cases, and would therefore not be a significant contributor to a large deviation from HWE.

Errors within the PCR assays, and subsequent result calling, must also be considered. PCR errors could occur if both alleles are not equally amplified or if primer pairs bind to other regions in the genome (creating a so-called pseudo-SNP). Observation from the initial design and optimisation stage does not support unequal amplification of alleles at the PCR stage. Pseudo-SNPs should not be 'called' as the primer and probes system used for the LightTyper system should avoid this. Multiple repetition of the assays should also help to exclude any 'miscalls' (such as those due to sample contamination).

The LightTyper system relies upon 'peak'-identification to be able to identify and call genotypes. In the ideal situation all of the samples would have had their DNA samples equalised and thus peak height for the two homozygous groups would be equal and the peaks for the heterozygote group would be half the height of the homozygotes. The samples in this study were observed to exhibit varying peak heights, and in the heterozygote group the two peaks were often of different heights. It is therefore possible that some heterozygote samples have been incorrectly called as homozygotes, leading to the large deviation from HWE.

In summary there are a number of reasons to explain why such a high degree of variation from the HWE has been observed in these samples. The most likely are that population selection has occurred and the variation is 'real' or, more likely, genotyping error (or at least error in the calling of results) has led to an apparent deviation from the HWE.

4.4.1 Reassessment of results

To assess the impact of possible genotype calling error on our initial results it was decided to review the genotyping data with the purpose of determining firstly whether error had occurred and secondly, if error had occurred, whether this was due to error in the assay itself or due to 'calling' errors. All of the results for each assay were systematically re-examined. Bearing in mind that the HWE results suggested that I may have scored too many samples as homozgotes when, in fact, they were heterozygotes and also that peak heights in the heterozygote group were not always equal it was possible that samples which seemingly had a single small peak (and were originally scored as homozygotes) were actually heterozygotes and that the second, smaller, peak had been lost within the baseline noise.

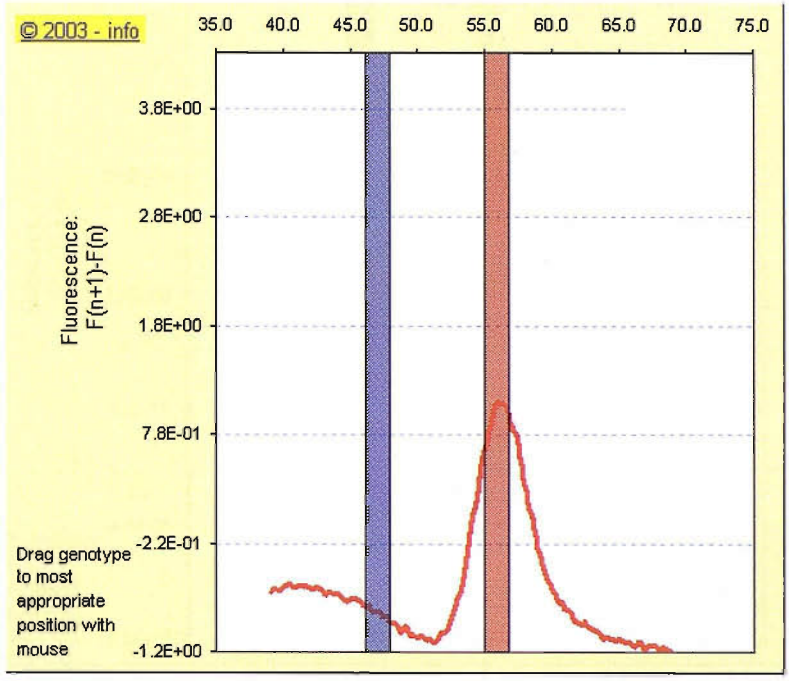
During the reassessment it became apparent that for a number of samples there had been a potential calling error. As predicted some samples had been called as homozygotes on the basis of a definite small single peak, but one that was only just above the baseline 'noise'. For these samples it could not therefore be determined if they were true homozygotes or heterozygotes with a 'missing' (*i.e.* lost within the baseline noise) second peak. Some samples had also been initially called as homozygotes when in fact two peaks were visible, although one was much taller than the other. Due to the nature of the DNA and its preparation it was felt, in retrospect that this was incorrect and the sample should be called as a heterozygote. Examples of clear calls and samples where the correct genotype was unclear are shown in Figure 4.2.

All results were reviewed on the basis of these findings and a revised set of genotypes produced. For those samples where the SNPtype could not be definitively determined due to a low peak height it was decided to reassign these as 'no-calls'. Samples with two definite peaks were reassigned as heterozygotes, whatever the height of the two peaks. Genotypes were reassigned by re-examining the original LightTyper data for each assay run. The melt curves obtained for each sample were reloaded into the LightTyper software and stratified into one of the five groups as had originally occurred (see section 4.2.5). This data was then loaded into the Excel macro and each result manually checked or, for those samples that the LightTyper

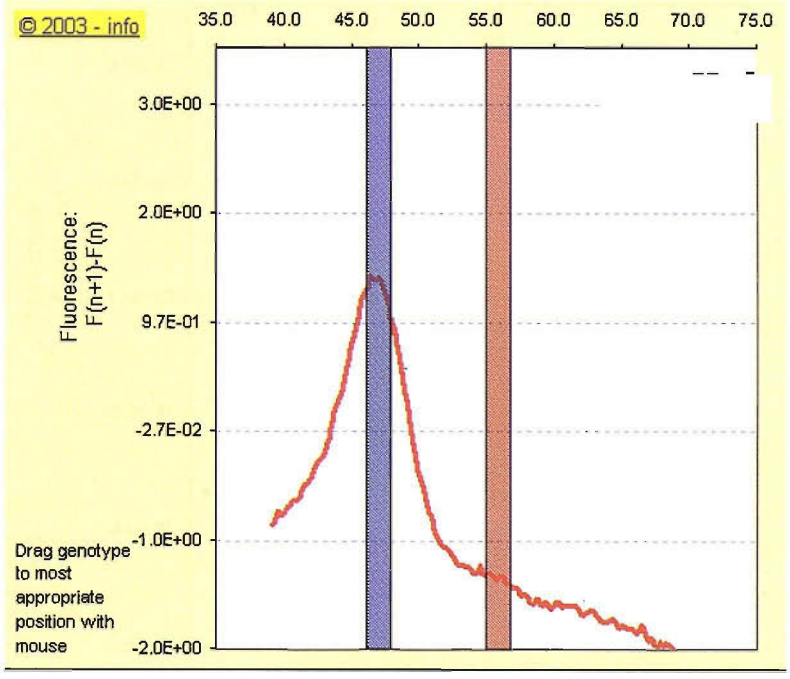
software had been unable to determine, manually assigned, using the new criteria determined above (examples of which are shown in Figure 4.2). On this basis a 'revised' set of SNPtypes was produced for each sample and each assay. These are shown in section 4.5.

In an ideal situation all of the samples would have been diluted to the same concentration after the pre-amplification stage and the peak heights for each sample would have been similar. This would have enabled me to set stricter criteria as to the degree of change in fluorescence that constituted a 'peak'. Those samples which fell into the groups illustrated in parts E and F in Figure 4.2 would then have been easier to categorise. This was not the case and therefore a slightly less robust system had to be utilised whereby the peak height was judged against the baseline to assess whether the 'peak' in question should be truly assessed as a peak.

Figure 4.2 – Examples of how genotype calling was performed, based upon melt curve analysis

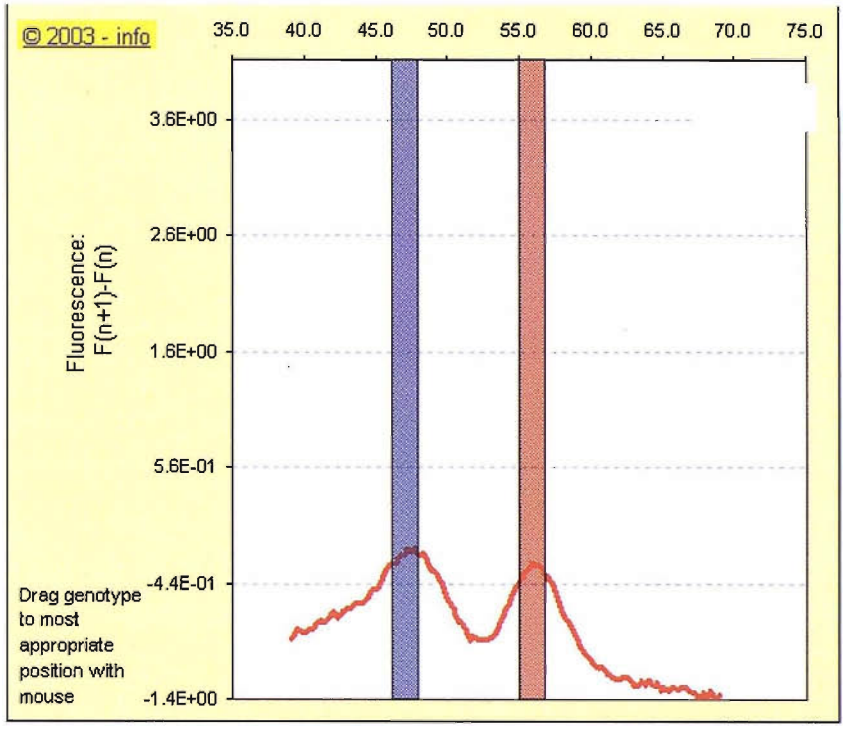


A – Homozygote for the allele that the probe is designed to bind to. There is a clear single peak at the higher melt point, indicated by the red band.

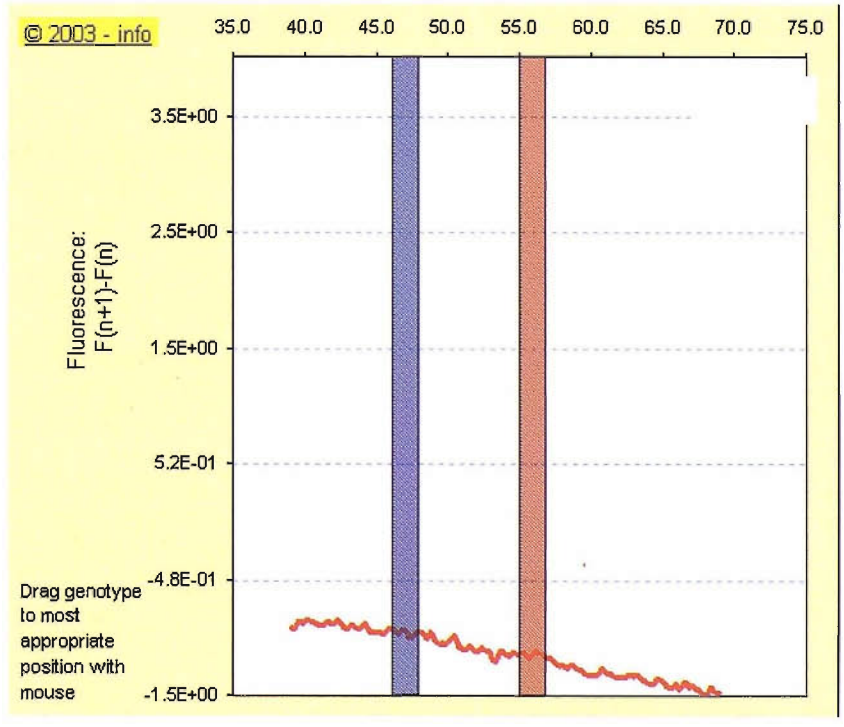


B – Homozygote for the alternative allele. As the probe is not as tightly bound the melt point is lower, and indicated by the blue band

Figure 4.2 – Continued

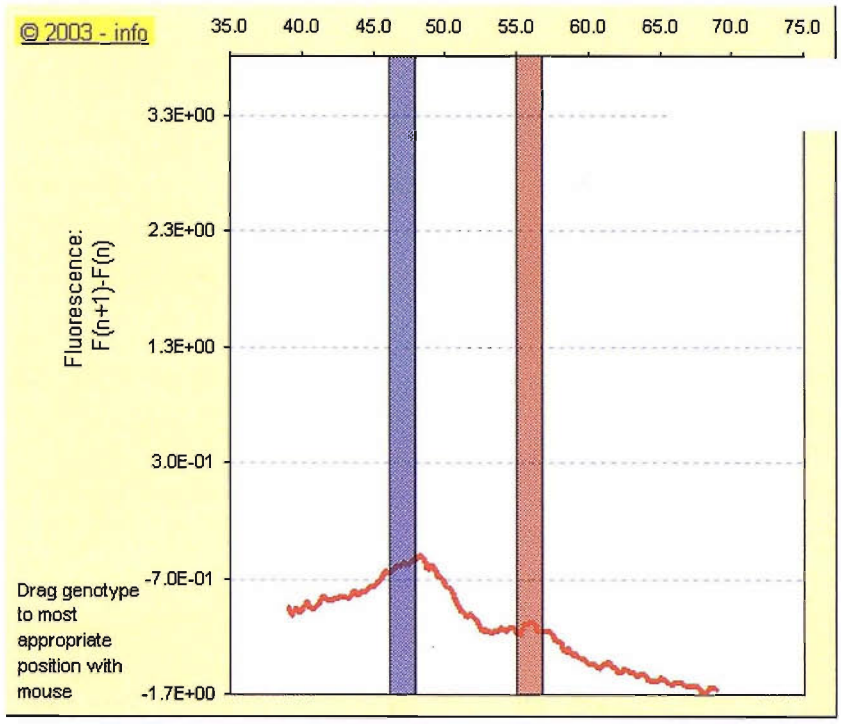


C – Heterozygote, with two clear peaks at each of the melting points

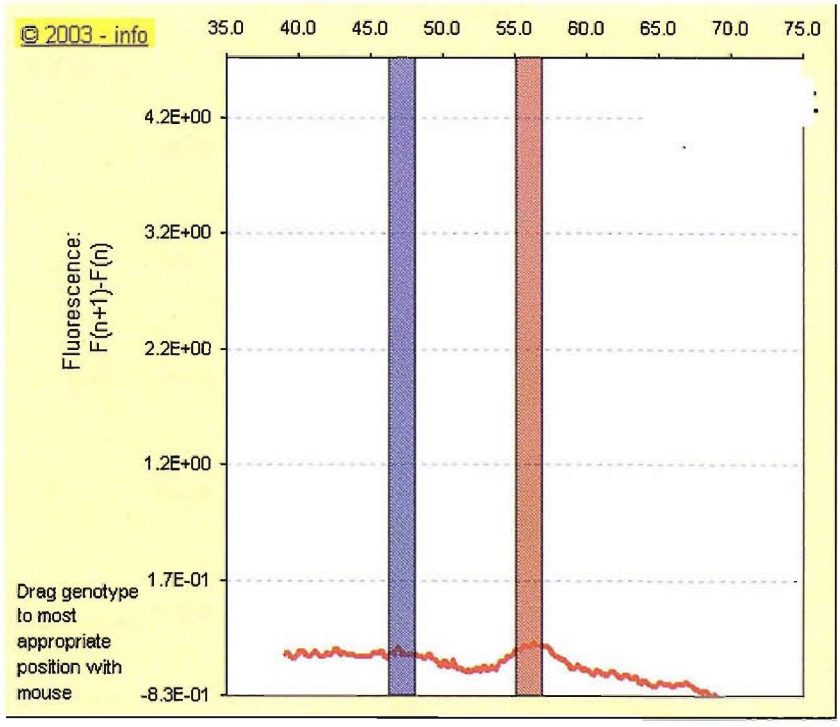


D – No call. No peaks visible, indicating either a blank control or a sample 'no-call'.

Figure 4.2 – Continued

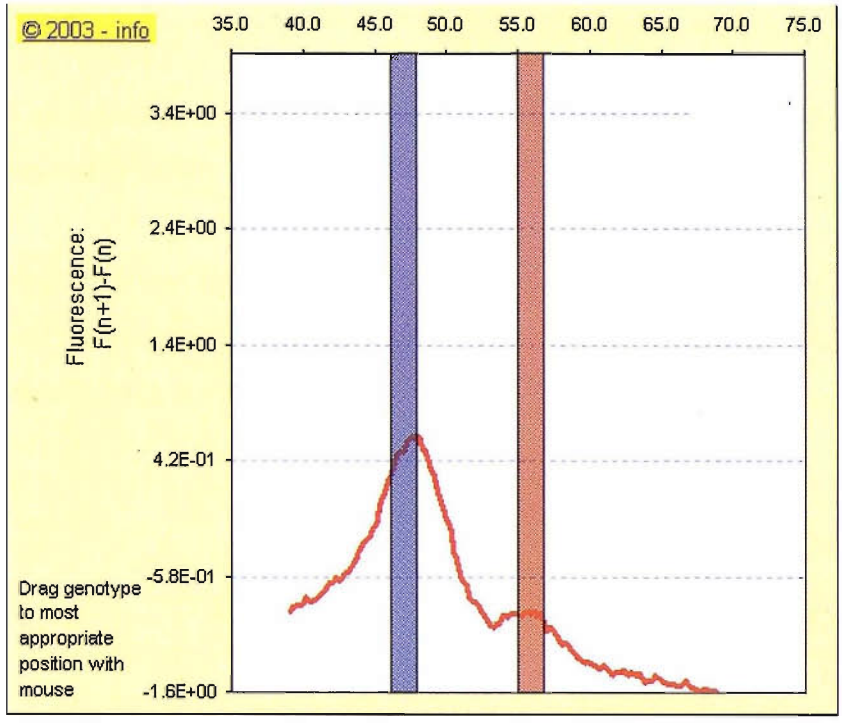


E – Heterozygote, illustrating that the peak heights were often not equal. In cases where the signal was less strong then the second peak would be lost within the baseline noise.

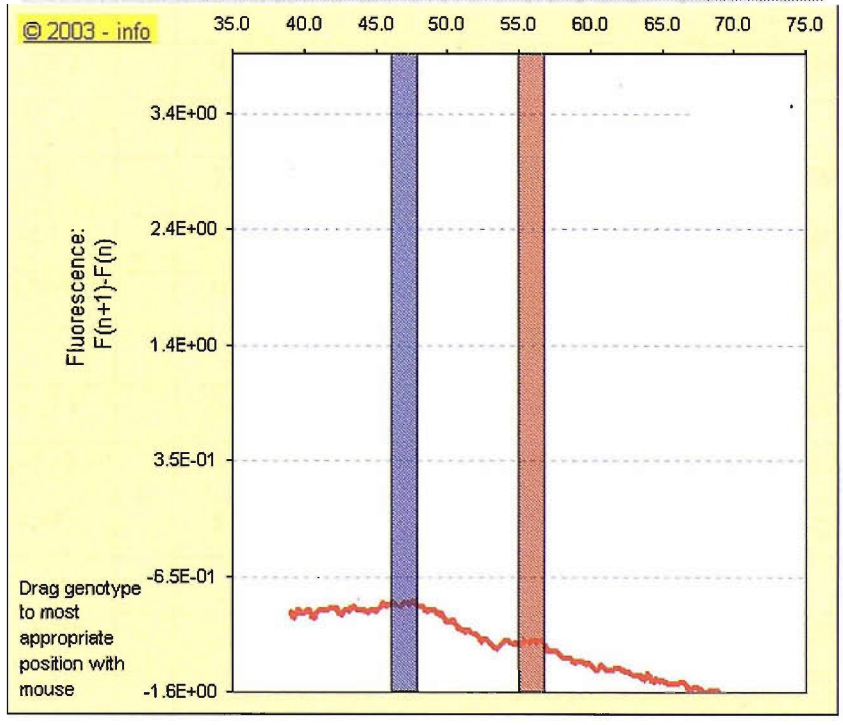


F – Single small peak visible. This was called as a homozygote in the original analysis. The presence of a second small peak cannot be excluded, so was called as a 'no-call' in the revised set of results.

Figure 4.2 – Continued



G – Heterozygote. In the initial analysis samples similar to this were called as homozygotes as the second peaks were so small. In retrospect this was an incorrect assumption, and was resolved during the revision of results



H– No-call. Although there are changes in fluorescence at the correct temperatures for this to be a heterozygote sample there are no actual peaks. These samples were called as 'no-calls' in the revision.

4.5 Revised Results

4.5.1 SNPtype results

The revised genotyping results after completion of review are shown in Table 4.6, divided according to cohort and also combined.

4.5.2 SNPtyping success by assay

A summary of the genotyping success rate per assay is shown in table 4.7, in order to compare with table 4.2.

Gene	SNP		Prospective		Retrospective		Combined
			n = 1085		n = 425		n = 1510
TNFA	-238		985 (90.8%)		411 (96.7%)		1396 (92.5%)
	-308		936 (86.3%)		391 (92.0%)		1327 (87.9%)
IL-1A	-889		939 (86.5%)		403 (94.8%)		1342 (88.9%)
IL-1B	-31		733 (67.6%)		363 (85.4%)		1096 (72.6%)
	-511		875 (80.6%)		392 (92.2%)		1267 (83.9%)
	+3953		976 (90.0%)		401 (94.4%)		1377 (91.2%)
IL-6	-174		601 (55.4%)		290 (68.2%)		891 (59.0%)
	-572		813 (74.9%)		380 (89.4%)		1193 (79.0%)
	-597		847 (78.1%)		393 (92.5%)		1240 (82.1%)
TGFB	-509		732 (67.5%)		327 (76.9%)		1059 (70.1%)
	-800		858 (79.1%)		373 (87.8%)		1231 (81.5%)

Table 4.7 – Quantification of SNPtyping success by individual SNP assay, after revision. Figures show the number of samples successfully genotyped for the given SNP, broken down by cohort and combined. Highest combined success rate was 92.5% (TNFα -238). Lowest rate was 59.0% (IL6 -174).

Gene	SNP		Prospective		Retrospective		Combined
			n / (%)		n / (%)		n / (%)
TNFA							
	-238	GG	872 (88.5)		361 (87.8)		1233 (88.3)
		GA	108 (11.0)		47 (11.4)		155 (11.1)
		AA	5 (0.5)		3 (0.7)		8 (0.6)
	-308	GG	609 (65.1)		256 (65.5)		865 (65.2)
		GA	270 (28.8)		117 (30.0)		387 (29.2)
		AA	57 (6.1)		18 (4.6)		75 (5.7)
IL-1A							
	-889	CC	484 (51.5)		197 (48.9)		681 (50.7)
		CT	363 (38.7)		173 (42.9)		536 (39.9)
		TT	92 (9.8)		33 (8.2)		125 (9.3)
IL-1B							
	-31	CC	114 (15.6)		57 (15.7)		171 (15.6)
		CT	284 (38.7)		155 (42.7)		439 (40.1)
		TT	335 (45.7)		151 (41.6)		486 (44.3)
	-511	TT	152 (17.4)		68 (17.3)		220 (17.4)
		TC	322 (36.8)		155 (39.5)		477 (37.6)
		CC	401 (45.8)		169 (43.1)		570 (45.0)
	+3953	CC	580 (59.4)		246 (61.3)		826 (60.0)
		CT	334 (34.2)		133 (33.1)		467 (33.9)
		TT	62 (6.4)		22 (5.5)		84 (6.1)
IL-6							
	-174	GG	221 (36.8)		110 (37.9)		331 (37.1)
		GC	254 (42.3)		127 (43.8)		381 (42.8)
		CC	126 (21.0)		53 (18.3)		179 (20.1)
	-572	GG	477 (58.7)		245 (64.5)		722 (60.5)
		GC	324 (39.9)		126 (33.2)		450 (37.7)
		CC	12 (1.5)		9 (2.4)		21 (1.8)
	-597	GG	355 (41.9)		160 (40.7)		515 (41.5)
		GA	352 (41.6)		182 (46.3)		534 (43.1)
		AA	140 (16.5)		51 (13.0)		191 (15.4)
TGFB							
	-509	CC	407 (55.6)		155 (47.4)		562 (53.1)
		CT	241 (32.9)		132 (40.4)		373 (35.2)
		TT	84 (11.5)		40 (12.2)		124 (11.7)
	-800	GG	713 (83.1)		322 (86.3)		1035 (84.1)
		GA	133 (15.5)		46 (12.3)		179 (14.5)
		AA	12 (1.4)		5 (1.3)		17 (1.4)

Table 4.6 – Revised SNPtyping results by cohort.
Percentage figures are percentage of genotyped samples, not percentage of whole cohort.

4.5.3 Quantity of samples with missing data points

It can be seen from the results in sections 4.5.1 and 4.5.2 that during the revision of result calling there has been an increase in the number of samples not genotyped. This obviously affects the completeness of the genotyping data – see table 4.8.

Missing Data / Sample	Prospective Cohort	Retrospective Cohort	Combined
	n = 1085	n = 425	n = 1510
0	436 (40.2%)	243 (57.2%)	679 (45.0%)
1	196 (18.1%)	79 (18.6%)	275 (18.2%)
2	114 (10.5%)	39 (9.2%)	153 (10.1%)
3	55 (5.1%)	14 (3.3%)	69 (4.6%)
4	38 (3.5%)	9 (2.1%)	47 (3.1%)
5	44 (4.1%)	8 (1.9%)	52 (3.4%)
6	38 (3.5%)	6 (1.4%)	44 (2.9%)
7	42 (3.9%)	5 (1.2%)	47 (3.1%)
8	36 (3.3%)	7 (1.6%)	43 (2.8%)
9	24 (2.2%)	5 (1.2%)	29 (1.9%)
10	29 (2.7%)	6 (1.4%)	35 (2.3%)
11	33 (3.0%)	4 (0.9%)	37 (2.5%)

Table 4.8 – Completeness of revised data, illustrated by categorization of samples according to the amount of data points missing from the maximum of 11.

4.5.4 Hardy-Weinberg Equilibrium

χ^2 values, testing for conformation to the Hardy-Weinberg equilibrium, for the revised genotyping data are shown in tables 4.9 (prospective cohort) and 4.10 (retrospective cohort).

SNP Assay Missing Data	IL-1 -889	IL1 -31	IL1 -511	IL1 +3953	TGF -509	TGF -800	IL6 -174	IL6 -572	IL6 -597	TNF -238	TNF-308
0	0.38	2.33	3.47	0.15	12.16	1.16	2.77	29.18	8.73	0.11	0.09
1	0.17	5.27	10.18	0.01	15.72	2.85	4.33	33.00	5.46	2.03	1.04
2	0.04	8.14	19.29	0.03	19.10	4.42	6.28	29.19	4.57	1.01	2.62
3	0.10	10.60	20.12	0.04	20.52	3.60	7.35	27.08	7.07	0.59	4.21
4	0.25	12.73	25.98	0.13	21.84	3.61	8.34	26.55	10.39	0.52	3.87
5	0.83	13.50	30.89	0.06	23.75	3.80	9.16	26.59	9.74	1.06	6.30
6	1.28	14.73	34.49	0.26	25.02	3.89	9.98	27.14	10.18	1.05	4.87
7	2.17	15.50	33.95	0.93	23.39	3.80	9.98	27.51	10.06	0.94	7.57
8	3.17	15.66	33.93	1.97	23.53	3.90	10.31	27.69	10.61	0.77	12.41
9	3.44	15.97	34.09	2.21	24.33	3.90	10.31	27.69	10.16	0.77	12.47
10	3.80	15.97	34.70	2.17	24.33	3.92	10.64	27.69	10.55	0.69	12.47

Table 4.9 – revised χ^2 values assessing conformation to Hardy-Weinberg equilibrium for the prospective cohort (n=1085). Missing data groups contain samples with up to that amount of missing data (e.g. the analysis of the '2' group contains samples with 2 or less pieces of missing data). Highlighting indicates significant deviation ($p<0.05$).

SNP Assay Missing Data	IL-1 -889	IL1 -31	IL1 -511	IL1 +3953	TGF -509	TGF -800	IL6 -174	IL6 -572	IL6 -597	TNF -238	TNF-308
0	0.67	0.27	0.95	1.60	0.32	2.43	1.04	6.38	0.02	0.98	0.26
1	1.10	0.94	3.54	0.17	0.44	6.02	1.43	5.71	0.00	0.09	0.01
2	1.34	2.07	5.30	0.02	1.03	5.23	1.94	1.97	0.00	0.14	0.19
3	1.31	1.71	6.15	0.03	1.26	5.52	2.18	2.23	0.02	0.14	0.29
4	1.01	1.59	7.30	0.04	1.59	5.30	2.29	2.37	0.00	0.18	0.24
5	0.96	1.66	7.48	0.14	2.03	5.36	2.29	2.30	0.00	0.21	0.58
6	0.74	2.15	8.25	0.13	2.03	4.61	2.29	2.30	0.01	0.17	0.90
7	0.70	2.24	8.37	0.17	2.03	4.67	2.29	2.27	0.00	0.13	0.92
8	0.45	2.50	8.99	0.12	2.03	4.67	2.29	2.27	0.02	0.11	0.95
9	0.48	2.50	8.99	0.30	2.03	4.67	2.29	2.40	0.00	1.09	0.95
10	0.28	2.50	8.99	0.30	2.03	4.67	2.29	2.40	0.00	1.11	0.95

Table 4.10 – revised χ^2 values assessing conformation to Hardy-Weinberg equilibrium for the retrospective cohort (n=425). Missing data groups contain samples with up to that amount of missing data (e.g. the analysis of the '2' group contains samples with 2 or less pieces of missing data). Highlighting indicates significant deviation ($p<0.05$).

4.6 Discussion of revised results

After reviewing the genotype data there was a rise in the number of samples with missing data. This is unsurprising, as the review process re-classified some of the original homozygotes as 'no-calls'. This is because a number of samples only had very small single peaks that were barely above baseline, meaning that they could not be clearly identified as heterozygotes. These samples were therefore not called as results. The rise in heterozygotes in the revised results set occurred because a number of samples originally classified as homozygotes actually proved to have small second peaks that had originally been overlooked (see Figure 4.2).

The genotype and allele frequencies observed in the two cohorts are similar. This is interesting as one might expect to see a difference as the retrospective group has been selected by having survived beyond six months post injury and have a GOS of at least 3. If any single genotype or allele had a large impact on survival (or predisposed to a very poor outcome at 6-months) then these would be seen at a lower frequency in the retrospective cohort. The results therefore suggest that none of the SNPs under study have a critically significant effect on survival between 6-months and 'long-term'.

The genotype and allele frequencies observed are generally similar to those observed in other cohorts. Table 4.11 can be used to compare genotype frequencies from the cohorts under study with those used in the SNP500 cancer database. Although some differences may be noted it is also interesting to see how genotypes vary according to the ethnic mix of the cohort under study. Care should be taken when comparing different populations as considerable variation in genotype frequencies may occur between different ethnic groups. In all cases the results from the Glasgow cohorts lie within the ranges demonstrated by the SNP500 database. This again supports the results I have demonstrated to be true results and not significantly skewed by any genotyping error.

Gene	SNP		Pro	Retro	All	Canc	Cauc1	Cont	Cauc 2
			%	%	%	%	%	%	%
TNFA									
	-238	GG	88.5	87.8	88.3	81.4	93.5		
		GA	11.0	11.4	11.1	18.6	6.5		
		AA	0.5	0.7	0.6	0	0		
	-308	GG	65.1	65.5	65.2	84.2	67.7	77.8	76.6
		GA	28.8	30.0	29.2	15.8	32.3	21.1	21.9
		AA	6.1	4.6	5.7	0	0	1.1	1.6
IL-1A									
	-889	CC	51.5	48.9	50.7	50.5	38.7		
		CT	38.7	42.9	39.9	41.4	51.6		
		TT	9.8	8.2	9.3	8.1	9.7		
IL-1B									
	-31	CC	15.6	15.7	15.6	28.4	9.7	37.0	12.9
		CT	38.7	42.7	40.1	44.1	32.3	40.1	35.5
		TT	45.7	41.6	44.3	27.5	58.1	23.0	51.6
	-511	TT	17.4	17.3	17.4	26.7	9.7		
		TC	36.8	39.5	37.6	42.6	32.3		
		CC	45.8	43.1	45.0	30.7	58.1		
	+3953	CC	59.4	61.3	60.0	70.3	54.8		
		CT	34.2	33.1	33.9	21.8	25.8		
		TT	6.4	5.5	6.1	7.9	19.4		
IL-6									
	-174	GG	36.8	37.9	37.1	66.7	25.8	76.6	37.1
		GC	42.3	43.8	42.8	22.5	48.4	19.0	50.0
		CC	21.0	18.3	20.1	10.8	25.8	4.4	12.9
	-572	GG	58.7	64.5	60.5	64.4	90.0		
		GC	39.9	33.2	37.7	19.8	10.0		
		CC	1.5	2.4	1.8	15.8	0		
	-597	GG	41.9	40.7	41.5	66.3	23.3	77.8	39.0
		GA	41.6	46.3	43.1	22.8	50.0	18.5	50.8
		AA	16.5	13.0	15.4	10.9	26.7	3.6	10.2
TGFB									
	-509	CC	55.6	47.4	53.1	35.3	22.6		
		CT	32.9	40.4	35.2	52.0	67.7		
		TT	11.5	12.2	11.7	12.7	9.7		
	-800	GG	83.1	86.3	84.1	95.0	96.7		
		GA	15.5	12.3	14.5	5.0	3.3		
		AA	1.4	1.3	1.4	0	0		

Table 4.11 – Comparison of SNPtype frequencies between the Glasgow head injury cohorts and data from the SNP500 database (SNP500cancer.nci.nih.gov). **Pro**=prospective Glasgow cohort **Retro**=retrospective Glasgow cohort **All**=combined Glasgow cohorts **Canc**=102 cancer sufferers included in SNP500 **Cauc1**=Caucasian subpopulation of 23 from the Canc group **Cont**=control population of 280 subjects included for some SNPs in SNP500 **Cauc2**=Caucasian subpopulation of Cont group.

In the retrospective cohort nine out of eleven genotype frequencies fit the HWE. The degree of deviation is much reduced, in comparison to the original results, in the other assays. In the larger prospective cohort seven out of eleven genotype frequencies lie outside the HWE, but by a considerably reduced amount in comparison to the initial results. One set of results lies just on the boundary of significant deviation, at the 0.05 level, and the remaining assays provide results acceding to the HWE. Due to the effects of population selection one may have expected the retrospective cohort to have demonstrated more deviation from HWE than the prospective cohort (as this is more a random selection from the general population) but in fact the opposite is true. This reassures us that the there is no significant genotyping error, at least in the retrospective group. As the same assays were performed at the same time on the larger prospective cohort it is unlikely that there is any systemic error within these samples. The population must therefore not fulfil the criteria to impose the HWE or the DNA from this cohort has degenerated to such a degree that the results are no longer reliable. This second possibility is highly unlikely as the samples have been used only recently to determine APOE genotypes and have since been stored in a $-80^{\circ}C$ freezer.

Tables 4.9 and 4.10 illustrate that some assays are more sensitive than others and also that as the number of samples with increasing amounts of missing data are included the degree of deviation increases (reflected in the increasing χ^2 values), suggesting that the results from these samples may be less accurate. It was decided to include all samples as it was unclear at what level to draw a cut-off, and this would have introduced bias into the study.

The reasons for the variation in success of genotyping by assay (table 4.7) were discussed in section 4.4, and apply equally to these revised results.

The revised results will now be combined with the clinical database in order to explore any associations between SNP genotype and outcome.

Chapter 5

Outcome at 6-months after TBI

5.1 Introduction

With the genotyping of all of the samples now complete we are now able to move on to the analysis in conjunction with the clinical data. Examples of the type of clinical data have already been presented (in Section 1.8). One of the strengths of this study is the large amount of data, both clinical and genetic, that is available for analysis but it is important for this to be done logically and correctly. The successes and pitfalls of gene association studies have been outlined in Section 1.5 and in a number of reviews (Gambaro *et al.*, 2000; Botstein & Risch, 2003).

Many previous association studies have searched for links between outcome and a single SNP within a given gene. This has the advantage of being relatively simple to analyze but has a number of potential pitfalls. Using this approach it is imperative to select the SNP of interest with great care, as even if you are searching the correct gene it is easy to overlook a causative SNP. Alternately an association may be detected but this may occur because the SNP under investigation is in linkage disequilibrium with the causative SNP. When considering the cytokine genes and their SNPs as part of the inflammatory reaction it is unlikely that a single gene or SNP will be the only genetic factor influencing outcome. Due to the interactions between cytokines during inflammation it is more likely that a combination of SNPs within the genes will determine the overall degree of inflammation, the temporal profile and whether a chronic low level of inflammation will be induced. Whilst there may be some benefit in searching for associations between outcome and individual genes (a strong association may indicate a more important role in the overall mechanism for the specific gene) it will be more important to examine haplotype groups, as discussed by Ollier (2004). Multiple SNPs exist across a whole gene, and many of these will exist in linkage disequilibrium. It is therefore possible to construct a number of SNP haplotypes that characterise the gene. In addition it may then be possible to identify specific 'tagging' SNPs that identify a particular haplotype and reduce the need to genotype every SNP within the haplotype (Johnson *et al.*, 2001).

The use of haplotypes in gene association studies is discussed in detail by Clark (2004), and is usefully summarised; *"1) that the unit of biological function, the*

protein-coding gene, produces proteins whose sequences correspond to maternal and paternal haplotypes, 2) that variation in a population is in fact structured into haplotypes that are likely to be transmitted as a unit, and 3) that regardless of the population genetic reasons, haplotypes serve to reduce the dimensionality of the problem of testing association, and so they may increase the power of those tests." Therefore, although examination of individual SNPs will be performed, another important part of the data analysis will be the construction and analysis of haplotype groups for each gene.

5.2 General Methods

The collection and initial analysis of the genotyping data has been covered in the preceding chapters. Examples of the clinical data are shown in Section 1.8.

Databases were constructed using Microsoft Excel. Statistical analyses were performed using SPSS 13.0 & 14.0 for Windows.

Clinical outcome at 6-months was determined using the Glasgow Outcome Scale (see Appendix A2). Outcome was then dichotomised into unfavourable (death, vegetative state, severe disability) or favourable (moderate disability or good recovery). This approach was used when this cohort was analysed in respect of potential association with APOE genotype (Teasdale *et al.*, 2005).

Haplotype estimation was performed using SNPAnalyzer (Yoo *et al.*, 2005), available at http://www.istech.info/istech/board/login_form.jsp.

5.3 Results

The data analysis has been separated according to the cohort being considered. The larger prospective cohort is most useful for assessing the impact of the cytokine gene polymorphisms on 6-month outcome, and this group shall be considered first. Any associations that are identified can then be tested upon the retrospective cohort. The retrospective cohort can then also be used to assess any associations with general 'long-term' outcome and more specific neuropsychological outcome some years after injury.

5.3.1 Initial Analysis

5.3.1.1 Association between SNPtype and dichotomised outcome

The relationship between SNP genotype and outcome at 6-months (as assessed by dichotomised GOS) is shown in Table 5.1. The *n* number varies for each SNP as only those patients who were successfully genotyped for the given SNP were included in the analysis. *p*-values were determined using the chi-squared test. This analysis does not take into account any other clinical variables, such as the degree of initial injury or patient age.

No SNPs reached statistical significance at the 0.05 level, although the TNFA -308 and TGFB -800 polymorphisms trended towards significance (-308; p=0.07, -800; p=0.1). For TNFA -308 39.8% of those patients with a poor outcome had a genotype containing the rarer A allele compared with 32.0% in those who had a good outcome. Those patients with the TGFB -800 AA genotype were more likely to have a favourable rather than an unfavourable outcome at 6-months.

GENOTYPE	OUTCOME			*p*-value
	unfavourable	favourable	Total	
TNF 238				
GG	295 (88.3%)	570 (88.9%)	865	0.73
GA	38 (11.4%)	67 (10.5%)	105	
AA	1 (0.3%)	4 (0.6%)	5	
Total	334 (100%)	641 (100%)	**975**	
TNF 308				
GG	186 (60.2%)	416 (67.4%)	602	0.07
GA	104 (33.7%)	164 (26.6%)	268	
AA	19 (6.1%)	37 (6.0%)	56	
Total	309 (100%)	617 (100%)	**926**	
IL1 889				
CC	147 (47.9%)	333 (53.3%)	480	0.30
CT	127 (41.4%)	233 (37.3%)	360	
TT	33 (10.7%)	59 (9.4%)	92	
Total	307 (100%)	625 (100%)	**932**	
IL1 31				
CC	35 (15.0%)	78 (15.9%)	113	0.27
CT	82 (35.0%)	199 (40.4%)	281	
TT	117 (50.0%)	215 (43.7%)	332	
Total	234 (100%)	492 (100%)	**726**	
IL1 511				
TT	49 (17.1%)	103 (17.8%)	152	0.83
TC	102 (35.7%)	216 (37.2%)	318	
CC	135 (47.2%)	261 (45.0%)	396	
Total	286 (100%)	580 (100%)	**866**	
IL1 3953				
CC	197 (61.0%)	376 (58.5%)	573	0.65
CT	108 (33.4%)	223 (34.7%)	331	
TT	18 (0.6%)	44 (0.7%)	62	
Total	323 (100%)	643 (100%)	**966**	

GENOTYPE	OUTCOME			*p*-value
	unfavourable	favourable	Total	
TGF 509				
CC	124 (54.9%)	281 (56.2%)	405	0.18
CT	82 (36.3%)	155 (31.0%)	237	
TT	20 (0.9%)	64 (12.8%)	84	
Total	226 (100%)	500 (100%)	**726**	
TGF 800				
GG	220 (81.5%)	484 (83.6%)	704	0.1
GA	49 (18.1%)	84 (14.5%)	133	
AA	1 (0.4%)	11 (1.9%)	12	
Total	270 (100%)	579 (100%)	**849**	
IL6 174				
GG	68 (34.9%)	148 (37.1%)	216	0.78
GC	83 (42.6%)	170 (42.6%)	253	
CC	44 (22.6%)	81 (20.3%)	125	
Total	195 (100%)	399 (100%)	**594**	
IL6 572				
GG	152 (56.5%)	318 (59.3%)	470	0.39
GC	111 (41.3%)	212 (39.6%)	323	
CC	6 (2.2%)	6 (1.1%)	12	
Total	269 (100%)	536 (100%)	**805**	
IL6 597				
GG	115 (40.9%)	234 (42.0%)	349	0.86
GA	117 (41.6%)	234 (42.0%)	351	
AA	49 (17.4%)	89 (16.0%)	138	
Total	281 (100%)	557 (100%)	**838**	

Table 5.1 (previous page) – SNP genotype vs. dichotomised 6-month outcome. *p*-values measured using χ^2 test.

The same data is used in Figure 5.1 to examine the relationship between alleles and dichotomised outcome (i.e. whether possession of a particular allele is associated with outcome). The results are presented as odds ratios, with only TNFA -308 showing an odds ratio that does not cross 1. TGFB -800 had one of the lowest odds ratios but had large confidence intervals so it is difficult to assess the importance of

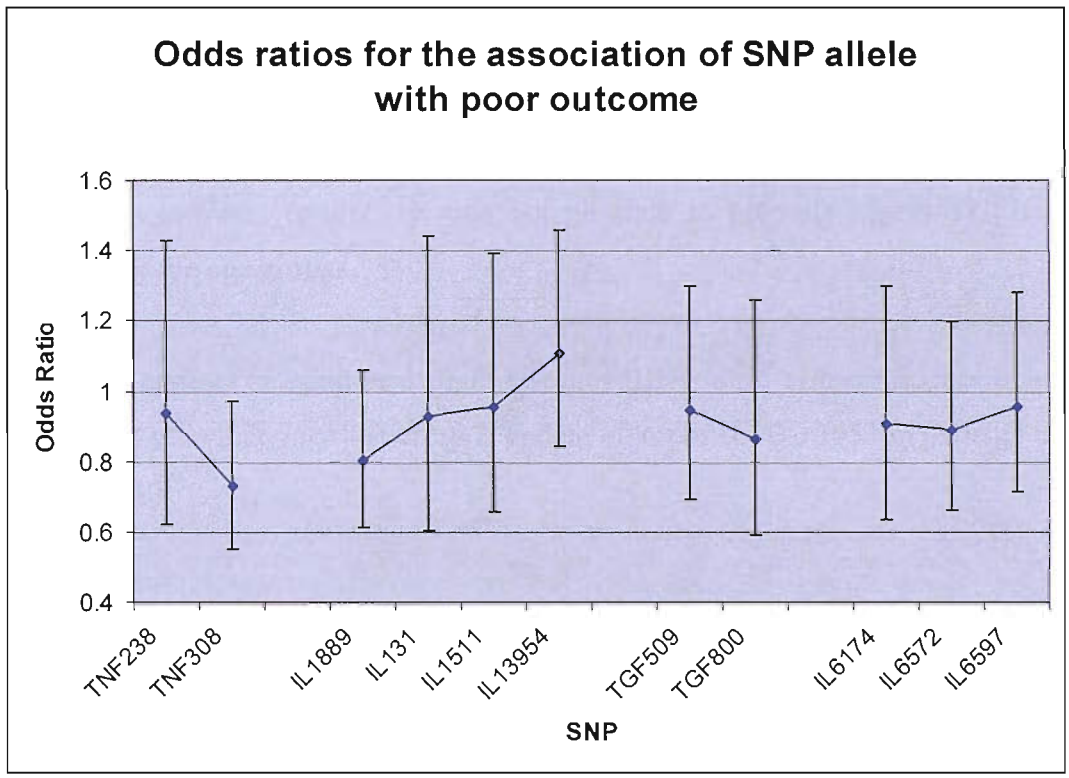


Figure 5.1 – Odds ratios for the association between SNP allele and outcome. An odds ratio of <1 indicates possession of the rarer allele is associated with poorer outcome.

this within this cohort. Although no significant genotype association was shown for IL1A -889 (see table 5.1) an allele effect may be present with an odds ratio of 0.806 (CI 0.613-1.059). In all three cases possession of the rarer allele is associated with a poorer outcome.

5.3.1.2 Association between SNP alleles and poor outcome in different age groups

Association between SNP alleles and poor outcome in different age groups was assessed. This analysis was designed to assess the interaction between the cytokine SNPs and age, as the effects of APOE $\epsilon 4$ is more pronounced in people of younger age (see Teasdale *et al.*, 2005). Results are shown in Appendix C as it was not possible to completely assess the SNPs with rare alternative alleles due to insufficient numbers in some groups.

The previously noted effect of TNFA -308 was fairly consistent (with similar odds ratios) through all of the age groups, although the confidence intervals meant these were non-significant results. It was not possible to properly assess TGFB -800 across all of the age groups.

There also appears to again be a slight effect of IL1A -889, with confidence intervals of the odds ratio that only just cross 1, and an effect of IL1B +3953 in patients under the age of 30.

5.3.1.3 Haplotype frequencies

The estimated haplotype frequencies within the prospective cohort are shown in Table 5.2.

	Haplotype	Frequency		Haplotype	Frequency
TNFA			**TGFB**		
	GG	71.96 %		CG	57.05 %
	GA	21.22 %		TG	29.73 %
	AG	5.92 %		CA	8.99 %
	AA	0.9 %		TA	4.24 %
IL1			**IL6**		
	CTCC	30.19 %		GGG	34.32 %
	CCTC	22.34 %		CGA	18.58 %
	TTCT	11.88 %		CGG	15.63 %
	CCCC	6.18 %		CCA	11.95 %
	TTCC	5.92 %		GGA	6.62 %
	CTTC	4.75 %		GCG	5.53 %
	CTCT	3.05 %		CCG	5.31 %
	TCTC	2.81 %		GCA	2.24 %
	TCCC	2.72 %			
	TCTT	2.17 %			
	TCCT	1.83 %			
	CCCT	1.51 %			
	TTTC	1.41 %			
	TTTT	1.12 %			
	CCTT	1.09 %			
	CTTT	1.04 %			

Table 5.2 – Estimated haplotype frequencies within the prospective cohort. Haplotypes are arranged as follows; TNFA:-238/-308, TGFB: -509/-800, IL1: ILIA-889/IL1B -31/-511/+3953, IL6: -174/-572/-597.

5.3.1.4 Haplotype association with 6-month outcome

The presence of an association between dichotomised clinical outcome and possession of a particular haplotype was examined by means of a Chi-squared test. Haplotypes with a frequency of less than 5% in the cohort were not considered.

Haplotype	**Outcome**			***p*-value**
	unfavourable	favourable		
TNFA				
GG negative	43	82	125	
GG positive	323	625	948	0.942
	366	707	1073	
GA negative	222	477	699	
GA positive	144	230	374	0.026
	366	707	1073	
AG negative	323	620	943	
AG positive	43	87	130	0.791
	366	707	1073	
TGFB				
CG negative	96	190	286	
CG positive	270	517	787	0.821
	366	707	1073	
TG negative	190	382	572	
TG positive	176	325	501	0.510
	366	707	1073	
CA negative	299	594	893	
CA positive	67	113	180	0.334
	366	707	1073	

Table 5.3 – Haplotype association with dichotomised outcome. Patients were assessed as to whether they possessed the haplotype or not and this was compared to outcome.

Haplotype	Outcome			*p*-value
	unfavourable	favourable		
IL1				
CTCC negative	206	367	573	
CTCC positive	160	340	500	0.173
	366	707	1073	
CCTC negative	228	393	621	
CCTC positive	138	314	452	0.035
	366	707	1073	
TTCT negative	271	524	795	
TTCT positive	95	183	278	0.980
	366	707	1073	
CCCC negative	321	650	971	
CCCC positive	45	57	102	0.025
	366	707	1073	
TTCC negative	318	632	950	
TTCC positive	48	75	123	0.222
	366	707	1073	
IL6				
GGG negative	154	276	430	
GGG positive	212	431	643	0.336
	366	707	1073	
CGA negative	243	481	724	
CGA positive	123	226	349	0.587
	366	707	1073	
CGG negative	277	543	820	
CGG positive	89	164	253	0.682
	366	707	1073	
CCA negative	254	512	766	
CCA positive	112	195	307	0.299
	366	707	1073	

Table 5.3 - cont'd

Haplotype	Outcome			*p*-value
	unfavourable	favourable		
IL6				
GGA negative	337	654	991	
GGA positive	29	53	82	0.803
	366	707	1073	
GCG negative	335	653	988	
GCG positive	31	54	85	0.632
	366	707	1073	
CCG negative	346	669	1015	
CCG positive	20	38	58	0.951
	366	707	1073	

Table 5.3 - cont'd

Three haplotypes demonstrate significant results at the 0.05 level; GA in TNFA (p=0.026) and CCTC (p=0.035) and CCCC (p=0.025) in IL1.

5.3.2 Subgroup analysis

Deviation from the Hardy-Weinberg equilibrium within the cohorts was discussed in section 4.4. Although the reasons for continuing to use the whole of the prospective cohort were explained there it would be useful to consider the results we have obtained so far in those samples where HWE is achieved. Consulting Table 4.9 we can see that if only samples with 2 or fewer pieces of missing data are considered then we still maintain a relatively large number of samples (n=746) in the analysis but minimise the deviation from the HWE. This will act as a further validation of the results from the whole cohort.

5.3.2.1 Association between SNPtype and dichotomised outcome

The chi-squared analysis, as performed in section 5.3.1.1, was repeated for the subgroup. As before the total n number for each SNP analysis varies due to the varying success of each genotyping assay. Results are shown in Table 5.4.

GENOTYPE	OUTCOME			P-value		GENOTYPE	OUTCOME			P-value
	unfavourable	favourable	Total				unfavourable	favourable	Total	
TNF 238						**TGF 509**				
GG	204 (87.2%)	446 (89.7%)	650	0.51		CC	117 (55.5%)	257 (56.2%)	374	0.26
GA	29 (12.4%)	48 (9.7%)	77			CT	76 (36.0%)	144 (31.5%)	220	
AA	1 (0.4%)	3 (0.6%)	4			TT	18 (8.5%)	56 (12.3%)	74	
Total	234 (100%)	497 (100%)	**731**			Total	211 (100%)	457 (100%)	**668**	
TNF 308						**TGF 800**				
GG	144 (61.5%)	332 (66.3%)	476	0.16		GG	187 (81.7%)	407 (82.1%)	594	0.18
GA	81 (34.6%)	141 (28.1%)	222			GA	41 (17.9%)	78 (15.7%)	119	
AA	9 (3.8%)	28 (5.6%)	37			AA	1 (0.4%)	11 (2.2%)	12	
Total	234 (100%)	501 (100%)	**735**			Total	229 (100%)	496 (100%)	**725**	
IL1 889						**IL6 174**				
CC	103 (44.2%)	268 (53.5%)	371	0.06		GG	63 (34.2%)	143 (37.4%)	206	0.65
CT	107 (45.9%)	193 (38.5%)	300			GC	81 (44.0%)	167 (43.7%)	248	
TT	23 (9.9%)	40 (8.0%)	63			CC	40 (21.7%)	72 (18.8%)	112	
Total	233 (100%)	501 (100%)	**734**			Total	184 (100%)	382 (100%)	**566**	
IL1 31						**IL6 572**				
CC	30 (14.5%)	69 (15.2%)	99	0.41		GG	125 (54.6%)	291 (59.5%)	416	0.37
CT	77 (37.2%)	190 (41.9%)	267			GC	100 (43.7%)	193 (39.5%)	293	
TT	100 (48.3%)	194 (42.8%)	294			CC	4 (1.7%)	5 (1.0%)	9	
Total	207 (100%)	453 (100%)	**660**			Total	229 (100%)	489 (100%)	**718**	
IL1 511						**IL6 597**				
TT	39 (16.7%)	84 (16.7%)	123	0.67		GG	84 (37.3%)	195 (41.3%)	279	0.43
TC	85 (36.3%)	199 (39.6%)	284			GA	99 (44.0%)	205 (43.4%)	304	
CC	110 (47.0%)	220 (43.7%)	330			AA	42 (18.7%)	72 (15.3%)	114	
Total	234 (100%)	503 (100%)	**737**			Total	225 (100%)	472 (100%)	**697**	
IL1 3953										
CC	130 (55.3%)	304 (60.6%)	434	0.37						
CT	92 (39.1%)	170 (33.9%)	262							
TT	13 (5.5%)	28 (5.6%)	41							
Total	235 (100%)	502 (100%)	**737**							

Table 5.4 (previous page) – SNP genotype vs. dichotomised 6-month outcome in the sub-group. *p*-values measured using χ^2 test.

Within the subgroup the results for the TNFA -308 and TGFB -800 become less significant (p=0.16 and p=0.18, respectively) than observed within the whole cohort (p=0.07 and p=0.1, respectively). However they remain among those which trend more towards an association than the other SNPs. The IL1A -889 SNP becomes closer to achieving significance within the subgroup (p=0.06) than within the whole cohort (p=0.3), mirroring the findings of the odds ratio analysis of the whole cohort. The other SNPs remain non-significant.

5.3.2.2 Haplotype association with 6-month outcome

The haplotype association analysis was also repeated to examine whether the same haplotypes associations identified in the whole cohort analysis remain significant. The same haplotypes examined in section 5.3.1.4 were used, and are shown in Table 5.5.

Haplotype	**Outcome**			***p*-value**
	unfavourable	favourable		
TNFA				
GG negative	20	47	67	
GG positive	215	457	672	0.719
	235	504	739	
GA negative	144	335	479	
GA positive	91	169	260	0.169
	235	504	739	
AG negative	204	450	654	
AG positive	31	54	85	0.326
	235	504	739	

Table 5.5 – Haplotype association with dichotomised outcome. Patients were assessed as to whether they possessed the haplotype or not and this was compared to outcome.

Haplotype	Outcome			*p*-value
	unfavourable	favourable		
IL1				
CTCC negative	115	231	346	
CTCC positive	120	273	393	0.431
	235	504	739	
CCTC negative	135	251	386	
CCTC positive	100	253	353	0.053
	235	504	739	
TTCT negative	153	355	508	
TTCT positive	82	149	231	0.145
	235	504	739	
CCCC negative	225	481	706	
CCCC positive	10	23	33	0.850
	235	504	739	
TTCC negative	200	449	649	
TTCC positive	35	55	90	0.123
	235	504	739	
IL6				
GGG negative	80	149	229	
GGG positive	155	355	510	0.220
	235	504	739	
CGA negative	149	336	485	
CGA positive	86	168	254	0.385
	235	504	739	
CGG negative	192	412	604	
CGG positive	43	92	135	0.989
	235	504	739	
CCA negative	152	349	501	
CCA positive	83	155	238	0.216
	235	504	739	

Table 5.5 - cont'd

Haplotype	Outcome			*p*-value
	unfavourable	favourable		
IL6				
GGA negative	220	474	694	
GGA positive	15	30	45	0.820
	235	504	739	
GCG negative	222	475	697	
GCG positive	13	29	42	0.903
	235	504	739	
CCG negative	228	488	716	
CCG positive	7	16	23	0.886
	235	504	739	
TGFB				
CG negative	41	106	147	
CG positive	194	398	592	0.256
	235	504	739	
TG negative	124	277	401	
TG positive	111	227	338	.0577
	235	504	739	
CA negative	193	419	612	
CA positive	42	85	127	0.735
	235	504	739	

Table 5.5 - cont'd

The analysis of the complete cohort indicated significant ($p<0.05$) associations between dichotomised outcome and three of the haplotypes: GA in TNFA and CCTC and CCCC in IL1. In the subgroup analysis the GA haplotype no longer remains significant ($p=0.169$ vs. $p=0.026$ in the full cohort). The IL1 CCTC haplotype remains borderline significant ($p=0.053$ vs. $p=0.035$) while the IL1 CCCC haplotype becomes markedly non-significant ($p=0.850$ vs. $p=0.025$). None of the other haplotypes become significantly associated with outcome in the subgroup analysis.

5.3.3 Discussion

A number of analyses have been undertaken to assess the presence of any association between our selected cytokine SNPs and dichotomised outcome (based upon the Glasgow Outcome Scale) after head injury. These initial analyses are admittedly rather statistically crude, as they disregard much of the other clinical information available for these patients, but they provide a good guide as to whether any associations are likely to become apparent in further analyses.

Patients were first considered according to their genotype (i.e. were they homo- or heterozygotes – see section 5.3.1.1). No statistically significant results were obtained at the 0.05 level but two SNPs showed a trend towards significance. Those patients who were heterozygotes or homozygous for the rarer A allele at TNFA -308 seemed to show a trend towards poorer outcome, falling just short of significance (p=0.07). This is an encouraging finding as this would 'fit' with our initial hypotheses as the A allele has been previously associated with increased levels of TNFα (Kroeger *et al.*, 1997; Wilson *et al.*, 1997), and TNFα is generally considered to have pro-inflammatory effects (see Section 1.9.1). The next most important result is that for TGFB -800, with a p-value of 0.1, suggesting a trend towards an association. However if the results are examined closely it becomes apparent that there is no clear trend (or additive effect) of the rarer allele. In table 5.1 we can see that being homozygous for the A allele appears to show an association with a much higher chance of having a good outcome when compared to those patients who are homozygous for the G allele. This effect disappears in those who are heterozygotes. There is therefore no evidence of a 'dose' effect of the allele but the possibility of an effect in A homozygotes. If this effect of the A allele is true then this again fits with our knowledge of the effects of these SNPs. The A allele has previously been associated with increased levels of TGFβ (Grainger *et al.*, 1999) and this is believed to exert a neuroprotective effect (see Section 1.9.4). None of the SNPs in the IL-1 or IL-6 clusters appeared to be exerting any effect on outcome.

In Figure 5.1 I have tested the allele effect on outcome. Patients were categorised in to one of two groups by asking the question 'Do they possess at least one copy of the

rarer allele at each SNP position?' The only allele to show a clear effect was the TNFA -308 A allele, again being associated with poorer outcome. No other SNPs showed a statistical association, although two other results are of interest. When examining allele effects the rarer A allele at TGFB -800 actually appears to predispose to a poorer outcome, the opposite of what was being suggested in the genotype analysis. Only 12 patients are homozygous for the A allele and therefore any potential protective effect of having two copies of the A allele (as identified in the genotype analysis) is being swamped in this allele analysis by the much larger number of patients with a single copy of the allele. A potential effect of the IL1A -889 SNP should also be considered on the basis of this analysis. Although again the effect does not reach significance the confidence intervals only just cross 1 and therefore a potential effect may be present.

In these first sets of analyses we have not taken account of other confounding variables, such as patient age, which we know has an effect on outcome after TBI. It may also be that the SNPs have differential effects in various age groups, such as has been shown for the APOE genotype (Teasdale *et al.*, 2005). It was not possible to properly assess all of the SNPs across all of the age groups because of insufficient numbers, even within this large cohort. The effect of TNFA -308 was again detected and seems to be a consistent, although non-significant, effect across all age groups. There is a strikingly different effect of the IL-1A -889 in the youngest age group as compared to the other age groups. Again this is non-significant but may highlight the possibility of differential effects of the SNPs according to age.

In the introduction to this section I highlighted the need to not only assess individual SNPs but to consider interaction between SNPs and the importance of considering haplotype groups within genes. By analysing these haplotypes we may detect gene interactions that would otherwise be missed if SNPs were considered independently. The initial analysis of the haplotypes present in this cohort has revealed a number of potential associations. Again an association is noted in the TNFα gene. However we must consider whether this is a true haplotype effect or as a result of the individual alleles in a haplotype which contains only two SNPs. When we examine the results we see that that the GA haplotype identified as significant is the only one of the three haplotypes considered which contains the A allele in the -308 position. We have

already identified this allele as having a potential association with outcome. It is therefore likely that this is not a true haplotype effect but an allele effect which masquerades as a haplotype effect in this analysis. There is no haplotype effect within the TGFβ gene, where we have already identified the -800 position as having a potential effect. Two haplotypes within the IL-1 gene cluster are identified using this analysis as having potential association with outcome. Both the CCTC and CCCC haplotypes have *p*-values less than 0.05. This suggests that there may be some interaction between the SNPs in the IL-1 genes which subsequently influences outcome, but the association needs to be explored further to establish this.

In the previous chapter we examined the Hardy-Weinberg Equilibrium within our prospective cohort. The deviation from this equilibrium among some of the SNPs was highlighted and the reasons for this discussed. Based upon this it was decided to continue to use the whole cohort for analysis. To a certain extent my results for the whole cohort can be validated by examining the subset of the cohort with the 'best fit' with the HWE. By including only those patients who are missing two or fewer pieces of SNP data in the subset this can be achieved relatively well. When the analysis is carried out on this subgroup the results in general stay very similar. In the genotype analysis the three SNPs with the smallest p-values remain the TNFA -308, TGFB -800 and IL1A -889, as found in the analysis of the full cohort. The -308 and -800 SNPs become less significant whilst the -889 SNP becomes more so. As we would expect, based upon the discussion above, the TNFA GA haplotype becomes less significant in tandem with the TNFA -308 SNP becoming less significant. Within the other genes only one of the IL1 haplotypes remains significant (CCTC, as identified in the full cohort) whilst the CCCC haplotype becomes marked non significant. The CCCC haplotype is one of the rarer haplotypes considered, with relatively few patients possessing it. By reducing the numbers still further within the subgroup it is unsurprising that the haplotype becomes non-significant. When assessing this subgroup we must remember that we are only considering two pieces of information – the dichotomised outcome and genetic results, taking no account of other factors such as age and degree of initial injury. The subgroup will not be directly comparable to the full cohort for this reason.

5.4 Regression Analysis

Having performed my initial analysis I now have a good idea as to which SNPs are likely to be exerting an effect on clinical outcome. I now need to take account of some of the other factors we know affect outcome after TBI. This can be best achieved by binary logistic regression analysis.

5.4.1 Methods

Outcome measures were dichotomised for the regression analysis. For overall outcome at six months the GOS was dichotomised in the standard manner (GOS 1-3 = poor outcome, 4-5 = good outcome). More specific outcome measures (such as seizures and raised intracranial pressure (ICP)) were assessed according to presence or absence of the specific outcome.

'Seizures' were seizures requiring treatment at any stage during admission, 'raised ICP' was raised intracranial pressure requiring treatment during admission and 'infection' was life-threatening infection at any point during admission.

Co-variates used in the analysis were age (continuous variable), GCS (categorised into mild, moderate or severe), initial CT scan findings (categorised, from the Marshall classification (Marshall *et al.*, 1991), into 3 groups), and APOE genotype (presence or absence of the $\epsilon 4$ allele).

As previously only haplotypes with a frequency of at least 5% within the cohort were analysed.

5.4.2 Results

5.4.2.1 Association between SNP allele and overall outcome

Genotype results for each SNP were dichotomised according to possession or not of at least one copy of the rarer allele. Table 5.6 demonstrates the odds ratios obtained

when each SNP was added to the logistic regression model with the co-variates outlined in section 5.4.1. Only the -308 SNP in TNFA had a significant impact upon the model. This is in keeping with the results obtained in the earlier analysis (see section 5.3.1.1). Previous analysis had also suggested that there may be a role for the TGFB -800 and IL1A -889 SNPs. This regression analysis has shown that these SNPs have a less significant effect when considered with the other factors that influence outcome and therefore do not appear to be associated with outcome after TBI.

SNP	n	Odds Ratio	C.I.	*p*-value
IL1A -889	836	1.210	0.855 - 1.712	0.283
IL1B -31	656	0.716	0.482 - 1.064	0.098
IL1B -511	777	0.897	0.629 - 1.279	0.549
IL1B +3953	871	0.867	0.612 – 1.229	0.423
IL6 -174	534	1.118	0.708 – 1.764	0.663
IL6 -572	726	1.150	0.789 – 1.677	0.467
IL6 -597	756	1.114	0.771 – 1.608	0.566
TGFB -509	649	1.093	0.728 – 1.641	0.667
TGFB -800	760	1.270	0.777 – 2.074	0.340
TNFA -238	876	1.142	0.667 – 1.954	0.628
TNFA -308	835	1.612	1.126 – 2.309	0.009

Table 5.6 – Odds ratio of achieving a good outcome if you do not possess the rare allele at each of the given SNP positions.
n=number of patients considered for the regression analysis, due to missing genetic or clinical data.
C.I. = 95% confidence intervals

5.4.2.2 Haplotype association with dichotomised overall outcome

In a similar manner to the previous haplotype analysis patients were dichotomised as to whether they possessed at least one copy of the haplotype under consideration or not. Haplotypes which occurred with a frequency of less than 5% in the cohort were not analysed. Results are shown in Table 5.7.

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	968	0.973	0.707 – 1.339	0.866
CCTC	968	0.774	0.559 – 1.070	0.121
TTCT	968	0.968	0.671 – 1.397	0.862
CCCC	968	1.685	0.998 – 2.847	0.051
TTCC	968	1.196	0.746 – 1.917	0.457
TGFB				
CG	968	0.964	0.674 – 1.379	0.839
TG	968	1.119	0.813 – 1.539	0.491
CA	968	1.320	0.863 – 2.020	0.201
IL6				
GGG	968	0.876	0.635 – 1.209	0.422
CGA	968	0.918	0.653 – 1.290	0.621
CGG	968	0.963	0.664 – 1.396	0.840
CCA	968	1.300	0.912 – 1.853	0.146
GGA	968	1.088	0.605 – 1.957	0.777
GCG	968	0.872	0.492 – 1.546	0.639
CCG	968	1.519	0.772 – 2.990	0.226
TNFA				
GG	968	0.988	0.603 – 1.619	0.962
GA	968	1.426	1.023 – 1.987	0.036
AG	968	0.976	0.595 – 1.600	0.923

Table 5.7 – Odds ratio of achieving a good outcome if you do not possess the given haplotype in the gene(s) indicated.
n=number of patients considered for the regression analysis, due to missing genetic or clinical data.
C.I. = 95% confidence intervals

5.4.2.3 SNP association with seizure occurrence

Results from the binary logistic regression analysis of SNP association with seizure occurrence are shown in table 5.8. Only one SNP, IL-1A -889, showed a significant effect, and this was only slight. Possession of at least one copy of the rarer T allele at the –889 position appears to be associated with an increased risk of a seizure after head injury.

SNP	*n*	Odds Ratio	C.I.	*p*-value
IL1A -889	843	0.692	0.479 – 0.999	0.049
IL1B -31	662	1.227	0.815 – 1.847	0.326
IL1B -511	784	1.108	0.766 - 1.605	0.586
IL1B +3953	879	1.018	0.705 – 1.470	0.923
IL6 -174	540	1.536	0.973 – 2.426	0.066
IL6 -572	733	0.773	0.523 – 1.142	0.195
IL6 -597	763	1.224	0.832 – 1.800	0.306
TGFB -509	656	0.701	0.464 – 1.058	0.091
TGFB -800	767	1.008	0.600 – 1.694	0.976
TNFA -238	884	1.175	0.643 – 2.149	0.600
TNFA -308	842	1.092	0.741 – 1.610	0.656

Table 5.8 – Odds ratio of a seizure occurring post TBI if you do not possess the rarer allele. n=number of subjects included in the analysis, C.I=95% confidence interval.

5.4.2.4 Haplotype association with seizure occurrence

Haplotype association with seizures after TBI are shown in table 5.9. Only the IL1 TTCC haplotype shows a significant association with seizure occurrence after TBI, and may be related to the significant association of the IL1A -889 SNP with seizure occurrence (see section 5.4.2.3).

Table 5.9 – Association between haplotype and post TBI seizure occurrence. n= subjects included in analysis, C.I. =95% confidence interval

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	976	0.994	0.706 – 1.399	0.973
CCTC	976	1.261	0.889 – 1.790	0.194
TTCT	976	0.950	0.645 – 1.399	0.795
CCCC	976	0.857	0.491 – 1.494	0.585
TTCC	976	0.555	0.348 – 0.886	0.014
TGFB				
CG	976	1.188	0.816 – 1.732	0.368
TG	976	0.859	0.611 – 1.206	0.380
CA	976	0.796	0.513 – 1.236	0.310
IL6				
GGG	976	1.015	0.718 – 1.436	0.932
CGA	976	1.289	0.886 – 1.874	0.184
CGG	976	1.460	0.948 – 2.251	0.086
CCA	976	0.849	0.585 – 1.231	0.387
GGA	976	0.764	0.425 – 1.373	0.368
GCG	976	0.719	0.404 – 1.280	0.263
CCG	976	1.332	0.587 – 3.023	0.492
TNFA				
GG	976	1.014	0.596 – 1.724	0.959
GA	976	0.988	0.692 – 1.410	0.947
AG	976	1.187	0.684 – 2.061	0.541

5.4.2.5 SNP association with raised ICP

Two SNPs show a potential association with significantly raised intracranial pressure after TBI, as shown in table 5.10. The more rare alleles at the –889 position in IL-1A and +3953 in IL-1B appear to be associated with a predisposition to raised intracranial pressure, with –889 having a more marked effect.

SNP	n	Odds Ratio	C.I.	*p*-value
IL1A -889	836	0.517	0.312 – 0.857	0.010
IL1B -31	656	1.280	0.738 – 2.222	0.380
IL1B -511	777	1.216	0.728 – 2.033	0.455
IL1B +3953	871	0.572	0.349 – 0.938	0.027
IL6 -174	534	0.838	0.437 – 1.605	0.593
IL6 -572	726	0.749	0.443 – 1.269	0.283
IL6 -597	756	0.714	0.409 – 1.244	0.234
TGFB -509	649	1.106	0.616 – 1.984	0.736
TGFB -800	760	0.809	0.402 – 1.629	0.553
TNFA -238	876	1.090	0.516 – 2.302	0.821
TNFA -308	835	0.819	0.497 – 1.348	0.432

Table 5.10 – Odds ratio of raised ICP if you do not possess the rarer allele. n=number of subjects included in the analysis, C.I=95% confidence interval.

5.4.2.6 Haplotype association with raised ICP

Haplotype association with raised ICP are shown in table 5.11. The IL1 TTCT haplotype is the only significant association identified (p=0.018).

Haplotype	n	Odds Ratio	C.I.	p-value
IL1				
CTCC	968	0.947	0.604 – 1.483	0.810
CCTC	968	0.984	0.625 – 1.549	0.944
TTCT	968	0.549	0.334 – 0.902	0.018
CCCC	968	1.345	0.646 – 2.804	0.428
TTCC	968	0.790	0.417 – 1.497	0.470
TGFB				
CG	968	1.380	0.848 – 2.246	0.195
TG	968	1.141	0.724 – 1.800	0.569
CA	968	0.770	0.437 – 1.356	0.365
IL6				
GGG	968	0.995	0.631 – 1.569	0.982
CGA	968	1.207	0.744 – 1.958	0.447
CGG	968	0.986	0.587 – 1.656	0.957
CCA	968	0.720	0.443 – 1.170	0.185
GGA	968	0.608	0.288 – 1.281	0.190
GCG	968	0.828	0.391 – 1.753	0.622
CCG	968	1.261	0.417 – 3.817	0.682
TNFA				
GG	968	1.564	0.846 – 2.893	0.154
GA	968	0.884	0.557 – 1.402	0.600
AG	968	0.963	0.494 – 1.874	0.911

Table 5.11 – Association of haplotype with raised ICP. n=number of subjects included in the analysis, C.I=95% confidence interval.

5.4.2.7 SNP association with infection

As we have seen for the other more specific outcome measures above it is again a SNP in the IL-1 gene family that has a significant association with severe infection after TBI (see table 5.12 below). In this instance the SNP at IL-1B -31 is responsible and the rarer C allele seems to exert a protective against infection.

SNP	n	Odds Ratio	C.I.	*p*-value
IL1A -889	836	0.591	0.324 – 1.076	0.085
IL1B -31	656	2.127	1.091 – 4.147	0.027
IL1B -511	777	1.218	0.661 – 2.245	0.527
IL1B +3953	871	0.731	0.411 – 1.299	0.286
IL6 -174	534	0.948	0.457 – 1.967	0.887
IL6 -572	726	0.629	0.344 – 1.151	0.133
IL6 -597	756	1.039	0.552 – 1.955	0.907
TGFB -509	649	0.534	0.273 – 1.042	0.066
TGFB -800	760	2.229	0.766 – 6.490	0.141
TNFA -238	876	1.022	0.413 – 2.525	0.963
TNFA -308	835	1.579	0.822 – 3.033	0.170

Table 5.12 – Odds ratio of a severe infection occurring post TBI if you do not possess the rarer allele. n=number of subjects included in the analysis, C.I=95% confidence interval.

5.4.2.8 Haplotype association with infection

Results are shown in table 5.13. Only the IL1B -31 SNP was shown to be associated with severe infection after TBI in the analysis of individual SNPs (section 5.4.2.7). In this haplotype analysis none of the IL1 haplotypes maintain this association. Interestingly the TG haplotype in TGFB appears to be associated with a significant protective effect (p=0.035).

Table 5.13 – Haplotype association with severe infection post TBI. n=number of subjects included in the analysis, C.I=95% confidence interval.

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	968	0.828	0.488 – 1.404	0.484
CCTC	968	1.352	0.778 – 2.349	0.285
TTCT	968	0.641	0.361 – 1.137	0.129
CCCC	968	2.203	0.761 – 6.380	0.145
TTCC	968	0.611	0.301 – 1.240	0.173
TGFB				
CG	968	1.064	0.587 – 1.928	0.838
TG	968	0.558	0.325 – 1.959	0.035
CA	968	2.035	0.847 – 4.893	0.112
IL6				
GGG	968	1.080	0.634 – 1.840	0.777
CGA	968	0.877	0.507 – 1.514	0.637
CGG	968	1.199	0.628 – 2.289	0.582
CCA	968	0.800	0.453 – 1.413	0.443
GGA	968	1.807	0.538 – 6.066	0.338
GCG	968	1.091	0.441 – 2.704	0.850
CCG	968	0.397	0.155 – 1.016	0.054
TNFA				
GG	968	1.350	0.649 – 2.810	0.422
GA	968	1.379	0.777 – 2.447	0.272
AG	968	0.947	0.428 – 2.098	0.894

5.5 Inflammatory Profiles

To this point in the analysis I have looked at the effect of individual SNPs and the effect of combined SNPs, in the form of haplotypes. However, the haplotype analysis only enables us to look for SNP interactions within a given gene or gene cluster. The interaction between genes is also important, especially if we consider that the inflammatory reaction will depend upon the interaction of a number of cytokines. The SNPs chosen for this study were selected because there was evidence that they either affected gene transcription or had been shown to influence disease susceptibility in other conditions. The SNPs can therefore be categorised into those that evidence suggests should have a neurotoxic effect, those that may be neuroprotective and those that have no clear effect. The interaction between these SNPs can then be investigated.

5.5.1 Methods

Based upon the literature (the evidence for selecting these SNPs and haplotypes is summarised in section 1.9) four factors were identified that I hypothesised would promote neuroinflammation; possession of the TNFA -308 A allele, the IL1A -889 T allele, the IL1B +3953 T allele and the IL6 GGA haplotype. Similarly three factors that would promote neuroprotection were identified. These were the -509 T and -800 A alleles in TGFB and the IL6 GGG haplotype.

To assess the effect of these SNPs in combination the cohort was stratified according to the number of 'high risk' or 'protective' SNPs or haplotypes possessed. The effect was assessed by performing a χ^2 test and calculating the odds ratio. Again outcome was measured using the dichotomised 6-month GOS. A total of 605 subjects who had a full set of genotyping data for the relevant SNPs were entered into the analysis

5.5.2 Results

5.5.2.1 Risk of poor outcome at 6-months according to the number of 'high risk' genetic factors possessed

Results are shown in table 5.14. Possession of one or more of the high risk factors increases the likelihood of having a poor outcome at 6-months post head injury. Possession of two of the factors significantly increases the risk (p=0.007). If this trend continues (and I have hypothesised the factors correctly) then possession of three or four factors would be expected to increase the risk further. It is slightly surprising therefore that possession of three factors does not also significantly increase the risk. In order to investigate this finding a further analysis was performed to investigate which combinations have the greatest effect (see section 5.5.2.2).

No of high risk factors	n	Poor Outcome	Good Outcome	χ^2 p-value	OR (95% confidence interval)
0	159	38	121	referent	referent
1	190	54	136	0.340	1.264 (0.781-2.047)
2	173	65	108	0.007	1.916 (1.189-3.088)
3	81	25	56	0.246	1.420 (0.783-2.580)
4	2	2	0	-	-

Table 5.14 – Odds ratio of a poor outcome according to the number of high risk factors possessed. n=number of subjects, OR=odds ratio.

5.5.2.2 Risk of poor outcome at 6-months according to the profile of 'high risk' genetic factors possessed

Subjects with one, two or three 'high risk' genetic factors were then classified according to their genetic profile and compared with those subjects with none of the risk factors (table 5.15). The TNFA -308, IL1A -889 and IL6 haplotype are all associated with an increased risk of a poor outcome at 6-months, as expected from the literature evidence. Possession of the +3953 T allele in IL1B is more likely to

exert a protective effect than a neurotoxic one. This is in keeping with the findings of the earlier analyses in this study, but not what would have been predicted from the literature. However when the allele is combined with possession of the -889 T allele in IL1A the effect is to increase the neurotoxic effect of -889 T (OR of -889 T alone = 1.33, but rises to 1.88 if -889 T and +3953 T are combined). This is a significant effect (p=0.01). The other significant result occurs when both the TNFA -308 and IL1A -889 are combined together (OR 2.73, p=0.02).

Genetic Profile								
-308 A	-889 T	+3953 T	GGA	**n**	**Poor**	**Good**	χ^2	**OR (95% C.I.)**
-	-	-	-	159	38	121	-	-
+	-	-	-	101	29	72	0.387	1.28 (0.73-2.26)
-	+	-	-	51	15	36	0.430	1.33 (0.66-2.68)
-	-	+	-	21	4	17	0.621	0.75 (0.24-2.36)
-	-	-	+	17	6	11	0.302	1.73 (0.60-5.01)
+	+	-	-	26	12	14	0.018	2.73 (1.16-6.40)
+	-	+	-	9	2	7	0.909	0.91 (0.18-4.57)
+	-	-	+	2	1	1	0.392	3.18(0.19-52.14)
-	+	+	-	132	49	83	0.014	1.88 (1.13-3.12)
-	+	-	+	3	0	3	-	-
-	-	+	+	1	1	0	-	-
+	+	+	-	69	22	47	0.208	1.49 (0.8-2.78)
+	+	-	+	2	0	2	-	-
+	-	+	+	0	0	0	-	-
-	+	+	+	10	3	7	0.662	1.36 (0.34-5.54)
+	+	+	+	2	2	0	-	-

Table 5.15 – Risk of poor outcome according to the genetic profile of high risk factors. n=number of subjects, OR=odds ratio, CI=confidence interval.

5.5.2.3 Risk of poor outcome at 6-months according to the number of 'protective' genetic factors possessed

In a similar manner to the analysis in section 5.5.2.1 the effect of the three protective genetic factors identified from the literature can be assessed. The results are shown in table 5.16. With increasing number of 'protective' factors possessed then the risk of a poor outcome decreases. The effects, though, are non-significant.

No of protective factors	n	Poor outcome	Good Outcome	χ^2 *p*-value	OR (95% confidence interval)
0	82	26	56	referent	referent
1	268	80	188	0.749	0.917 (0.538-1.563)
2	240	75	165	0.939	0.979 (0.571-1.679)
3	15	3	12	0.362	0.538 (0.140-2.073)

Table 5.16 – Odds ratio of a poor outcome according to the number of protective factors possessed. n=number of subjects, OR=odds ratio.

5.5.2.4 Risk of poor outcome at 6-months according to the profile of 'protective' genetic factors possessed

In the same fashion as the 'high risk' genetic factors (see section 5.5.2.2) the 'protective' factors can be categorised in order to investigate whether the literature evidence is correct and these are 'anti' inflammatory factors and which combination provides the greatest protection. Results are shown in table 5.17. Alone the SNPs in TGFB do not appear to be protective but in combination with the IL6 GGG haplotype they become so. When possessed in combination they are also protective. None of the combinations exert a significant effect on overall 6-month outcome.

Genetic Profile							
-509 T	-800 A	GGG	n	Poor	Good	χ^2	OR (95% C.I.)
-	-	-	82	26	56	-	-
+	-	-	71	24	47	0.783	1.10 (0.56-2.16)
-	+	-	21	8	13	0.579	1.32 (0.49-3.59)
-	-	+	175	47	128	0.422	0.79 (0.45-1.40)
+	+	-	14	3	11	0.439	0.59 (0.12-2.29)
+	-	+	168	52	116	0.904	0.97 (0.55-1.70)
-	+	+	58	20	38	0.731	1.13 (0.56-2.31)
+	+	+	15	3	12	0.362	0.54 (0.14-2.07)

Table 5.17 – Risk of poor outcome according to the genetic profile of protective factors. n=number of subjects, OR=odds ratio, CI=confidence interval.

5.6 Discussion

Binary logistic regression has been useful in defining the potential genetic associations with outcomes after TBI in this cohort and set of SNPs. In the initial analysis of dichotomised overall outcome, measured using the GOS, three of our eleven SNPs were identified as likely candidates to show an association (TNFA –308, TGFB –800 & IL1A –889), although none were significant at the 0.05 level. In the further analysis, controlling for other variables known to influence outcome after head injury, the effect of TNFA -308 became more significant (p=0.009), and the other two SNPs appear to not truly influence outcome. As already discussed the TNFA -308 association would seem a biologically plausible effect as the A allele has been previously associated with increased levels of TNFα (Kroeger *et al.*, 1997; Wilson *et al.*, 1997), is associated with a number of disease conditions (see section 1.9.1) and TNFα is generally considered to have pro-inflammatory effects (see Section 1.9.1). However the role of TNFα in inflammation, and especially neuroinflammation, is still not clearly defined, as it probably has a role in both the promotion and resolution of inflammation. The association between the TNFA –308 SNP and six-month outcome after TBI has not previously been demonstrated, although recent studies have implicated TNFA –308 SNP as having a role in other

neurodegenerative conditions, such as determining age of onset in Alzheimer's disease (Lio *et al.*, 2006) and also the risk of lacunar infarction within the brain (Harcos *et al.*, 2006). It must be remembered that studies of the TNFA gene should be interpreted with care. The gene sits within the MHC cluster on chromosome 6, and is closely related to the lymphotoxin-α and -β genes (LTA & LTB). The probability is that TNFA SNPs form part of a haplotype that stretches across these closely related genes, and therefore it is difficult to subscribe an apparent association to a single causative SNP within this region (see Ruuls & Sedgwick, 1999, for a fuller discussion of the interactions of TNF with other elements of the MHC cluster). Further genetic studies would be required to identify haplotype associations within this extended region to delineate the role of other SNPs within these closely related genes.

A recent study (Uzan *et al.*, 2005) identified a possible association between the –511 and +3953 SNPs within the IL-1β gene and outcome after head injury. The study was small, with 69 patients admitted to a neurosurgical unit after TBI included. Fourteen out of twenty-five patients (56%) with the +3953 T allele had a poor outcome compared with only eight out of forty four (18.1%) in those patients who did not possess the T allele (p=0.0004). Twenty out of twenty eight patients (71.4%) with the -511 T allele had a poor outcome compared with two out of forty one (4.8%) without the rarer allele (p=0.005). These findings have not been confirmed in our study. None of the IL1 SNPs studied in this cohort showed an association with overall outcome at six-months. This does confirm the negative association between the IL-1 –889 SNP and overall six-month outcome identified in a small study by Tanriverdi *et al.* (2006). This study was performed by the same group that performed the IL1β study described above (Uzan *et al.*, 2005). 71 patients admitted to a neurosurgical unit after TBI were studied. Although those patients who possessed the IL1A -889 T allele were more likely to have a poor outcome (eighteen out of forty (45%)) compared to those without (seven out of thirty one (22.5%)) the association did nor reach statistical significance (p=0.08).

Microdialysis studies have suggested that IL-6 may have a neuroprotective effect after severe TBI (Singhal *et al.*, 2002; Winter *et al.*, 2004), although the single

previous study of the influence of IL-6 SNPs on outcome after TBI did not identify any association (Minambres *et al.*, 2003). The current study has confirmed the finding of no association between the three IL-6 SNPs investigated and six-month outcome after TBI.

A very limited haplotype for the TNFA gene can be constructed from the two SNPs studied in this cohort. The GA haplotype was identified as having a likely association in the initial analysis (see table 5.3). This finding is confirmed in the regression analysis. As discussed in section 5.3.3 this may not be a true haplotype effect, but the manifestation of an allele effect at the –308 SNP. The two haplotypes in the IL1 gene cluster identified in the initial analysis as having a potential effect do not have a significant influence on outcome in the regression analysis. The CCCC haplotype (p=0.051) is almost significant, but the haplotype is rare within the cohort and the result would require further investigation in a larger cohort in order to increase the number of patients possessing the CCCC haplotype.

Using the clinical data available in this cohort it is possible to study other more specific outcome measures that may be related to the process of neuroinflammation, and therefore possibly associated with SNPs within the cytokine genes. After TBI seizures may be provoked by the initial damage to the brain parenchyma at the time of the injury. However brain inflammation and swelling may lower the threshold for seizures in the acute phase after injury. Raised intracranial pressure after TBI may be related to an intracranial mass lesion but also results from the cerebral oedema and swelling which occurs as part of the neuroinflammatory response after TBI. The response to sepsis occurring after TBI may also be influenced by cytokine gene polymorphisms (Gordon *et al.*, 2004; Arcaroli *et al.*, 2005; Watanabe *et al.*, 2005).

The IL-1A -889 polymorphism is the only SNP which appears to be associated with seizure occurrence after TBI, and this result is of borderline significance. Seizures after TBI are relatively common (Frey, 2003) but to date only one study has investigated the possible genetic risk factors for post traumatic epilepsy (Diaz-Arrastia *et al.*, 2003) The finding of a putative association with the -889 SNP will need further investigation before it could conclusively be felt to be a risk factor for posttraumatic seizures.

The -889 also has an association with significantly raised ICP after TBI. There also appears to be a role for the +3953 SNP in IL1B. The associations between cytokine SNPs and raised ICP after TBI have not previously been investigated. One may speculate that patients who have significantly raised ICPs during their inpatient treatment would be more likely to sustain brain damage, and thus have poorer outcomes. Neither of the SNPs identified here have a significant association with outcome so this may not be correct. The TTCT haplotype also shows a positive association, although this is a rare haplotype and it contains both the -889 and +3953 alleles that have been individually identified, so may not be a true haplotype effect.

A SNP in the IL1 gene cluster is again identified as having a positive association in the analysis of severe infection occurring after TBI, although this time at the IL1B -31 position. No haplotype association are identified. Although the importance of cytokines (notably TNFα and IL6) in determining the response to sepsis is well known (Gordon *et al.*, 2004; Arcaroli *et al.*, 2005; Watanabe *et al.*, 2005) the association between this particular SNP and sepsis post-TBI has not been previously elucidated.

In order to try to assess the importance of interaction between genes, rather than simply within genes, 'inflammatory profiles' were created. These profiles were based upon whether the specific SNP or haplotype would exert a 'pro' or 'anti' inflammatory action during the process of inflammation. The profiles were created on the basis of perceived action of the specific cytokine and whether the SNP or haplotype increased or decreased expression. Much of this information is not yet definitively known so this model is somewhat theoretical. The interaction between the genetic factors can then be assessed, in a similar manner to the analysis performed by Flex *et al.* (2004) in their study of ischemic stroke. Four 'pro'-inflammatory factors were identified from the available evidence. The analysis confirms that three of the factors do promote neurotoxicity (or at least predispose to a poorer outcome at 6-months). However the rarer T allele at the +3953 position in IL1B has the reverse effect to that expected. When the factors are looked at in combination the picture becomes less clear. The TNFA -308 A allele is associated with poor outcome (significantly so in the whole cohort) but this effect is reversed if

-308 A is inherited together with +3953 T. This suggests the +3953 T allele has a more protective effect than -308 A has damaging. When +3953 T is held together with -889 T there is a combined effect, increasing the neurotoxicity of the -889 T allele. Of the protective factors the -509 T and -800 A alleles did not show the expected result when looked at individually (both in this analysis and the analysis of the complete cohort), however when combined together there was a large swing to a protective effect. This TA haplotype is uncommon and was not analysed in the full cohort. The GGG haplotype in IL6 is the most common haplotype in this cohort. This analysis therefore confirms that generally the IL6 cytokine acts as a neuroprotective factor, although its effect may be relatively weak and is easily 'drowned out' by other neurotoxic factors.

When interpreting these results I have not so far included a correction for the effect of multiple testing. It is vital to consider these important effects, and interpret the results bearing this in mind. The reasons for not including a Bonferroni correction within the results is covered in detail in Perneger's review (1998). The main aim of correcting for multiple testing is to avoid Type I errors (false positives). This occurs because a significant difference will be observed simply through chance in 1 in 20 tests if a *p*-value of 0.05 is used. Therefore the more comparisons that are made the higher the chance of observing a false positive. The Bonferroni correction is used to reduce this possibility. In the case of this study each of the eleven SNPs were not selected randomly but on the basis that each was likely, based upon biological activity, to have a potential effect on outcome after TBI. Thus a hypothesis could be set for each individual SNP. It was not practicable or sensible to perform the study on each SNP independently, so they were investigated as part of a single study. Any associations identified within the context of this study will have to remain preliminary. In order to confirm the associations further studies will be required in other populations. The possibility that I have identified a false positive result remains.

In summary, of the eleven SNPs identified for investigation in this study only one (308 G/A in TNFA) has been identified as having a significant association with outcome at 6-months after TBI. There may also be a haplotype association within the TNFA gene, but this may be a reflection of the allele effect. SNPs within the IL1

gene cluster are potentially important in determining the risk of inflammation-related complications, such as raised ICP, seizures and severe infection, after TBI. This is possible related to the important role IL1 plays in controlling the neuroinflammatory reaction. There are important interactions between genes, which also influence outcome. As expected the influence of genetic polymorphisms on outcome at 6-months is small but still important. In order to confirm these results, and to ensure they are not false positives, the findings will need to be replicated in other cohorts. Within the clinical data collected from the 'long-term' outcome group (see section 1.8.2) there is a record of the GOS at 6-months. It may therefore be possible to use this second cohort to confirm the findings identified in the 6-month follow-up patients. This will be demonstrated in Chapter 6.

Chapter 6

‘Long-term’ outcome after TBI

6.1 Introduction

Within my second cohort of patients I will be able to detect associations between the cytokine SNPs under study and outcome after TBI a number of years after the insult. This 'long-term' follow-up group is described in section 1.8.2. These patients were re-contacted some years after their injury and were assessed according to a number of outcome measures (see Millar *et al.*, 2003). They are an ideal cohort in which to study outcome after TBI in the much longer term (*i.e.* years as opposed to months). They are an entirely separate cohort from the '6-month' follow-up group described in Chapter 5. Utilising this cohort it will be possible to investigate associations between any of the eleven chosen cytokine SNPs and 'long-term' outcome after TBI, and also to assess the effect on improvement or decline in function between 6-months post injury and the time of re-assessment some years after injury.

Outcome data (in the form of GOS measurements) at 6-months post TBI is available for the 'long-term' follow-up cohort. Using this second cohort it may be possible to confirm the findings of the '6-month' follow-up cohort, shown in Chapter 5. However it must be considered that the 'long-term' follow-up group were highly selected and are not directly analogous to the '6-month' follow-up cohort. In order to be eligible for entry into the 'long-term' follow up cohort it was necessary to have survived for at least 6-months after the TBI. Therefore those with the poorest six-month outcome were excluded. In addition 65% of those patients who were alive after 6-months had either died or were unable to be assessed at the time of further follow-up (Millar *et al.*, 2003). The two cohorts are not directly comparable, but useful secondary information may be obtained by searching for associations with 6-month outcome in the 'long-term' follow-up group.

6.2 General Methods

The collection and initial analysis of the genotyping data has been covered in the preceding chapters, and allele frequencies can be seen in section 4.5. Examples of the clinical data are shown in Section 1.8.

Databases were constructed using Microsoft Excel. Statistical analyses were performed using SPSS 14.0 for Windows. Binary logistic regression was performed using dichotomised GOS as the outcome measure. Age, categorised GCS at time of injury (mild, moderate & severe) and possession of the apolipoprotein E $\epsilon 4$ allele were entered as covariates.

Haplotype estimation was performed using SNPAnalyzer (Yoo *et al.*, 2005), available at http://www.istech.info/istech/board/login_form.jsp.

6.3 Results

In a similar manner to the prospective cohort the potential association between genotype, allele or haplotype has been investigated. The results for long-term outcome are shown first, and then the influence on change in functional level, then the results at 6-months, to correlate with the results from the previous chapter.

6.3.1 Association with 'long term' outcome

Patients were reassessed a mean of 18 years after their initial TBI (see section 1.8.2). Overall clinical outcome was assessed on the extended 8-point GOS, and this was dichotomised by including categories 1-4 in the poor outcome group and 5-8 in the good outcome group.

GENOTYPE	OUTCOME			*p*-value		GENOTYPE	OUTCOME			*p*-value
	unfavourable	favourable	Total				unfavourable	favourable	Total	
TNF 238						**TGF 509**				
GG	108 (85.0%)	245 (88.8%)	353	0.31		CC	49 (46.2%)	104 (48.4%)	153	0.57
GA	17 (13.4%)	30 (10.9%)	47			CT	46 (43.4%)	82 (38.1%)	128	
AA	2 (1.6%)	1 (0.3%)	3			TT	11 (10.4%)	29 (13.5%)	40	
Total	127 (100%)	276 (100%)	**403**			Total	106 (100%)	215 (100%)	**321**	
TNF 308						**TGF 800**				
GG	70 (57.9%)	183 (69.3%)	253	0.08		GG	96 (82.8%)	223 (88.1%)	319	0.37
GA	43 (35.5%)	71 (26.9%)	114			GA	18 (15.5%)	27 (10.7%)	45	
AA	8 (6.6%)	10 (3.8%)	18			AA	2 (1.7%)	3 (1.2%)	5	
Total	121 (100%)	264 (100%)	**385**			Total	116 (100%)	253 (100%)	**369**	
IL1 889						**IL6 174**				
CC	62 (48.8%)	135 (50.0%)	197	0.91		GG	28 (31.1%)	79 (40.1%)	107	0.34
CT	54 (42.5%)	115 (42.6%)	169			GC	44 (48.9%)	83 (42.1%)	127	
TT	11 (8.7%)	20 (7.4%)	31			CC	18 (20.0%)	35 (17.8%)	53	
Total	127 (100%)	270 (100%)	**397**			Total	90 (100%)	197 (100%)	**287**	
IL1 31						**IL6 572**				
CC	19 (16.5%)	38 (15.7%)	57	0.90		GG	70 (58.8%)	172 (67.5%)	242	0.16
CT	47 (40.9%)	105 (43.4%)	152			GC	47 (39.5%)	76 (29.8%)	123	
TT	49 (42.6%)	99 (40.9%)	148			CC	2 (1.7%)	7 (2.7%)	9	
Total	115 (100%)	242 (100%)	**357**			Total	119 (100%)	255 (100%)	**374**	
IL1 511						**IL6 597**				
CC	57 (47.1%)	109 (41.3%)	166	0.52		GG	46 (37.1%)	109 (41.7%)	155	0.45
TC	46 (38.0%)	107 (40.5%)	153			GA	58 (46.8%)	121 (46.4%)	179	
TT	18 (14.9%)	48 (18.2%)	66			AA	20 (16.1%)	31 (11.9%)	51	
Total	121 (100%)	264 (100%)	**385**			Total	124 (100%)	261 (100%)	**385**	
IL1 3953										
CC	73 (59.3%)	171 (63.1%)	244	0.30						
CT	46 (37.4%)	84 (31.0%)	130							
TT	4 (3.3%)	16 (5.9%)	20							
Total	123 (100%)	271 (100%)	**394**							

Table 6.1 (previous page) – Genotype association with long term dichotomised outcome. p-value assessed by χ^2 test.

6.3.1.1 SNPtype association with dichotomised long term outcome

Results are shown in Table 6.1. Although none of the results in this initial analysis reach significance it is interesting to note that again the TNFA -308 polymorphism trends towards an association with outcome, again with the A allele appearing to promote a poor outcome (p=0.08).

6.3.1.2 Allele association with dichotomised long term outcome

When possession of the rarer allele at each of the SNP positions is examined (Table 6.2) it can be seen that the -308 polymorphism in TNFA does show an association with outcome, as expected from the analysis in section 6.3.2.2. To confirm that this is an effect of the allele logistic regression analysis shall again be performed in the next section. No significant associations are identified among the other SNPs.

Table 6.2 (following page) – Allele association with long term dichotomised outcome. OR=odds ratio, CI=95% confidence interval.

Allele	Outcome			OR	C.I.
	poor	good			
IL1A -889					
T negative	62	135	197		
T positive	65	135	200	0.954	0.626-1.454
	127	270	397		
IL1B -31					
C negative	49	99	148		
C positive	66	143	209	1.072	0.684-1.681
	115	242	357		
IL1B -511					
T negative	57	109	166		
T positive	64	155	219	1.266	0.821-1.953
	121	264	385		
IL1B +3953					
T negative	73	171	244		
T positive	50	100	150	0.854	0.552-1.321
	123	271	394		
IL6 -174					
C negative	28	79	107		
C positive	62	118	180	0.675	0.397-1.145
	90	197	287		
IL6 -572					
C negative	70	172	242		
C positive	49	83	132	0.689	0.440-1.080
	119	255	374		
IL6 -597					
A negative	46	109	155		
A positive	78	152	230	0.822	0.530-1.276
	124	261	385		
TGFB -509					
T negative	49	104	153		
T positive	57	111	168	0.918	0.576-1.462
	106	215	321		
TGFB -800					
A negative	96	223	319		
A positive	20	30	50	0.646	0.349-1.194
	116	253	369		
TNFA -238					
A negative	108	245	353		
A positive	19	31	50	0.719	0.389-1.329
	127	276	403		
TNFA -308					
A negative	70	183	253		
A positive	51	81	132	0.608	0.389-0.949
	121	264	385		

6.3.1.3 Allele association with dichotomised long outcome assessed by binary logistic regression

Table 6.3 shows that the TNFA -308 polymorphism is associated with long term outcome after TBI, with possession of the A allele being a risk factor for poor outcome (p=0.018). None of the other SNPs shows a statistically significant effect.

SNP	n	Odds Ratio	C.I.	p-value
IL1α-889	336	0.984	0.602 – 1.610	0.950
IL1β-31	301	1.024	0.603 – 1.738	0.931
IL1β-511	323	0.831	0.500 – 1.379	0.473
IL1β+3953	332	1.261	0.756 – 2.103	0.374
IL6 -174	245	1.413	0.774 – 2.583	0.260
IL6 -572	315	1.461	0.864 – 2.470	0.157
IL6 -597	323	1.528	0.908 – 2.573	0.111
TGFβ-509	272	1.237	0.723 – 2.118	0.438
TGFβ-800	312	1.566	0.742 – 3.306	0.240
TNFα-238	341	1.325	0.653 – 2.689	0.436
TNFα-308	326	1.877	1.116 – 3.155	0.018

Table 6.3 – Odds ratio of achieving a good outcome if you do not possess the rare allele at each of the given SNP positions.
n=number of patients considered for the regression analysis, due to missing genetic or clinical data.
C.I. = 95% confidence intervals

6.3.1.4 Haplotype association with dichotomised long term outcome

As performed previously for both the prospective cohort and the retrospective cohort it is important to examine for SNP interactions within genes. Haplotypes will be examined for a potential association with dichotomised long term outcome. Results are demonstrated in table 6.4 and 6.5 (logistic regression analysis).

Haplotype	Outcome			*p*-value	OR	CI
	poor	good				
IL1						
CTCC negative	61	144	205			
CTCC positive	71	141	212	0.412	0.841	0.556-1.272
	132	285	417			
CCTC negative	70	140	210			
CCTC positive	62	145	207	0.458	1.169	0.774-1.768
	132	285	417			
TTCT negative	91	208	299			
TTCT positive	41	77	118	0.394	0.822	0.523-1.291
	132	285	417			
TTCC negative	113	243	356			
TTCC positive	19	42	61	0.927	1.028	0.572-1.847
	132	285	417			
IL6						
GGG negative	42	87	129			
GGG positive	90	198	288	0.791	1.062	0.681-1.657
	132	285	417			
CGA negative	79	174	253			
CGA positive	53	111	164	0.815	0.951	0.624-1.450
	132	285	417			
CGG negative	107	226	333			
CGG positive	25	59	84	0.676	1.117	0.663-1.882
	132	285	417			
CCA negative	92	221	313			
CCA positive	40	64	104	0.085	0.666	0.419-1.059
	132	285	417			

Haplotype	Outcome			*p*-value	OR	CI
	poor	good				
TNFA						
GG negative	11	18	29			
GG positive	121	267	388	0.451	1.348	0.618-2.942
	132	285	417			
GA negative	78	197	275			
GA positive	54	88	142	0.044	0.645	0.420-0.990
	132	285	417			
AG negative	113	255	368			
AG positive	19	30	49	0.254	0.700	0.378-1.295
	132	285	417			
TGFB						
CG negative	23	67	90			
CG positive	109	218	327	0.160	0.687	0.406-1.162
	132	285	417			
TG negative	66	135	201			
TG positive	66	150	216	0.617	1.111	0.735-1.679
	132	285	417			
CA negative	110	251	361			
CA positive	22	34	56	0.187	0.677	0.379-1.211
	132	285	417			

Table 6.4 – Haplotype association with dichotomised long term outcome. Patients were assessed as to whether they possessed the haplotype or not and this was compared to outcome. *p*-value calculated using χ^2 test, OR=odds ratio, CI=95% confidence interval

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	353	1.303	0.805 – 2.109	0.281
CCTC	353	0.866	0.536 – 1.397	0.555
TTCT	353	1.191	0.696 – 2.037	0.523
TTCC	353	0.914	0.470 – 1.777	0.791
TGFB				
CG	353	1.345	0.724 – 2.496	0.348
TG	353	1.196	0.737 – 1.941	0.468
CA	353	1.546	0.772 – 3.095	0.218
IL6				
GGG	353	0.945	0.564 – 1.582	0.829
CGA	353	1.120	0.689 – 1.820	0.649
CGG	353	0.698	0.371 – 1.313	0.698
CCA	353	1.634	0.953 – 2.800	0.074
TNFA				
GG	353	0.553	0.222 – 1.378	0.204
GA	353	1.806	1.095 – 2.978	0.021
AG	353	1.370	0.678 – 2.768	0.381

Table 6.5 – Logistic regression analysis of haplotype association with long term outcome. CI= 95% confidence interval.

As found in the analysis of 6-month outcome in the prospective cohort there is a significant association between the TNFA GA haplotype and long term outcome. However, for the same reasons as already outlined, this is more likely to represent an allele effect rather than a haplotype effect as it has also been shown that the A allele at the -308 position is associated with poorer outcome. The CCA haplotype in IL6 approaches significance in the regression analysis but not in the standard analysis.

6.3.2 Association with change in dichotomised outcome from 6-months to long term

In addition to searching for associations between the cytokine SNPs and outcome at two specific time points (6-months or 'long term') it may be hypothesised that the SNPs may be important determinants of an ongoing inflammatory reaction that may influence a decline (or improvement) in overall function between these time points. In the next set of results this possibility is explored. In order to do this the overall outcome of each patient, measured by GOS category, was compared between the two time points. Any change in category, either decline or improvement, was noted.

6.3.2.1 SNPtype association with change in GOS category

Table 6.6 shows the genotype associations with change in outcome. In this analysis none of the SNPs shows a significant association with change in overall outcome.

Table 6.6 (following page) – Genotype association with change in dichotomised outcome. p-value assessed by χ^2 test.

GENOTYPE	OUTCOME				*p*-value		GENOTYPE	OUTCOME				*p*-value
	improvement	none	decline					improvement	none	decline		
TNF 238							**TGF 509**					
GG	58	190	105	353	0.20		CC	20	88	45	153	0.51
GA	3	30	14	47			CT	18	73	37	128	
AA	0	3	0	3			TT	8	17	15	40	
	61	223	119	**403**				46	178	97	**321**	
TNF 308							**TGF 800**					
GG	40	142	71	253	0.67		GG	47	180	92	319	0.34
GA	16	61	37	114			GA	6	21	18	45	
AA	1	10	7	18			AA	1	4	0	5	
	57	213	115	**385**				54	205	110	**369**	
IL1 889							**IL6 174**					
CC	36	110	51	197	0.22		GG	12	67	28	107	0.38
CT	19	92	58	169			GC	23	65	39	127	
TT	4	19	8	31			CC	6	32	15	53	
	59	221	117	**397**				41	164	82	**287**	
IL1 31							**IL6 572**					
CC	10	33	14	57	0.57		GG	33	134	75	242	0.23
CT	25	86	41	152			GC	24	70	29	123	
TT	17	83	48	148			CC	0	5	4	9	
	52	202	103	**357**				57	209	108	**374**	
IL1 511							**IL6 597**					
CC	17	95	54	166	0.16		GG	19	94	42	155	0.42
TC	27	82	44	153			GA	32	92	55	179	
TT	13	39	14	66			AA	6	29	16	51	
	57	216	112	**385**				57	215	113	**385**	
IL1 3953												
CC	43	133	68	244	0.16							
CT	14	73	43	130								
TT	2	15	3	20								
	59	221	114	**394**								

6.3.2.2 Allele association with change in GOS category

Although no significant association was identified in the assessment of genotype effect allele association analysis has also been performed. As can be seen in Table 6.7 the rarer alleles at two of the SNP positions in the IL1 gene cluster approach significance (IL1A -889, p=0.09, and IL1B -511, p=0.07).

Binary logistic regression has also been performed. In order to do this the patients were categorised into those who had declined in function from 6-months to long term and those who had improved or remained static were treated as the second category (Table 6.8). The analysis was then repeated but this time including those patients who had improved in function in one group and those who had remained static or declined in function in another (Table 6.9).

Table 6.7 (following page) – Allele association with change in dichotomised outcome. p-value assessed by χ^2 test.

Allele	Outcome				*p*-value
	improvement	none	decline		
IL1A -889					
T negative	36	110	51	197	
T positive	23	111	66	200	0.092
	59	221	117	397	
IL1B -31					
C negative	17	83	48	148	
C positive	35	119	55	209	0.249
	52	202	103	357	
IL1B -511					
T negative	17	95	54	166	
T positive	40	121	58	219	0.069
	57	216	112	385	
IL1B +3953					
T negative	43	133	68	244	
T positive	16	88	46	150	0.170
	59	221	114	394	
IL6 -174					
C negative	12	67	28	107	
C positive	29	97	54	180	0.306
	41	164	82	287	
IL6 -572					
C negative	33	134	75	242	
C positive	24	75	33	132	0.324
	57	209	108	374	
IL6 -597					
A negative	19	94	42	155	
A positive	38	121	71	230	0.265
	57	215	113	385	
TGFB -509					
T negative	20	88	45	153	
T positive	26	90	52	168	0.737
	46	178	97	321	
TGFB -800					
A negative	47	180	92	319	
A positive	7	25	18	50	0.583
	54	205	110		
TNFA -238					
A negative	58	190	105	353	
A positive	3	33	14	50	0.113
	61	223	119	403	
TNFA -308					
A negative	40	142	71	253	
A positive	17	71	44	132	0.498
	57	213	115	385	

SNP	n	Odds Ratio	C.I.	*p*-value
IL1A -889	336	0.636	0.394 – 1.028	0.064
IL1B -31	301	1.193	0.718 – 1.984	0.495
IL1B -511	323	1.217	0.747 – 1.982	0.430
IL1B +3953	332	0.822	0.502 – 1.347	0.438
IL6 -174	245	0.820	0.459 – 1.465	0.502
IL6 -572	315	1.320	0.784 – 2.224	0.296
IL6 -597	323	0.689	0.416 – 1.141	0.148
TGFB -509	272	0.958	0.563 – 1.630	0.874
TGFB -800	312	0.538	0.271 – 1.066	0.075
TNFA -238	341	1.175	0.574 – 2.405	0.659
TNFA -308	326	0.819	0.497 – 1.348	0.432

Table 6.8 – Logistic regression analysis of allele association with decline in outcome from 6-months to long term. CI= 95% confidence interval.

SNP	n	Odds Ratio	C.I.	*p*-value
IL1A -889	336	1.986	1.044 – 3.778	0.037
IL1B -31	301	0.868	0.442 – 1.705	0.681
IL1B -511	323	0.636	0.326 – 1.240	0.184
IL1B +3953	332	2.419	1.151 – 5.085	0.020
IL6 -174	245	0.665	0.299 – 1.482	0.318
IL6 -572	315	0.752	0.394 – 1.434	0.386
IL6 -597	323	0.733	0.375 – 1.433	0.365
TGFB -509	272	0.690	0.339 – 1.407	0.308
TGFB -800	312	1.603	0.539 – 4.772	0.396
TNFA -238	341	8.032	1.076 – 59.946	0.042
TNFA -308	326	1.155	0.586 – 2.277	0.678

Table 6.9 – Logistic regression analysis of allele association with improvement in outcome from 6-months to long term. CI= 95% confidence interval.

6.3.2.3 Haplotype association with change in GOS category

The TTCT haplotype in the IL1 gene cluster shows a significant (p=0.01) association with change in outcome, with TTCT possessors being less likely to show any improvement (Table 6.10).

Haplotype	Outcome				p-value
	improvement	none	decline		
IL1					
CTCC negative	31	117	57	205	
CTCC positive	31	113	68	212	0.631
	62	230	125	417	
CCTC negative	25	114	71	210	
CCTC positive	37	116	54	207	0.099
	62	230	125	417	
TTCT negative	54	160	85	299	
TTCT positive	8	70	40	118	0.014
	62	230	125	417	
TTCC negative	53	200	103	356	
TTCC positive	9	30	22	61	0.510
	62	230	125	417	
IL6					
GGG negative	18	75	36	129	
GGG positive	44	155	89	288	0.714
	62	230	125	417	
CGA negative	35	147	71	253	
CGA positive	27	83	54	164	0.323
	62	230	125	417	
CGG negative	50	180	103	333	
CGG positive	12	50	22	84	0.641
	62	230	125	417	
CCA negative	45	171	97	313	
CCA positive	17	59	28	104	0.706
	62	230	125	417	

Haplotype	Outcome				*p*-value
	improvement	none	decline		
TNFA					
GG negative	2	16	11	29	
GG positive	60	214	114	388	0.370
	62	230	125	417	
GA negative	43	156	76	275	
GA positive	19	74	49	142	0.340
	62	230	125	417	
AG negative	59	199	110	368	
AG positive	3	31	15	49	0.172
	62	230	125	417	
TGFB					
CG negative	19	43	28	90	
CG positive	43	187	97	327	0.123
	62	230	125	417	
TG negative	27	115	59	201	
TG positive	35	115	66	216	0.642
	62	230	125	417	
CA negative	55	199	107	361	
CA positive	7	31	18	56	0.841
	62	230	125	417	

Table 6.10 – Haplotype association with change in outcome. Patients were assessed as to whether they possessed the haplotype or not and this was compared to change in outcome. *p*-value calculated using χ^2 test.

In a similar manner to the allele analysis binary logistic regression can be used to assess any haplotype association if the subjects are assessed according to either decline or improvement in outcome scores between 6-months and long term. Results when looking for an association with decline in function are shown in Table 6.11 and for improvement in function in Table 6.12.

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	353	0.938	0.593 – 1.482	0.782
CCTC	353	1.417	0.894 – 2.244	0.138
TTCT	353	0.776	0.469 – 1.284	0.324
TTCC	353	0.650	0.355 – 1.191	0.163
TGFB				
CG	353	1.017	0.576 – 1.793	0.955
TG	353	0.961	0.607 – 1.523	0.866
CA	353	0.645	0.338 – 1.232	0.185
IL6				
GGG	353	0.990	0.602 – 1.628	0.967
CGA	353	0.725	0.457 – 1.153	0.174
CGG	353	1.633	0.869 – 3.069	0.128
CCA	353	1.181	0.689 – 2.025	0.545
TNFA				
GG	353	1.319	0.534 – 3.260	0.549
GA	353	0.739	0.459 – 1.189	0.213
AG	353	1.070	0.532 – 2.151	0.849

Table 6.11 – Logistic regression analysis of haplotype association with decline in outcome from 6-months to long term. CI= 95% confidence interval.

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	353	0.914	0.500 – 1.670	0.769
CCTC	353	0.735	0.401 – 1.347	0.319
TTCT	353	3.229	1.325 – 7.870	0.010
TTCC	353	0.919	0.403 – 2.096	0.840
TGFB				
CG	353	1.388	0.689 – 2.793	0.359
TG	353	0.815	0.443 – 1.498	0.510
CA	353	1.897	0.644 – 5.590	0.245
IL6				
GGG	353	0.861	0.441 – 1.682	0.661
CGA	353	0.792	0.432 – 1.453	0.792
CGG	353	1.059	0.485 – 2.312	0.885
CCA	353	0.851	0.430 – 1.686	0.643
TNFA				
GG	353	0.280	0.037 – 2.131	0.219
GA	353	1.087	0.572 – 2.066	0.798
AG	353	7.887	1.059 – 58.764	0.044

Table 6.12 – Logistic regression analysis of haplotype association with improvement in outcome from 6-months to long term. CI= 95% confidence interval.

The association between the TTCT haplotype in the IL1 gene cluster shown in Table 6.10 is confirmed in the regression analysis, with TTCT possessors significantly less likely to show an improvement (p=0.01, Table 6.12). A similar effect for possessors of the AG haplotype in TNFA may also be present (p=0.04). No significant associations were identified when comparing those who had declined in function with those that were unchanged or improved (Table 6.11).

6.3.3 Association with 6-month outcome

6.3.3.1 SNPtype association with dichotomised 6-month outcome

Results are shown in Table 6.13. Within this smaller cohort the -889 SNP in IL1A reaches significance at the 0.05 level, but none of the other SNPs show an association. Importantly it appears that the finding of a significant association between TNFA -308 and six month outcome is not being replicated. However further analysis in the form of allele and regression analysis is required.

Table 6.13 (following page) – SNP genotype vs. dichotomised 6-month outcome in the retrospective group. *p*-values measured using χ^2 test.

GENOTYPE	OUTCOME			*p*-value		GENOTYPE	OUTCOME			*p*-value
	unfavourable	favourable					unfavourable	favourable		
TNF 238						**TGF 509**				
GG	90	271	361	0.21		CC	37	118	155	0.65
GA	14	33	47			CT	37	95	132	
AA	2	1	3			TT	9	31	40	
	106	305	**411**				83	244	**327**	
TNF 308						**TGF 800**				
GG	59	197	256	0.45		GG	82	240	322	0.51
GA	34	83	117			GA	9	37	46	
AA	5	13	18			AA	2	3	5	
	98	293	**391**				93	280	**373**	
IL1 889						**IL6 174**				
CC	55	142	197	0.04		GG	25	85	110	0.43
CT	35	138	173			GC	38	89	127	
TT	13	20	33			CC	13	40	53	
	103	300	**403**				76	214	**290**	
IL1 31						**IL6 572**				
CC	15	42	57	0.87		GG	56	189	245	0.06
CT	39	116	155			GC	43	84	126	
TT	42	109	151			CC	1	8	9	
	96	267	**363**				99	281	**380**	
IL1 511						**IL6 597**				
CC	47	122	169	0.64		GG	39	121	160	0.87
TC	36	119	155			GA	49	133	182	
TT	17	51	68			AA	13	38	51	
	100	292	**392**				101	292	**393**	
IL1 3953										
CC	66	180	246	0.65						
CT	30	103	133							
TT	6	16	22							
	102	299	**401**							

6.3.3.2 Allele association with dichotomised 6-month outcome

The finding that the -308 A allele was associated with poor outcome in the prospective cohort is not repeated in this cohort (see Table 6.14). No significant allele associations are identified within this cohort

Allele	Outcome			OR	C.I.
	poor	good			
IL1A -889					
T negative	55	142	197		
T positive	47	153	200	1.261	0.803-1.980
	102	295	397		
IL1B -31					
C negative	41	107	148		
C positive	54	155	209	1.100	0.684-1.768
	95	262	357		
IL1B -511					
T negative	46	120	166		
T positive	52	167	219	1.231	0.776-1.952
	98	287	385		
IL1B +3953					
T negative	65	179	244		
T positive	35	115	150	1.193	0.743-1.915
	100	294	394		
IL6 -174					
C negative	25	82	107		
C positive	51	129	180	0.771	0.444-1.340
	76	211	287		
IL6 -572					
C negative	55	187	242		
C positive	42	90	132	0.630	0.392-1.012
	97	277	374		
IL6 -597					
A negative	37	118	155		
A positive	62	168	230	0.850	0.531-1.360
	99	286	385		
TGFB -509					
T negative	36	117	153		
T positive	45	123	168	0.841	0.507-1.395
	81	240	321		
TGFB -800					
A negative	80	239	319		
A positive	11	39	50	1.187	0.580-2.427
	91	278	369		
TNFA -238					
A negative	88	265	353		
A positive	16	34	50	0.706	0.372-1.340
	104	299	403		
TNFA -308					
A negative	59	194	253		
A positive	37	95	132	0.781	0.484-1.260
	96	289	385		

Table 6.14 (previous page) – Allele association with 6-month dichotomised outcome. OR=odds ratio, CI=95% confidence interval.

6.3.3.3 Allele association with dichotomised 6-month outcome assessed by binary logistic regression

As expected from the results of the initial analysis of the allele effects none of the SNPs demonstrates a significant association with outcome from TBI at 6-months in the logistic regression analysis (see Table 6.15). The findings in the prospective cohort have not been confirmed.

SNP	n	Odds Ratio	C.I.	*p*-value
IL1α -889	341	0.672	0.406 – 1.113	0.123
IL1β -31	306	0.997	0.585 – 1.698	0.990
IL1β -511	328	0.847	0.505 – 1.420	0.528
IL1β +3953	336	0.741	0.443 – 1.266	0.272
IL6 -174	247	1.337	0.720 – 2.482	0.358
IL6 -572	319	1.456	0.859 – 2.469	0.163
IL6 -597	328	1.204	0.713 – 2.034	0.487
TGFβ -509	276	1.445	0.827 – 2.528	0.196
TGFβ -800	314	0.676	0.296 – 1.544	0.353
TNFα -238	346	1.470	0.728 – 2.968	0.283
TNFα -308	330	1.282	0.753 – 2.184	0.361

Table 6.15 – Odds ratio of achieving a good outcome if you do not possess the rare allele at each of the given SNP positions.
n=number of patients considered for the regression analysis, due to missing genetic or clinical data.
C.I. = 95% confidence intervals

6.3.3.4 Haplotype association with dichotomised 6-month outcome

Although no significant associations between individual SNPs and outcome at 6-months have been identified it is important to assess for haplotype effects, as previously discussed. The haplotype frequencies in the retrospective cohort are similar to those in the prospective cohort and shown in Table 6.16, for comparison.

	Hap	FreqR	FreqP		Hap	FreqR	FreqP
TNFα				**TGFβ**			
	GG	74.53%	71.96%		CG	59.30%	57.05%
	GA	19.12%	21.22%		TG	31.88%	29.73%
	AG	5.94%	5.92%		CA	6.7%	8.99%
	AA	0.41%	0.9%		TA	2.12%	4.24%
IL1				**IL6**			
	CTCC	31.49%	30.19%		GGG	43.99%	34.32%
	CCTC	28.12%	22.34%		CGA	20.88%	18.58%
	TTCT	13.84%	11.88%		CGG	12.44%	15.63%
	TTCC	8.89%	5.92%		CCA	10.74%	11.95%
	CTCT	3.46%	3.05%		GCG	4.34%	5.53%
	CCCC	3.15%	6.18%		GGA	3.62%	6.62%
	CTTC	2.08%			CCG	2.64%	5.31%
	TCTT	2.03%	2.17%		GCA	1.34%	2.24%
	TCTC	1.57%					
	TTTC	1.21%	1.41%				
	TCCC	1.03%	2.72%				
	CCTT	1.02%	1.09%				
	CCCT	0.79%	1.51%				
	TCCT	0.65%	1.83%				
	CTTT	0.48%	1.04%				
	TTTT	0.21%	1.12%				

Table 6.16 – Estimated haplotype frequencies within the retrospective cohort.
Haplotypes are arranged as follows; TNFA:-238/-308, TGFB: -509/-800, IL1: IL1A-889/IL1B -31/-511/+3953, IL6: -174/-572/-597. Hap=haplotype, FreqR=frequency in the retrospective cohort, FreqP=frequency in the prospective cohort.

Table 6.17 shows the haplotype associations with outcome at 6-months. As previously only those haplotypes with a frequency of at least 5% in the cohort are considered.

Haplotype	Outcome			*p*-value	OR	CI
	poor	good				
IL1						
CTCC negative	52	161	213			
CTCC positive	57	155	212	0.559	0.878	0.568-1.358
	109	316	425			
CCTC negative	58	157	215			
CCTC positive	51	159	210	0.525	1.152	0.745-1.781
	109	316	425			
TTCT negative	84	218	302			
TTCT positive	25	98	123	0.109	1.510	0.911-2.506
	109	316	425			
TTCC negative	89	273	362			
TTCC positive	20	43	63	0.230	0.701	0.392-1.254
	109	316	425			
IL6						
GGG negative	33	98	131			
GGG positive	76	218	294	0.886	0.966	0.602-1.550
	109	316	425			
CGA negative	66	195	261			
CGA positive	43	121	164	0.830	0.952	0.610-1.488
	109	316	425			
CGG negative	88	252	340			
CGG positive	21	64	85	0.824	1.064	0.614-1.844
	109	316	425			
CCA negative	77	243	320			
CCA positive	32	73	105	0.192	0.723	0.444-1.178
	109	316	425			

Table 6.17 – Haplotype association with dichotomised 6-month outcome. Patients were assessed as to whether they possessed the haplotype or not and this was compared to outcome. *p*-value calculated using χ^2 test, OR=odds ratio, CI=95% confidence interval

Haplotype	Outcome			*p*-value	OR	CI
	poor	good				
TNFA						
GG negative	8	22	30			
GG positive	101	294	395	0.894	1.059	0.457-2.452
	109	316	425			
GA negative	68	211	279			
GA positive	41	105	146	0.406	0.825	0.525-1.298
	109	316	425			
AG negative	93	283	376			
AG positive	16	33	49	0.232	0.678	0.357-1.287
	109	316	425			
TGFB						
CG negative	22	70	92			
CG positive	87	246	333	0.667	0.889	0.519-1.522
	109	316	425			
TG negative	53	151	204			
TG positive	56	165	221	0.880	1.034	0.669-1.599
	109	316	425			
CA negative	95	272	367			
CA positive	14	44	58	0.777	1.098	0.576-2.093
	109	316	425			

Table 6.17 - cont'd

No significant haplotype associations with 6-month outcome are identified within the retrospective cohort. A logistic regression analysis of the haplotypes in relation to dichotomised 6-month outcome was also performed (Table 6.18). A mildly significant result is seen for the TTCT haplotype. However this haplotype was not identified in the prospective cohort as having an effect, and it only just reaches significance at the 0.05 level in this analysis. In keeping with the findings shown in Table 6.16 none of the other haplotypes show a significant association.

Haplotype	n	Odds Ratio	C.I.	*p*-value
IL1				
CTCC	358	1.255	0.770 – 2.043	0.362
CCTC	358	0.918	0.565 – 1.491	0.729
TTCT	358	0.550	0.306 – 0.990	0.046
TTCC	358	1.447	0.764 – 2.742	0.257
TGFB				
CG	358	0.971	0.529 – 1.783	0.924
TG	358	1.255	0.767 – 2.054	0.366
CA	358	0.752	0.354 – 1.599	0.459
IL6				
GGG	358	1.305	0.760 – 2.240	0.334
CGA	358	1.065	0.649 – 1.746	0.804
CGG	358	0.922	0.492 – 1.730	0.801
CCA	358	1.345	0.778 – 2.324	0.289
TNFA				
GG	358	0.825	0.322 – 2.116	0.689
GA	358	1.165	0.701 – 1.935	0.555
AG	358	1.537	0.767 – 3.801	0.226

Table 6.18 – Logistic regression analysis of haplotype association with 6-month outcome. CI= 95% confidence interval.

6.4 Inflammatory profiles and change in outcome

In section 5.5 a model was constructed whereby the influence of 'inflammatory profiles' was investigated. There is some evidence linking the cytokine SNPs investigated there with long term chronic inflammation after TBI (see Gentleman *et al.*, 2004 for example). One may hypothesize that those patients with a 'neurotoxic' inflammatory profile would be more likely to decline in function from 6-months to 'long term', whilst those with a 'neuroprotective' profile would be more likely to improve in function. The same model used in section 5.5 has been used to assess this hypothesis.

6.4.1 Risk of decline in outcome from 6-months to long term according to the number of 'high risk' genetic factors

Possession of one or more of the 'risk' SNP or haplotypes increases the risk of decline in overall outcome from 6-months to long term. None of the results is significant at the 0.05 level (Table 6.19).

No of high risk factors	n	Decline in outcome	Improve/no change in outcome	χ^2 *p*-value	OR (95% confidence interval)
0	101	24	77	referent	referent
1	114	34	80	0.318	1.364 (0.742-2.507)
2	113	38	75	0.112	1.626 (0.891-2.968)
3	45	15	30	0.227	1.604 (0.742-3.467)
4	2	1	1	0.391	3.208 (0.193-53.262)

Table 6.19 – Odds ratio.of a decline in outcome category according to the number of high risk factors possessed. n=number of subjects, OR=odds ratio.

6.4.2 Risk of decline in outcome from 6-months to long term according to the profile of 'high risk' genetic factors

Subjects with one, two or three 'high risk' genetic factors were then classified according to their genetic profile and compared with those subjects with none of the risk factors (Table 6.20).

Genetic Profile								
-308 A	-889 T	+3953 T	GGA	**n**	**Decl**	**No Decl**	χ^2	**OR (95% C.I.)**
-	-	-	-	101	24	77	-	-
+	-	-	-	58	19	39	0.219	1.56 (0.76-3.19)
-	+	-	-	37	11	26	0.475	1.36 (0.59-3.15)
-	-	+	-	14	3	11	0.847	0.88 (0.23-3.40)
-	-	-	+	5	1	4	0.847	0.80 (0.09-7.52)
+	+	-	-	24	11	13	0.030	2.71 (1.08-6.84)
+	-	+	-	5	1	4	0.847	0.88 (0.23-3.40)
+	-	-	+	1	0	1	-	-
-	+	+	-	79	24	55	0.319	1.40 (0.72-2.72)
-	+	-	+	2	1	1	0.391	3.21 (0.19-53.26)
-	-	+	+	2	1	1	0.391	3.21 (0.19-53.26)
+	+	+	-	37	12	25	0.304	1.54 (0.67-3.52)
+	+	-	+	1	0	1	-	-
+	-	+	+	0	0	0	-	-
-	+	+	+	7	3	4	0.259	2.41 (0.50-11.51)
+	+	+	+	2	1	1	0.391	3.21 (0.19-53.26)

Table 6.20 – Risk of decline in outcome category according to the genetic profile of high risk factors. n=number of subjects, Decl=decline in category, No decline=improvement or no change in category, OR=odds ratio, CI=confidence interval.

The only significant effect is seen in those patients with both the TNFA -308 A allele and the IL1A -889 T allele. The combination of these two alleles was also significantly associated with poor outcome at 6-months (Table 5.15). It appears that they may continue to exert an effect which leads to a further decline in overall function. No other combination exerts a significant effect.

6.4.3 Improvement in overall outcome from 6-months to long term, according to the number of 'protective' genetic factors

The protective factors have been analysed to examine whether they have a significant effect in determining whether there is an improvement in function from 6-months to long term. Results are shown in Table 6.21. Possession of at least one protective factor makes an improvement in function more likely, although this is not a significant effect.

No of protective factors	n	Improvement in outcome	Decline/no change in outcome	χ^2 *p*-value	OR (95% confidence interval)
0	31	2	29	referent	referent
1	125	19	106	0.201	2.599 (0.572-11.811)
2	140	21	119	0.207	2.559 (0.567-11.539)
3	11	2	9	0.255	3.222 (0.395-26.256)

Table 6.21 – Odds ratio.of an improvement in outcome according to the number of protective factors possessed. n=number of subjects, OR=odds ratio.

6.4.4 Improvement in overall outcome from 6-months to long term, according to the profile of 'protective' genetic factors

No significant effects are found when the profile of protective factors is examined (Table 6.22), although each combination seems to favour an improvement in overall outcome from 6-months to long term.

Genetic Profile							
-509 T	-800 A	GGG	**n**	**Imp**	**No Imp**	χ^2	**OR (95% C.I.)**
-	-	-	31	2	29	-	-
+	-	-	31	5	26	0.229	2.79 (0.50-15.62)
-	+	-	8	1	7	0.567	2.07 (0.16-26.22)
-	-	+	86	13	73	0.216	2.58 (0.55-12.16)
+	+	-	6	1	5	0.401	2.90 (0.22-38.32)
+	-	+	115	17	98	0.221	2.52 (0.55-11.53)
-	+	+	19	3	16	0.285	2.72 (0.41-18.01)
+	+	+	11	2	9	0.255	3.22 (0.40-26.26)

Table 6.22 – Odds ratio of an improvement in outcome according to the genetic profile of protective factors. n=number of subjects, Imp=improvement in category, No Imp= Decline or no change in outcome category, OR=odds ratio, CI=confidence interval.

6.5 Discussion

In this chapter the results from the retrospective cohort have been analysed. This cohort is unique in being the only cohort in the published literature in which genetic polymorphisms have been examined to ascertain any influence on long term outcome after head injury (Millar *et al.*, 2003). In addition to the genetic information, this paper is useful as it demonstrates that a high proportion of those patients assessed 15 to 25 years after injury were severely impaired. Deterioration in outcome, as measured by the GOS, occurs in almost one third of patients between 6-months and their late assessment and improvement in this time period is relatively uncommon (but does occur). As discussed by Millar *et al.*, this supports previous findings of late deterioration after TBI (Corkin *et al.*, 1989; Plessman *et al.*, 2000).

The functional impact of TBI may have consequences far beyond the 6-month outcome period considered in the previous chapter. As already discussed, head injury is associated with cognitive decline some time after the injury. Cytokines play a role in chronic inflammatory states, such as may occur in Alzheimer's disease (see Section 1.3 and Gentleman *et al.*, 2004), and polymorphisms within the cytokine genes may trigger, exacerbate or ameliorate this chronic activation (Scola *et al.*, 2003; Laws *et al.*, 2005; Dickson *et al.*, 2005; Lio *et al.*, 2006). Thus cytokine polymorphisms may be associated with head injury outcome at a much later stage than the 6-months post injury considered to this point. The long-term outcome data from the retrospective cohort enables this to be assessed.

Intriguingly it is again the TNFA -308 polymorphism, together with the GA TNF haplotype, that shows a significant association with outcome many years after TBI. This suggests that TNFα, and specifically the -308 polymorphism, is not only associated with outcome relatively soon after TBI but also has an ongoing relationship with outcome. Again the rarer A allele is associated with poorer outcome long-term so the GA haplotype association that was detected may actually be a reflection of the allele effect, as previously discussed. Recent experimental evidence (see Section 1.9.1) suggests that lack of TNFα is detrimental in the short-term but beneficial in the long run. The A allele is associated with increased

transcription of TNFA, but there is no firm evidence to confirm whether this continues in states of chronic neuroinflammation. There is evidence to suggest this polymorphism contributes to chronic inflammation in other conditions, affecting a number of organ systems,. The rarer A allele is significantly overrepresented in patients who suffer from chronic bronchitis, suggesting a role in chronic inflammation of the respiratory tract (Huang *et al.*, 1997). Carriage of the rarer allele at position -308 is also associated with a significantly increased risk of fibrosing alveolitis (Whyte *et al.*, 2000). In the gastrointestinal system this TNFA polymorphism may influence disease activity in sufferers of Crohn's disease (Sykora *et al.*, 2006). Within the CNS the -308 SNP has recently been shown to affect the risk of late-onset AD, possibly by modifying the cerebral inflammatory response (Ramos *et al.*, 2006). An earlier study has also suggested a role for this polymorphism in AD (Culpan *et al.*, 2003). Despite this evidence a biological explanation linking the activity of the SNP with actual biological action is not yet clear, and requires further investigation. It must also be remembered that this is an association study and the -308 polymorphism may not be directly involved in the underlying processes, but merely acting as a marker. When examining the retrospective cohort alone the TNFA -308 SNP is not significantly associated with outcome at 6-months but becomes associated by the time of the 'late' assessment. One may therefore expect to see an association between the SNP and 'change' in outcome. This is not the case. This may be as a result of a methodological issue, as the 'change' is not between the dichotomised groups but between GOS categories. Alternatively this cohort may not be large enough to detect a small effect. Two SNPs – IL1A -889 and IL1B +3953 - do show a significant association with change in function between 6-months and 'long term'. IL-1 is known to have a role in a number of chronic neuroinflammatory conditions (see Section 1.9.2) and would be expected to have a major role in the control of ongoing inflammation after TBI (Section 1.3). The -889 SNP is associated with microglial activation and +3953 with increase IL1 expression. There is thus biological evidence to support these SNPs as being involved with ongoing neuroinflammation after TBI, which leads to a late decline in overall function.

As discussed in the previous chapter it will be important to replicate these findings in other cohorts before any concrete conclusions can be drawn. No other studies have

searched for associations between cytokine SNPs and outcome post-TBI at such a late stage after the injury. Until further studies are completed the findings of this study must remain speculative to some degree.

In a similar manner to the previous chapter I have created a series of 'toxic' and 'protective' inflammatory profiles. As has been shown there is little evidence to specifically support the role of these SNPs at such a long time course after TBI, although there is certainly a role for them in chronic neuroinflammation. The profiles should therefore again be treated as a model rather than definitive fact at this stage. As per Chapter 5 the models again reveal interesting results. Possession of at least one of the 'toxic' factors increases the chances of a decline in function whereas, conversely, possession of at least one 'protective' factor increases the chances of an improvement in overall function. Looking at the toxic group in more detail it can be seen that the +3953 SNP and the GGA IL6 haplotype, when possessed alone, appear to exert the reverse effect to that predicted from the literature, although the confidence intervals are wide. When these are possessed in combination with the other toxic factors then the protective effect disappears. The only statistically significant effect is seen when TNFA -308 and IL1A -889 are possessed together (p=0.03), increasing the risk of a decline in function. The +3953 SNP acts to ameliorate this effect when all three SNPs are possessed. This would seem to fit with the findings earlier in this chapter, where those three SNPs are the ones found to show significant effects.

As would be predicted the 'protective' model shows that possession of at least one of the 'protective' factors increases the chances of an improvement in overall function from 6-months to 'long-term'. No combination of the three factors appears to favour improvement over any of the other combinations, although the largest odds ratio is achieved by the group who possess all three of the factors. The number of patients whose overall function (as assessed by the GOS) improved during the period in question is much smaller than the number who declined, so the confidence intervals are much larger in this model as compared to the 'toxic' model.

If these results are compared to those obtained in the similar models in the previous chapter it can be seen that the results are strikingly similar in the effects of the

chosen SNPs, despite the use of two different cohorts and different outcome measures. This would seem to confirm the roles of these SNPs as either neuroprotective or neurotoxic, and that their effects (although possibly small) persist for some time.

Six month outcome data is available for this cohort so it can be potentially used to confirm the findings in the prospective cohort, demonstrated in Chapter 5. The main finding in the prospective cohort was that possession of the rarer A allele at the TNFA -308 SNP position was associated with worse outcome at 6-months. This finding has not been confirmed in the retrospective cohort, with the -308 SNP not being shown to be significant in any of the analyses of 6-month outcome. In fact none of the 11 SNPs examined shows an association with 6-month outcome in this cohort. This may be explained in a number of ways. Firstly this may mean that that the results obtained in the prospective cohort are false positives (as discussed in the previous chapter) and that none of these SNPs are associated with 6-month outcome. However another explanation may be that the retrospective cohort may not be as representative of the general population (of head injured patients) as the prospective cohort is (and thus not directly comparable), because of the selection criteria for entry into the retrospective cohort. The cohort included only those patients who had survived for 6-months after their injury and were alive and available for follow-up 15-25 years later. There may have been some selective loss of certain alleles in this process. As can be seen in Table 4.2 the genotype frequencies in the two cohorts are relatively similar, although small variations in the percentage of rare alleles may result in large differences when the cohorts are compared. Patients who had more severe injuries or had significantly declined could have been lost due to death prior to the study or being too unwell to participate. It is therefore possible that a real association of TNFA -308 is being masked in the retrospective cohort due to the manner in which the patients were selected.

The finding of no other association between any of the cytokine SNPs and 6-month outcome is in keeping with the findings from the prospective cohort. In the retrospective cohort the -889 SNP in IL1A just reaches significance in the analysis of dichotomised outcome and genotypes, but this finding is not repeated in any of the further analyses. The TTCT haplotype in IL1 also just reaches significance in the

logistic regression analysis. Although this needs to be acknowledged there is little evidence to support this being a true positive. This haplotype was non-significant in the prospective cohort and only just reaches significance in this analysis. It showed no significance in the standard analysis.

In this chapter I have used a unique cohort to evaluate the role of cytokine gene polymorphisms in determining both 'long-term' outcome after TBI, and change in outcome from 6-months to long-term. In addition I have utilised the available data to see if the 6-month outcome findings in the prospective cohort could be replicated. In contrast to the prospective cohort, where the TNFA -308 SNP was highlighted, no SNP associations with 6-month outcome were identified. This may have been due to the cohort selection criteria or may suggest the finding in the prospective cohort is a false positive. When concentrating on 'long term' outcome the TNFA -308 SNP is again identified as having an association with outcome. The fact that the same SNP shows a significant association is further evidence to suggest the finding in the prospective cohort is not simply a false positive. To fully understand these results further work is required. The findings must be replicated in other cohorts, that in the case of the 'long term' follow-up will be difficult due to the problems of recruiting sufficient participants and the length of time required to wait for the 'long term' data. It would be interesting to see if the results are replicated in other ethnic groups. If the association is confirmed then the underlying biology will need further investigation. Little is known about the long term effects of most of the cytokine SNPs after TBI, especially with respect to the initiation and continuation of a chronic inflammatory response. As discussed in the paper by Millar *et al.*, (2003) despite the long follow-up time in the retrospective cohort the subjects remain relatively young and it is to early to assess whether there is an association with further cognitive decline or the onset of Alzheimer's disease.

Appendices

Appendix A

A.1 Organotypic hippocampal slice culture models

A correlation between gene polymorphisms and clinical outcome is a strong indicator that the gene product may be acting as a disease- or condition-modifying agent. However, increased gene expression may also occur as a non-specific response to the injury itself and as such may not be influencing patient outcome at all. In order to test this possibility, the potential neuroprotective/neurotoxic effects of cytokines can be investigated in *in-vitro* models of neurodegeneration that reflect a number of the processes known to underlie traumatic brain injury.

A number of in-vitro techniques are available to study the properties, interactions and responses of neuronal tissue. Primary dissociated cultures have been used for many years (Brewer, 1997) but have the disadvantage that the complex neuronal networks, which to some degree determine the behaviour of the cells within the network, cannot be represented. Organotypic cell cultures are not dissociated and therefore cells within the culture maintain their relationship with surrounding cells. The *in-vitro* organotypic hippocampal slice culture (OHSC) model retains many of the intercellular interactions that occur within the *in-vivo* brain.

A.1.1 Organotypic hippocampal slice cultures

The hippocampus is an integral part of the limbic system, important for learning, memory and spatial orientation. In the human the hippocampal formation consists of the subiculum, the hippocampus and the dentate gyrus, located along the base of the lateral ventricle in the temporal lobe. The hippocampus is divided into four zones, labelled CA (*cornu ammonis*) 1 through 4. A number of intrinsic pathways exist within the hippocampus, and are preserved in the organotypic model. The best known are the so-called Schaffer collaterals, which run between CA3 and CA1.

Stoppini *et al.* published a method for culturing organotypic hippocampal slices in 1991 (Stoppini et al., 1991). This was an advance on previous techniques for producing organotypic cultures utilising the roller tube method (Gahwiler, 1984). Subsequent minor alterations have refined the method and it is now used as a

valuable *in-vitro* model (Finley *et al.*, 2004). Briefly, the technique entails rapid dissection of the hippocampus from Wistar rat pups, sectioning and then growth of the sections on a semi-permeable membrane. The OHSCs are well characterised as models for both cerebral ischaemia (Newell *et al.*, 1995; Pringle *et al.*, 1997) and hypoxia (Pringle *et al.*, 2001; Graulich *et al.*, 2002).

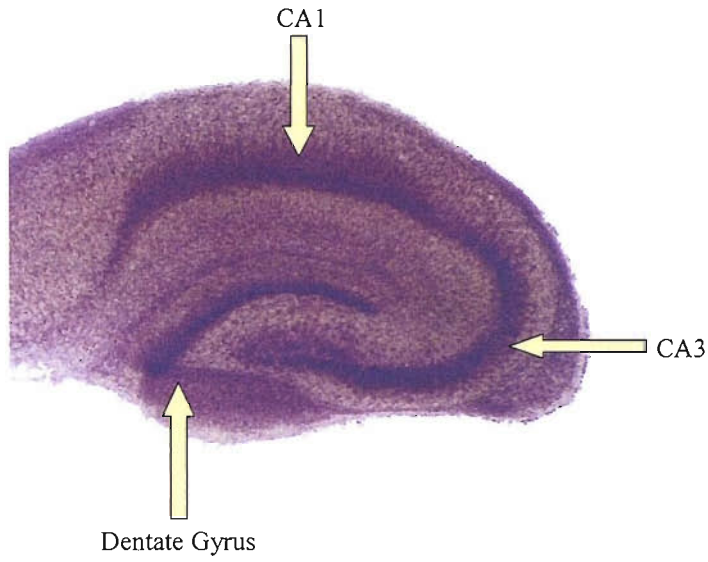


Figure A.1 – An organotypic hippocampal slice culture, clearly demonstrating the different cell layers

7.1.2 Cytokines and OHSC models of ischaemia and hypoxia

The neuroprotective/neurotoxic effects of cytokines in various in-vitro models were briefly described in Chapter 1. TNF-α has a crucial role in the control of the inflammatory response, and has been demonstrated to have both neurotoxic and neuroprotective actions depending upon the model and protocol used. Models of ischaemia and hypoxia best resemble the type of damage seen after TBI. In a hippocampal slice culture model of ischaemia TNF-α attenuated and augmented damage, dependent upon whether TNF-α was present before or after the ischaemic insult (Wilde *et al.*, 2000). When cultures were exposed to TNF-α for 24 hours prior to the ischaemic insult reduced cell death was observed. In contrast if the TNF-α was not added to the culture medium until after the insult then cell death was enhanced. This paper clearly demonstrates the biphasic effects of TNF-α.

Although considered to be a pro-inflammatory cytokine, interleukin-1 has some neuroprotective effects in an OHSC model of ischaemia, but not hypoxia. Pringle *et al.* (2001) demonstrated that high concentrations of IL-1 were neuroprotective in models of oxygen/glucose deprivation and N-methyl-D-aspartate (NMDA) excitotoxicity, whilst lower concentrations showed no effect. In contrast, in a model of hypoxia low concentrations of IL-1 potentiated neurodegeneration whilst higher concentrations had no effect.

IL-6 has been investigated in an OHSC model of NMDA excitotoxicity (Pizzi *et al.*, 2004), where it was shown that various concentrations of IL-6 were neuroprotective when administered before NMDA, although the response after oxygen/glucose deprivation or hypoxia has not been established.

The effects of TGFβ have not been investigated in OHSC models of ischaemia and hypoxia.

A.1.3 Aims

The OHSC studies were run in parallel to the genetic studies, and therefore commenced prior to any results from the genetic studies. IL-6 was chosen as the initial candidate cytokine for the reasons below, although subsequently the SNPs identified for the genetic studies did not prove to be associated with outcome after TBI. This, of course, does not exclude the possibility that IL-6 has an important role to play after TBI, but that SNPs other than those identified for study are relevant.

Clinical data from other work in our laboratory has suggested raised IL-6 levels may be associated with improved patient outcome (Winter *et al.*, 2004). It is conceivable that these patients have a genotype that favours increased IL-6 production. IL-6 is thus a prime candidate for initial investigation, especially as the response of the ischaemia and hypoxia models in OHSC has not been explored.

Whilst most of this work concentrates on the role of cytokines in outcome from brain injury I have also outlined the possible role of apolipoprotein E (and more

specifically the ε4 allele) in Chapter 1. In view of this evidence I will also explore the effects of apoE in our OHSC models.

In this chapter I will assess the neuroprotective/neurotoxic effects of IL-6 and apolipoprotein E in our ischaemia and hypoxia OHSC models.

A.2 Methods

A.2.1 Organotypic hippocampal slice culture preparation

OHSCs were prepared using the method described by Stoppini *et al.* (1991), and modified by Morrison *et al.* (2002). Eight to ten day old Wistar rat pups (Biomedical Research Facility, University of Southampton) were killed by decapitation and both hippocampi rapidly dissected out. Transverse sections, with a thickness of 415 microns, were then cut using a McIlwain tissue chopper (Mickle Laboratory Engineering Ltd, Surrey, UK) and placed into ice cold Geys salt solution (Sigma, Ayrshire, UK), supplemented with 28mM glucose (Sigma). Using plastic paddles the slices were then plated onto Millicell-CM semi-porous membranes (0.4μm pores; Millipore, Watford, UK). Four cultures were placed on each insert in a six well culture plate (Nunc, Roskilde, Denmark). Cultures were grown in 1ml horse serum based medium consisting of 50% minimum essential medium (MEM) (Gibco, Paisley, UK), 25% Hank's balanced salt solution (ICN Chemicals, Cambridge, UK), 25% heat inactivated horse serum (Gibco) supplemented with 1mM glutamine (Sigma) and 4.5mg/ml glucose (Gibco). Cultures were grown for 14 days in an incubator at 37°C with 100% humidity and an atmosphere of 5% CO_2, 20% O_2 and 75 N_2. Medium was changed every 3-4 days.

A.2.2 Visualisation of neuronal damage

Neuronal death was determined using propidium iodide (PI) (Molecular Probes, Leiden, Netherlands), a fluorescent exclusion dye. PI is highly polar and under normal circumstances is excluded from cells. After cell membrane damage PI is able to enter cells, where it binds to DNA and fluoresces.

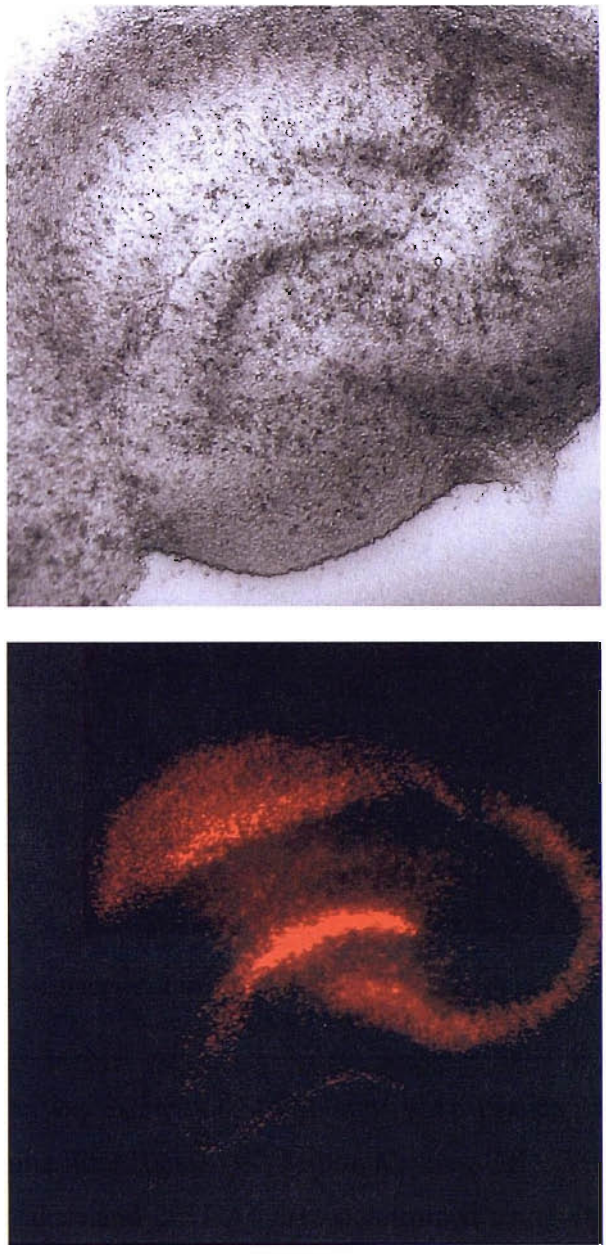


Figure A.2 – Examples of the transmission (upper) and PI (lower) images of hippocampal slice cultures (different slices)

PI fluorescence was excited at 515-560nm using a Leica inverted microscope fitted with a rhodamine filter (Leica UK, Milton Keynes, UK). Transmission images of the cultures were taken and the CA1 area determined using Openlab 3.0 (Improvision, Warwick, UK) image analysis software on an Apple Macintosh computer. The area of PI fluorescence above background in the CA1 area was determined using the 'density slice' feature of the software and the percentage area of PI fluorescence calculated.

A.2.3 Experimental protocols

All experiments were performed in serum free medium, consisting of 75% MEM, 25% Hanks balanced solution, 5mg/ml glucose and 1 mmol/L glutamine. Cultures were transferred into this medium 20 minutes prior to commencing experiments. Transmission images (see above) were taken during this period.

Three experimental protocols were used: ischaemia, hypoxia and NMDA excitotoxicity. Ischaemia (oxygen-glucose deprivation – OGD) was induced by quickly transferring the cultures into glucose-free SFM saturated with 95% N_2/5% CO_2 and then placing the culture plate in an airtight box, saturated with 95% N_2/5% CO_2, in an incubator. Cultures remained in this atmosphere for one hour, then were removed and returned to normoxic SFM.

Hypoxia was induced in a similar manner to ischaemia, except that cultures remained in SFM, and were kept in the 95% N_2/5% CO_2 saturated airtight box for three hours.

NMDA excitotoxicity was induced by exposing cultures to 10mM NMDA (Tocris) for 3 hours.

Cultures were exposed to various doses (10-100ng/ml) of recombinant rat IL-6 (Biosource) 24 hours prior to insult, during the insult or for the 24-hour recovery period post insult. Neuronal damage was assessed at 24 hours post insult.

Cultures were exposed to 1μg human apolipoprotein E (Calbiochem) in the apoE experiments. Either a mixed, plasma-derived, apolipoprotein E preparation or specific isoforms were used.

A.3 Results

A.3.1 IL-6 & Ischaemia (OGD)

Pre-treatment

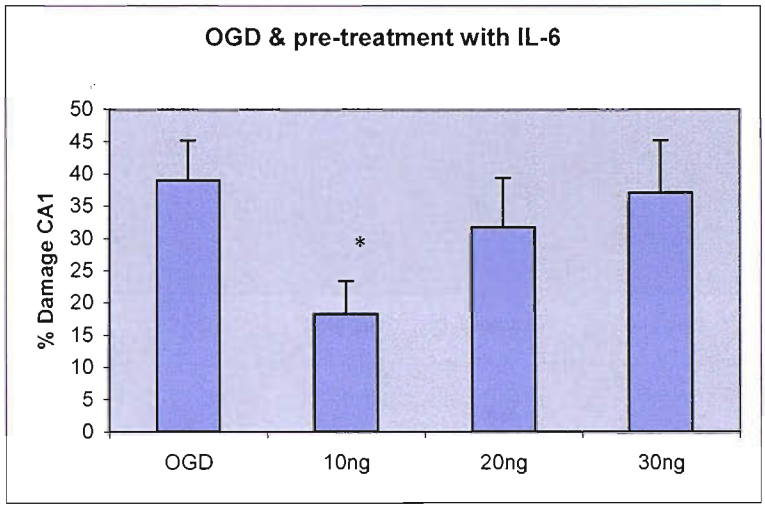


Figure A.3 – neuroprotective effect of low dose IL-6 when present pre-insult. 10ng/ml IL-6 provides significant protection (p=0.02). High doses did not differ significantly from controls. (OGD; *n*=43, 10ng; *n*=24, 20ng; *n*=25, 30ng; *n*=24)

During insult

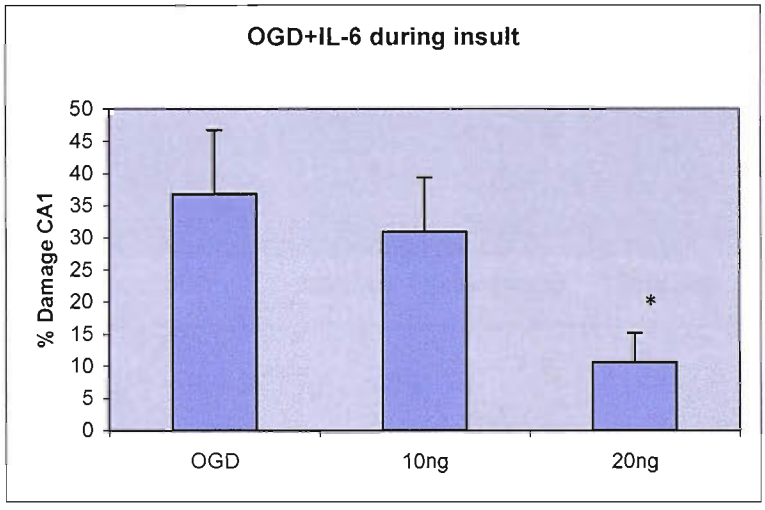


Figure A.4 – when administered during the period of insult IL-6 showed significant protection (p=0.02) at a dose of 20ng/ml. (OGD; *n*=16; 10ng; *n*=14, 20ng; *n*=16)

Post insult

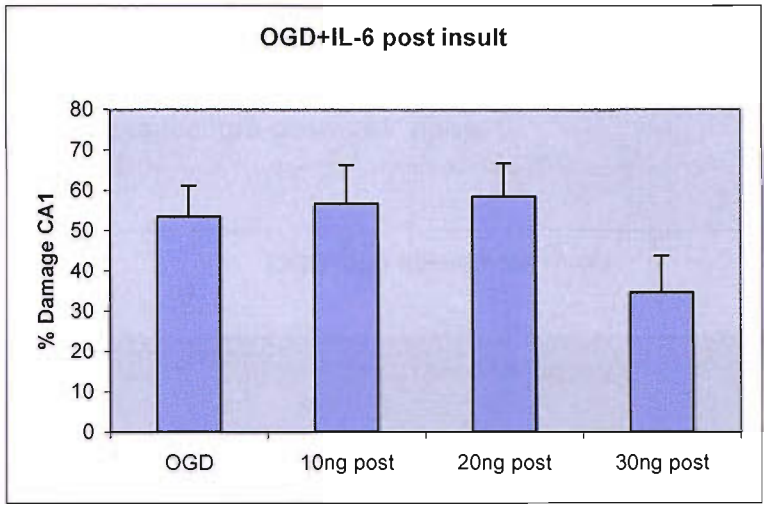


Figure A.5 – IL-6 did not provide significant neuroprotection when applied for 24 hours post ischaemic insult. (OGD; *n*=18, 10ng; *n*=14, 20ng; *n*=16, 30ng; *n*=16)

High dose IL-6

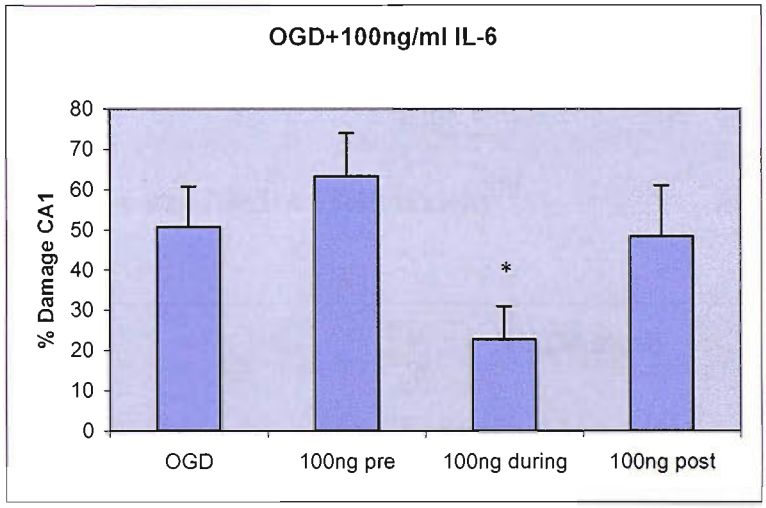


Figure A.6 – at a much higher dose of 100ng/ml IL-6 was not neuroprotective when applied pre or post ischaemic insult. However when applied at the same time it was significantly neuroprotective (p=0.04), in keeping with the results shown in Figure 7.3. (OGD; *n*=12, pre; *n*=10, during; *n*=12, post; *n*=11)

Summary

The neuroprotective effects of IL-6 in our OHSC model of ischaemia are displayed in the above figures (Figure A.3 – Figure A.6). No overt neurotoxic effects of IL-6 were demonstrated, although there may be a trend towards additional neurodegeneration occurring when cultures are exposed to high dose IL-6 prior to the ischaemic insult (Figure A.6). Neuroprotection was observed in two situations; i) pre-treatment with low dose IL-6 (see figure A.3) and ii) higher doses of IL-6 present during the period of insult (see figures A.4 and A.6). When IL-6 is applied throughout the whole time course of the experiment i.e. 24 hours pre-treatment,

during the insult and then for 24 hours post-insult, no significant protection is achieved, although there may be a trend towards neuroprotection at higher doses (see figure A.7 below). This suggests that most of the neuroprotective effects occur during the period of insult, and these outweigh any potential neurotoxic effects occurring in the 'pre-treatment' phase.

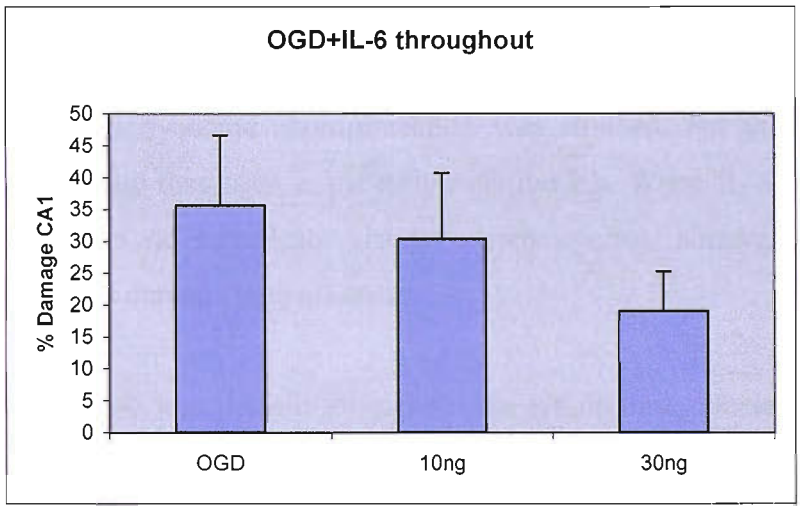


Figure A.7 – trend towards neuroprotection when increasing doses of IL-6 are present throughout the whole experimental protocol. (OGD; *n*=14, 10ng; *n*=14, 30ng; *n*=15)

A.3.2 IL-6 and NMDA excitotoxicity

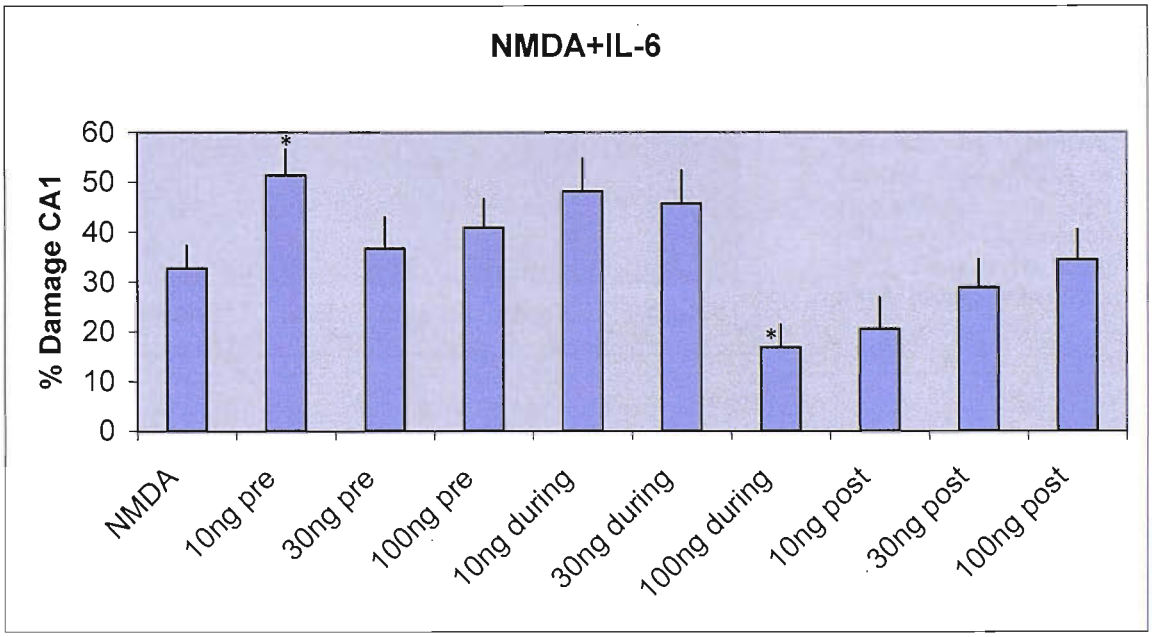


Figure A.8 – doses of IL-6 given pre, during, or post excitotoxic insult with 10mM NMDA. 10ng IL-6 treatment pre-insult resulted in significant increase in damage (p=0.01), while 100ng present during the insult was significantly neuroprotective (p=0.02). (*n*: NMDA=30, Pre; 10ng=27, 30ng=31, 100ng=24, During; 10ng=27, 30ng=24, 100ng=27, Post; 10ng=23, 30ng=33, 100ng=24)

The response to IL-6 in our NMDA model of excitotoxicity was less consistent than seen in the ischaemia model. The results are summarised in a single graph (Figure A.8). When IL-6 was used as a pre-treatment low dose increased the neurodegeneration induced by NMDA, but this did not occur at higher doses. This is the reverse of the situation seen in the ischaemia model, where low doses were protective. However there did not appear to be a dose response to higher doses so this finding may be questionable. When IL-6 was applied during the period of excitotoxicity some neuroprotection was attained, but this became apparent at a higher dose than seen in the ischaemia model. When IL-6 post-treatment was used there was no significant change versus control, although a slight trend toward increased damage with all doses.

When IL-6 was present throughout the whole time course of the experiment there was no significant change in damage (Figure A.9).

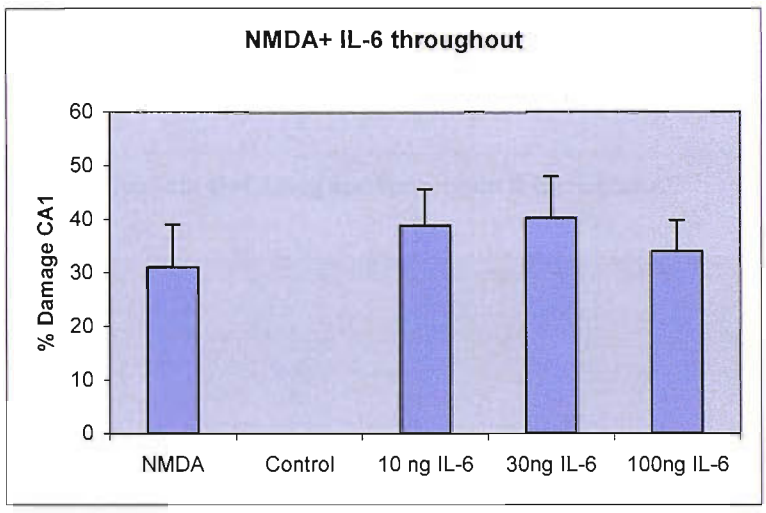


Figure A.9 – doses of IL-6 applied throughout the complete time course of the experiment. Addition of IL-6 did not significantly alter neuronal damage induced by NMDA. Control = no NMDA or IL-6 added.
(NMDA; n=12, Control; n=12, 10ng; n=16, 20ng; n=14, 100ng; n=14)

A.3.3 Response to apolipoprotein E

OHSCs were exposed to apoE throughout the full time course of the experiments (i.e. pre-, during, and post-insult). The response of the cultures was examined in both the ischaemia and the hypoxia models.

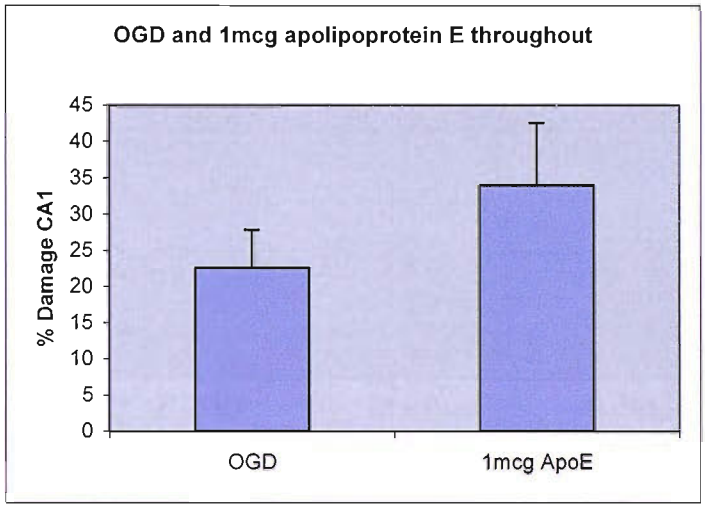


Figure A.10 – Apolipoprotein E shows neither neuroprotective nor neurotoxic effects when added to the OHSC model of ischaemia.
(OGD; n=12, ApoE; n=12)

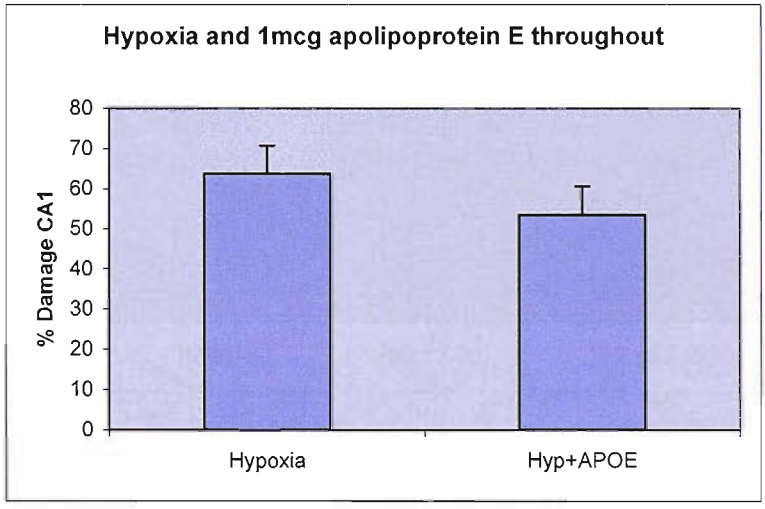


Figure A.11 – Apolipoprotein E shows neither neuroprotective nor neurotoxic effects when added to the OHSC model of hypoxia.
(Hyp; n=23, Hyp+ApoE; n=23)

In both the model of ischaemia and hypoxia apolipoprotein E did not alter the amount of neuronal damage sustained. Hypoxia is usually a 'softer' insult than ischaemia, so the degree of damage noted in this experiment (figure A.11) is unusually high.

A.3.4 Response to apolipoprotein E isoforms

OHSCs were exposed to apoE isoforms E3 and E4 throughout the full time course of the experiments (i.e. pre-, during, and post-insult). The response of the cultures was examined in the ischaemia, hypoxia and the excitotoxicity models.

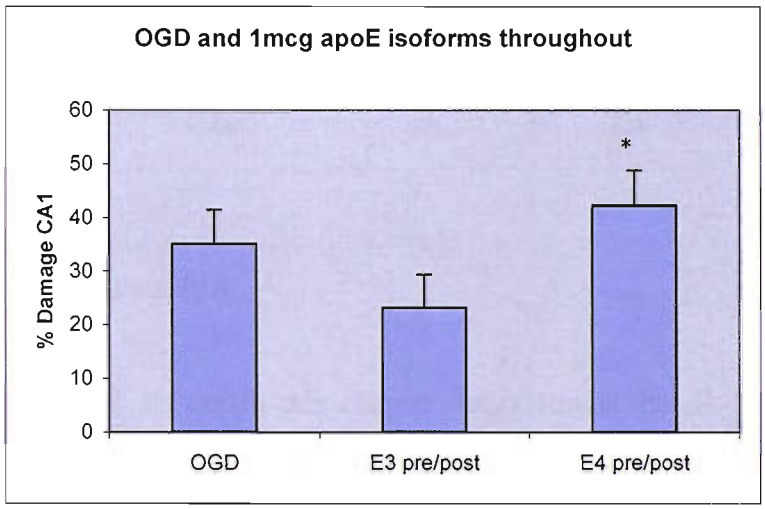


Figure A.12 – the ε3 and ε4 isoforms show no significant effect on CA1 damage after OGD when compared to controls. A significant increase in damage is observed when E4 treated cultures are compared to those treated with E3 ($p=0.04$). (OGD; $n=24$, E3; $n=22$, E4; $n=21$)

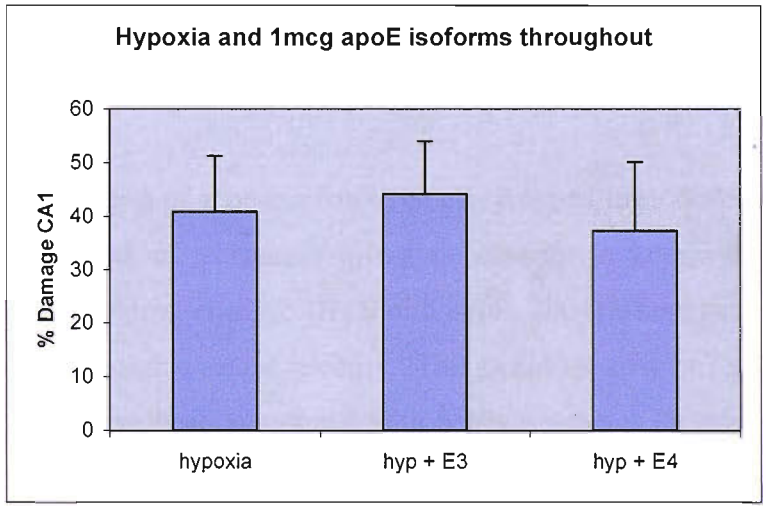


Figure A.13 – No differences are seen in the hypoxia model when comparing E3 and E4 treated cultures with controls. (Hypoxia; $n=15$, E3; $n=14$, E4; $n=12$)

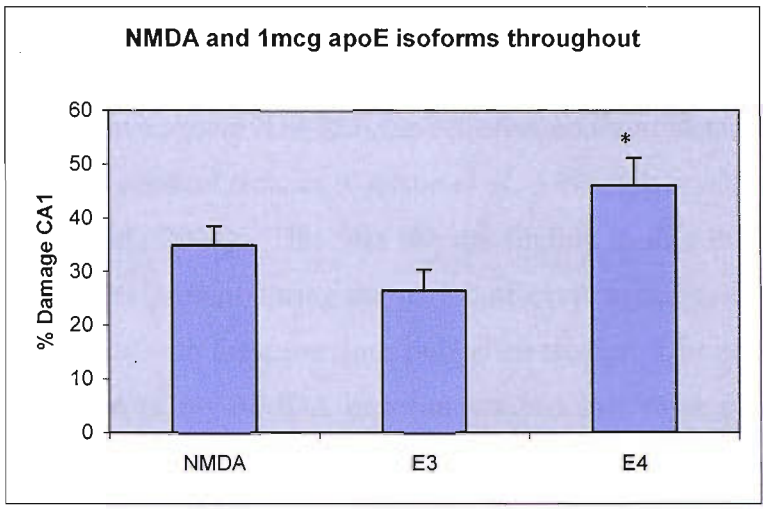


Figure A.14 – The NMDA model shows a similar pattern to the OGD model. There is no significant difference between isoform treated cultures and control. E4 treated cultures show a significant increase in damage in comparison to E3 ($p=0.003$). (NMDA; $n=45$, E3; $n=34$, E4; $n=36$)

A.4 Discussion

The work set out in this chapter demonstrates that IL-6 has some neuroprotective actions in our OHSC model of ischaemia. Results from the NMDA model were less conclusive. Early work exploring the role of apolipoprotein E has shown a significant difference between the E3 and E4 isoforms.

A.4.1 IL-6

The finding of a neuroprotective effect when high doses of IL-6 are present during the period of ischaemia is not necessarily in keeping with a number of clinical studies (Perini *et al.*, 2001; Smith *et al.*, 2004) where peak IL-6 levels are associated with ischaemic stroke severity. This is not the case in TBI patients where raised IL-6 levels have been associated with better outcomes (Winter *et al.*, 2004). The precise nature of the tissue injury is obviously one of the factors determining injury severity and outcome. As the IL-6 dose is the only variable in the OHSC models it must be assumed that the alteration of neuronal damage results as a direct effect of IL-6 or the effect of IL-6 on other induced inflammatory events. IL-6 has been acknowledged as both a pro- and anti-inflammatory cytokine. The variation in study results may be explained not only by the tissue concentrations of IL-6 but also the temporal change in these levels. The presence (or absence) of a critical level of IL-6 during the periods of ischaemia and then reperfusion may be the key to determining the ultimate degree of damage or cell survival.

The actions of IL-6 in the NMDA excitotoxicity model were less clear. Previous studies have shown IL-6 to be an effective neuroprotectant against NMDA toxicity in a dose-dependent manner (Carlson *et al.*, 1999; Ali *et al.*, 2000; Inomata *et al.*, 2003; Pizzi *et al.*, 2004). This was not the finding in this study, except when very high doses were present during the period of excitotoxicity. It is unclear why my results are at odds with these previous published studies. One possibility is that the batch of IL-6 used in my NMDA experiments had lost some of its potency. To test this possibility two additional batches of IL-6 were sourced, and compared with the batch used in the original experiments. No difference in response between the batches was noted in either the NMDA model or the OGD model. We must therefore assume that IL-6 truly failed to display any neuroprotective effect in the NMDA excitotoxicity model.

A.4.2 Apolipoprotein E

Experiments were performed using either serum derived apolipoprotein E or isoform specific preparations (either E3 or E4). There was no significant effect of the serum-deriveved apoE treatment on the degree of damage in either the hypoxia or OGD model, although apoE treatment appeared to enhance damage in the OGD model but not in the hypoxia model. This suggests that, similar to the cytokine experiments, there may be a model specific effect occurring i.e. apoE is able to effect and alter the mechanisms occurring in the OGD model, but not those occurring in the hypoxia model.

The same pattern is observed in the isoform-specific experiments, with the isoforms causing little variation in the amount of damage observed in the hypoxia model. In the OGD and excitotoxicity models the E3 isoform tends to reduce damage, while the E4 isoform tends to increase damage. This observation of similar effects of a substance in the OGD and excitoxicity models that differ from the hypoxia model has been seen previously (Pringle *et al.*, 2001).

In both the OGD and excitotoxicity models there is a significant difference in the damage observed when comparing the cultures treated with apolipoprotein E3 with

those treated with E4. In both cases E4 causes an increase in damage. This corroborates the genetic data that associates the ε4 allele with poorer outcome (see Section 1.6). The mechanism for this effect is unclear but the E4 isoform appears to be promoting cell death, whilst E3 is possibly exerting a neuroprotective effect (evidenced by the slight fall in observed damage in comparison with controls). If this is the case then it is interesting that a single polymorphism (ε3 differs from ε4 by a single polymorphism at codon 158) can lead to the production of a substance that in one form is neuroprotective but by changing a single amino acid this becomes neurotoxic. This finding is supported by other in-vitro studies (Miyata & Smith, 1996; Jordan *et al.*, 1998; Michikawa & Yanagisawa 1999; Veinbergs *et al.*, 2002; Ji *et al.*, 2002) but not universally (Lendon *et al.*, 2000).

This work is still at an early stage, and requires further validation before drawing any absolute conclusions. However the current findings are interesting and worthy of further investigation. Further work may include investigating the E2 isoform, to identify any further isoform specific effects, and to examine purely protein effects (Laskowitz *et al.*, 2001; Lynch *et al.*, 2005) or effects associated with lipoprotein particles (see Kay *et al.*, 2003 for example).

Appendix B

B1 Glasgow Coma Scale

The Glasgow Coma Scale (GCS) was first proposed in 1974 (Teasdale & Jennett, 1974). It is the most widely used scale for assessing consciousness after TBI. The level of consciousness is established by 'scoring' the patient according to three responses to stimulation (eye opening, verbal response & motor response - see below). A total score of between 3 (worst) and 15 (best) is established by adding the scores in the three categories.

The severity of a head injury can be gauged using the GCS, with patients with mild injuries having a GCS of 14-15, moderate a GCS of 9-13 and severe a GCS of 8 or less.

Glasgow Coma Scale:

Score	Best Eye Opening	Best Verbal	Best motor
6	-	-	obeys commands
5	-	oriented speech	localises pain
4	spontaneous	confused speech	withdraws to pain
3	to speech	inappropriate speech	abnormal flexion
2	to pain	incomprehensible	abnormal extension
1	none	none	none

B2 Glasgow Outcome Scale

The Glasgow Outcome Scale (Jennett & Bond, 1975) is a useful tool for assessing and scoring outcomes after brain injury. It is now often used in its extended form (Teasdale et al., 1998), wherein the three categories of severe disability, moderate disability and good recovery are split into two subcategories, scoring from 1 to 8. Assessment is usually in the form of structured interview (Wilson et al., 1998).

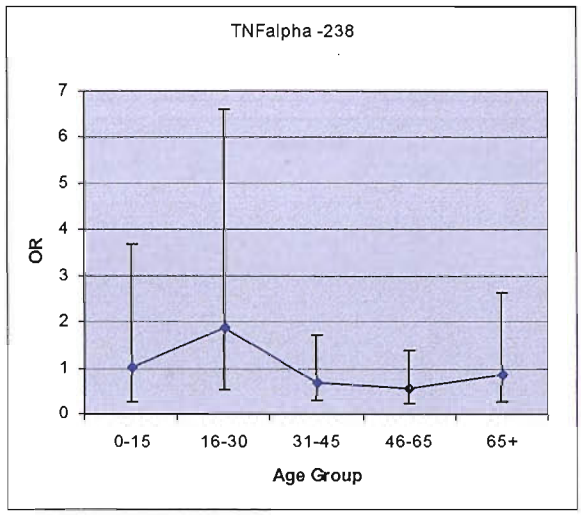
Glasgow Outcome Scale:

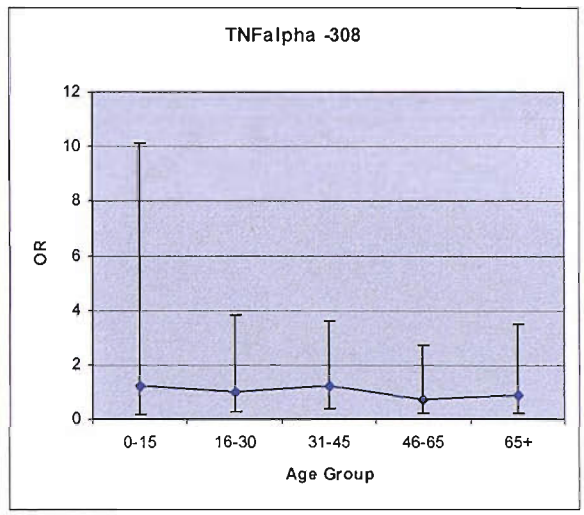
Score	Description
1	**Death**
2	**Persistent vegetative state** Patient exhibits no obvious cortical function
3	**Severe Disability** Patient depends upon others for daily care
4	**Moderate Disability** Patient is independent but has a degree of disability
5	**Good Recovery** Resumption of normal activities

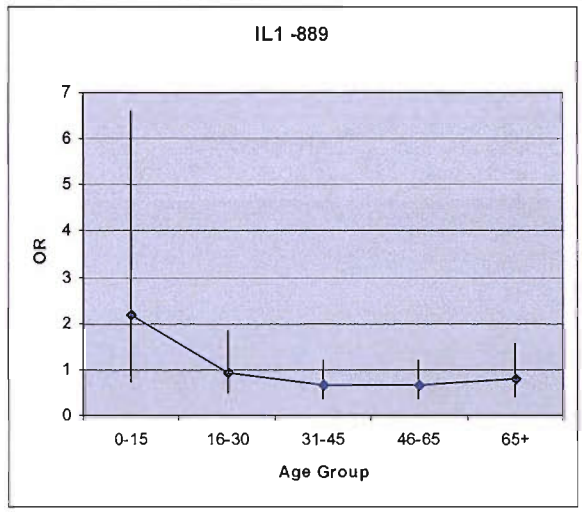
Appendix C

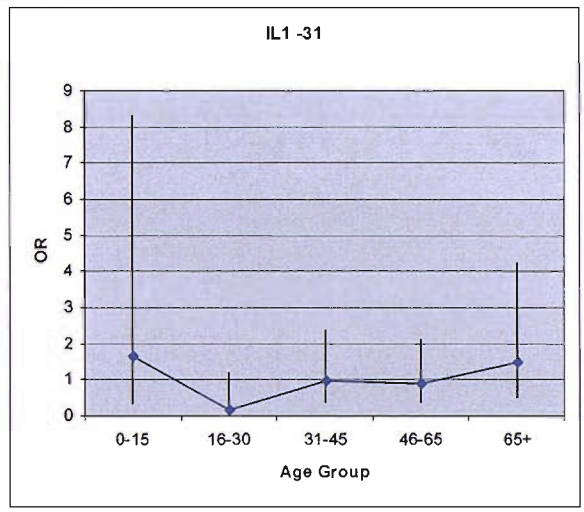
Association between SNP alleles and poor outcome in different age groups

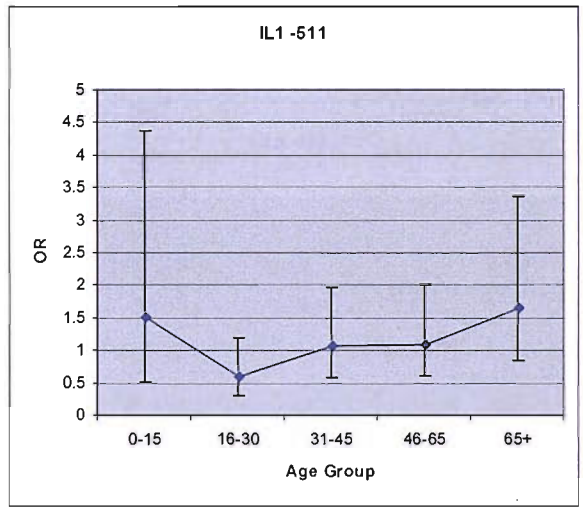
This section contains the analysis to detect potential association between cytokine SNP alleles and clinical outcome according to the age group of the patients. This is discussed in section 5.3.1.2.

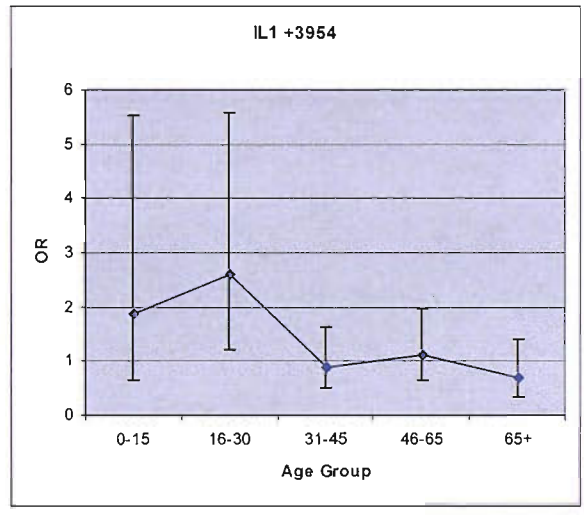




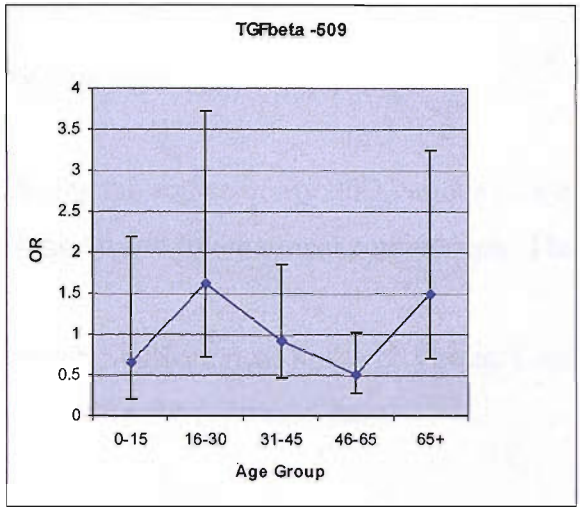


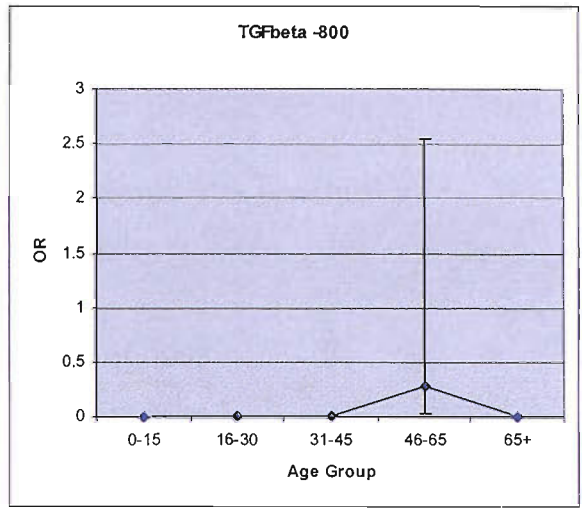


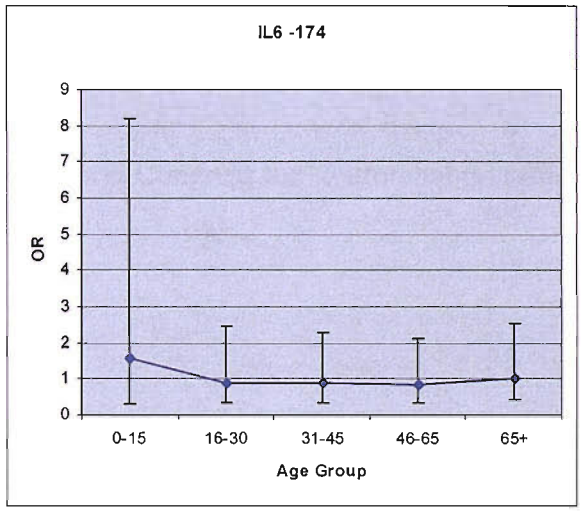


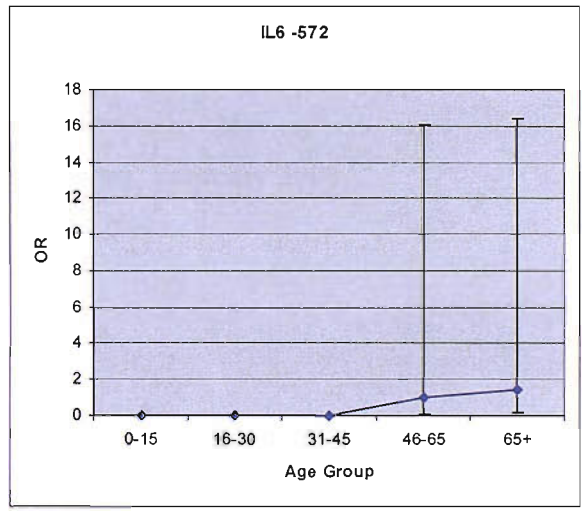


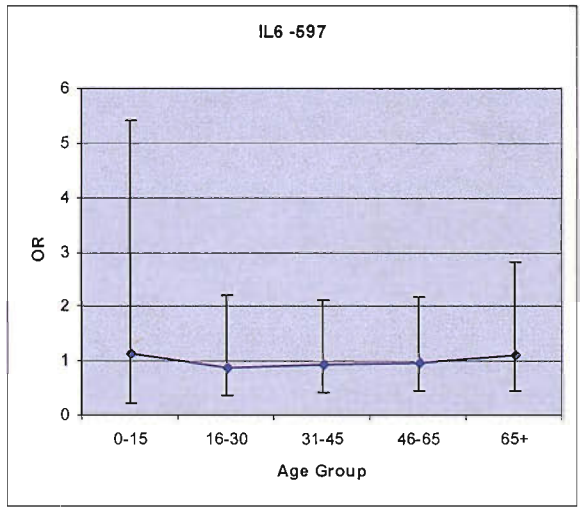
Association between SNP alleles and poor outcome according to age group, expressed as odds ratios.
Values of zero, with no confidence intervals indicate the group contained no rare alleles.
Figure continues overleaf.











Association between SNP alleles and poor outcome according to age group, expressed as odds ratios.
Values of zero, with no confidence intervals indicate the group contained no rare alleles.

Appendix D

Presentations

During the course of my PhD various elements of this work were presented at National and International conferences. These included;

British Neurosurgical Research Group, London, January 2005
(shown on the following page)

Neurotrauma 2005, Washington DC, October 2005
(shown as the final image in this section)

4th World Congress for Neurorehabilitation, Hong Kong, February 2006

Do cytokine gene polymorphisms influence outcome after head injury ?

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Division of Clinical Neurosciences, University of Southampton, UK

Introduction

Traumatic brain injury (TBI) is common, often occurs at a young age and can result in lifelong disability. Clinical outcome after head injury is variable and cannot easily be predicted, but there is now evidence to suggest there may be a genetic influence (1, 2). After TBI an inflammatory reaction is provoked within the central nervous system. Cytokines play an important role in mediating this response. Polymorphisms within cytokine genes may alter gene transcription and thereby alter the magnitude and duration of the inflammatory response, which in turn may affect clinical outcome.

We have agreed access to DNA samples collected from 1463 patients treated after TBI in Glasgow. A clinical database for these patients is available, including details of their injuries, outcome after 6 months and, for some, outcome 15-25 years after injury. These have been used previously to determine genetic influence on outcome (1, 3).

We have been typing the DNA samples for a number of single nucleotide polymorphisms (SNPs) within five cytokine genes. Combining this data with the clinical outcome measures we will be able to identify any significant influences.

Cytokine SNPs

We are studying the role of the following SNPs:

Gene	SNP
TNF-α	-238 G/A
	-308 G/A
IL-1α	-889 C/T
IL-1β	-31 C/T
	-511 C/T
	+3953 C/T
IL-4	-34 G/T
	-590 C/T
	-187 G/A
TGFβ	+869 C/T
	+915 G/C

1. DNA Quantitation

DNA samples were collected from patients in the form of buccal swabs. DNA preparation was carried out in Glasgow. DNA quantification was necessary to assess the need for pre-amplification of the DNA, in order to ensure sufficient DNA for multiple SNP assays and preservation of the resource for future studies.

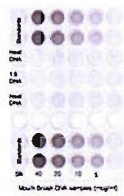


Figure 1.1 – DNA quantitation using PicoGreen fluorescence. Most DNA samples have lower fluorescence than the 5ng/ml standard, indicating a DNA concentration<5 µg/ml.

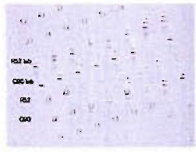


Figure 1.2 – Comparison of buccal DNA with blood derived DNA. Upper two rows demonstrate 'blood derived control' DNA samples. Lower two are buccal DNA samples. 432 and G90 are two polymorphisms within the Cytokine gene.

2. DNA Pre-amplification

By demonstrating that the buccal samples had DNA concentrations <5 µg/ml we had confirmed the need for a pre-amplification step, prior to genotyping.

DOP-PCR

A degenerate oligonucleotide primer (DOP) technique was used to achieve genome wide amplification. PCR was performed in a final volume of 50µl on a thermal cycler. Various quantities of template DNA were trialled. PCRs contained 1µM DOP primers (5'-CCGACTCGAGNNNNNNATGTGG-3'), 250µM dNTPs, 2.5µM MgCl, 1.3M Betaine, 0.02 units PWO, 5µl DOP Buffer and 2.5 units Taq polymerase. An initial denaturation at 94ºC for 5 minutes was followed by 8 cycles of 94ºC for 1 minute, 30ºC for one minute and 72ºC for 3 minutes, then 28 cycles of 94ºC for 1 minute, 60ºC for 1 minute and 72ºC for 3 minutes. The pre-amplified samples were then used as a template for further PCR. By performing these PCRs it became apparent that the DOP-PCR method was not providing sufficiently consistent amplification (see Figure 2.1).

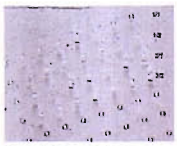


Figure 2.1 – G90 PCR run on DOP amplified samples

1/1=1µl buccal DNA used for DOP amplification then 1µl of amplified product used as template for PCR. 1/2=1µl buccal DNA used for DOP amplification then 2µl of amplified product used as template for PCR etc.

Figure 2.2 – Comparison of preamplification methods, using the G90 PCR. 'Lab DNA' are blood derived control DNAs. The same eight samples were used for the DOP and Phi29 amplification

Phi29

As an alternative to the DOP-PCR method we used the GenomiPhi DNA amplification kit (Amersham Biosciences). This kit utilises random hexamer primers and bacteriophage Phi29 polymerase (see Figures 2.3).

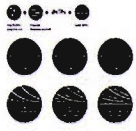


Figure 2.3 – How GenomiPhi pre-amplification works. Images from www.genomiphi.com

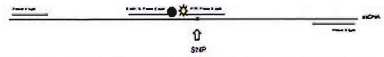
When compared with the DOP method GenomiPhi provided much improved and more consistent amplification. Figure 2.2 shows the comparison when running the G90 PCR on equal quantities of DOP and GenomiPhi amplified material. The GenomiPhi kit is now being used to amplify all of the buccal DNA samples.

3. SNP Genotyping

Due to our sample size and the number of SNPs we wish to type it was necessary to utilise a high-throughput method of SNP genotyping. We have designed assays which rely on 'Melting Curve' analysis, which can rapidly be achieved by the Odyssey LightTyper machine. A fluorescent probe, which binds precisely with one of the SNP alleles, is included during the PCR stage. Post PCR the sample plate is transferred to the LightTyper, where it is heated. During heating a probe which is imprecisely bound (i.e bound to the alternate allele of a SNP) will 'melt off' at a lower temperature than a precisely bound probe. A change in fluorescence (detected by the LightTyper) will therefore occur at a different temperature for each allele.

PCR design

A separate PCR is run for each SNP. Assays include asymmetric primers to amplify the SNP region, a fluorescein labelled probe specific for one of the SNP alleles and a DABCYL-labelled probe, which sits adjacent to the fluorescein, to quench the fluorescence whilst both probes are bound.



Each PCR uses the same cycle of 94ºC for 2 minutes, 55 cycles of 94ºC for 20 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds and finally 72ºC for 2 minutes.

4. Results

Results from the LightTyper are processed via specific LightTyper software (Idaho Technologies). This displays fluorescence peaks and can be used for automated sample calling.

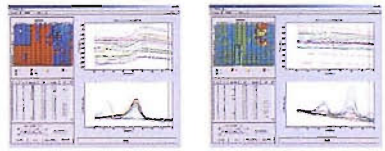


Figure 4.1 – Examples from the LightTyper analysis software, demonstrating the fluorescence peaks obtained from the pre-amplified Glasgow samples. Assays for the TNFα -238 and IL-4 -174 SNPs are shown. Probes have been designed so the higher temperature peak signify the more common SNP allele.

Data obtained from the LightTyper software are checked, via an Excel macro, to ensure correct genotype calling. This also enables easy visualisation of results.

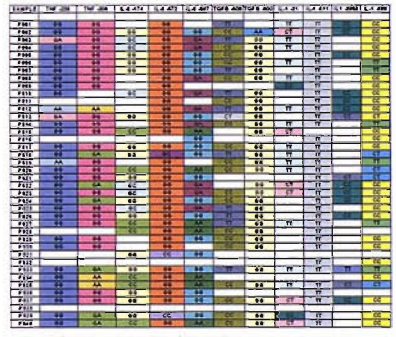


Figure 4.2 – Provisional genotyping data for first forty DNA samples. Blanks appear where samples have not yet been genotyped successfully. Work is currently ongoing to genotype the first 360 samples.

5. Further Work

A total of 450 samples are now available for genotyping in Southampton. This work is currently underway. Aliquots of the remaining samples will be transferred and typed in due course. Once the cytokine genotypes have been determined we plan to extend the study to include polymorphisms in the apolipoprotein E gene promoter and GAP43. Both are genes of importance in neuronal repair.

We anticipate the data analysis will comprise most of the second half of the project. The analysis will be performed in collaboration with a statistician familiar with the clinical database. We will be able to search for associations of individual SNPs, within-gene SNP haplotypes and haplotypes across genes with outcome after TBI.

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Do cytokine gene polymorphisms influence outcome after head injury ?

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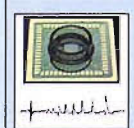


INTRODUCTION

Clinical outcome after traumatic brain injury (TBI) is variable and cannot easily be predicted, but there is now increasing evidence to suggest there may be genetic influences (1). Previous studies have explored an association between apolipoprotein E gene polymorphism and outcome six months after injury and at later follow-up with variable findings. This leaves scope for an effect of polymorphism of other genes. Cytokines play an important role in mediating the inflammatory response provoked within the central nervous system after TBI. Polymorphisms within cytokine genes may alter gene transcription and thereby alter the magnitude and duration of the inflammatory response, which in turn may affect clinical outcome.

DNA from buccal swabs has been collected from 1463 patients treated after TBI, for previous studies (2, 3). A clinical database for these patients is available, including details of their injuries, outcome (GOS) after 6 months (n=1067) or outcome 15-25 years after injury (n=396).

We have been typing the DNA samples for a number of single nucleotide polymorphisms (SNPs) within five cytokine genes. Combining this data with the clinical outcome measures we will be able to identify any significant influences.

CYTOKINE SNP TARGETS

Gene	SNP	dbSNP ref	Effect
TNFα	-238 G/A	361525	Multiple positive association studies in various conditions, but functional effects one in question
	-308 G/A	1800629	
IL-1α	-889 C/T	1800587	Influences microglial activation
IL-1β	-31 C/T	1143627	Multiple positive association studies in various conditions, +3953 shown to increase expression
	-511 C/T	16944	
	+3953 C/T	1143634	
IL-6	-174 G/C	1800795	Complex interactions, which influences transcription
	-572 G/C	1800796	
	-597 G/A	1800797	
TGFβ	-509 C/T	1800469	Both SNPs associated with raised plasma concentrations
	-800 G/A	1800468	

METHODS

DNA Preparation

Buccal swabs were collected by rubbing a cytology brush on the inner surface of the patient's cheek for 15 seconds. The brush was then agitated in 400µl ethanol. The samples were centrifuged at 13000 rpm for 30 seconds. The ethanol was then decanted and the pelleted samples dried. The samples were then digested with Proteinase K.

DNA Pre-amplification

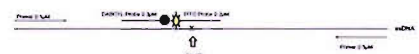
Pre-amplification was required to ensure sufficient template for multiple PCRs and preservation of the resource. The GenomiPhi amplification kit (GE Healthcare) was utilised.

SNP GENOTYPING

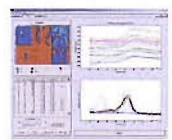
Due to our sample size and the number of SNPs we wish to type it was necessary to utilise a high-throughput method of SNP genotyping. We have designed assays which rely on 'Melting Curve' analysis, which can rapidly be achieved by the Odyssey LightTyper machine (Roche Diagnostics Ltd). A fluorescent probe, which binds precisely with one of the SNP alleles, is included during the PCR stage. Post PCR the sample plate is transferred to the LightTyper, where it is heated. During heating a probe which is imprecisely bound (i.e bound to the alternate allele of a SNP) will 'melt off' at a lower temperature than a precisely bound probe. A change in fluorescence (detected by the LightTyper) will therefore occur at a different temperature for each allele.

PCR design

A separate PCR is run for each SNP. Assays include asymmetric primers to amplify the SNP region, a fluorescein labelled probe specific for one of the SNP alleles and a DABCYL-labelled probe, which sits adjacent to the fluorescein, to quench the fluorescence whilst both probes are bound.



Each PCR uses the same cycle of 94ºC for 2 minutes, 55 cycles of 94ºC for 20 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds and finally 72ºC for 2 minutes.



Examples from the LightTyper analysis software (Roche), demonstrating the fluorescence peaks obtained from the pre-amplified samples. Assays for the TNFα -238 (left) and IL-6 -174 (right) SNPs are shown. Probes have been designed so the higher temperature peak signify the more common SNP allele.

PRELIMINARY RESULTS

GENOTYPE	OUTCOME unfavourable n (%)	OUTCOME favourable n (%)		P-value	GENOTYPE	OUTCOME unfavourable n (%)	OUTCOME favourable n (%)		P-value
TNF -238					**TGF -509**				
GG	295 (88.3)	570 (88.9)	865	0.73	CC	124 (54.9)	281 (56.2)	405	0.18
GA	38 (11.4)	67 (10.5)	105		CT	82 (36.3)	155 (31.0)	237	
AA	1 (0.3)	4 (0.6)	5		TT	20 (8.8)	64 (12.8)	84	
	334	641	975			226	500	726	
TNF -308					**TGF -800**				
GG	186 (60.2)	416 (67.4)	602	0.07	GG	220 (81.5)	484 (83.6)	704	0.1
GA	104 (33.7)	164 (26.6)	268		GA	49 (18.1)	84 (14.5)	133	
AA	19 (6.1)	37 (6.0)	56		AA	1 (0.4)	11 (1.9)	12	
	309	617	926			270	579	849	
IL1 -889					**IL6 -174**				
CC	147 (47.9)	333 (53.3)	480	0.30	GG	68 (34.9)	148 (37.1)	216	0.78
CT	127 (41.4)	233 (37.3)	360		GC	83 (42.6)	170 (42.6)	253	
TT	33 (10.7)	59 (9.4)	92		CC	44 (22.5)	81 (20.3)	125	
	307	625	932			195	399	594	
IL1 -31					**IL6 -572**				
CC	35 (15.0)	78 (15.9)	113	0.27	GG	152 (56.3)	318 (59.3)	470	0.39
CT	82 (35.0)	199 (40.4)	281		GC	111 (41.3)	212 (39.6)	323	
TT	117 (50.0)	215 (43.7)	332		CC	6 (2.2)	6 (1.1)	12	
	234	492	726			269	536	805	
IL1 -511					**IL6 -597**				
TT	49 (17.1)	103 (17.8)	152	0.83	GG	115 (40.9)	234 (42.0)	349	0.86
TC	102 (35.7)	216 (37.2)	318		GA	117 (41.6)	234 (42.0)	351	
CC	135 (47.2)	261 (45.0)	396		AA	49 (17.4)	89 (16.0)	138	
	286	580	866			281	557	838	
IL1 +3953									
CC	197 (61.0)	376 (58.5)	573	0.65					
CT	108 (33.4)	223 (34.7)	331						
TT	18 (5.6)	44 (6.8)	62						
	323	643	966						

Results for the prospective cohort (n=1067) only. 6-month outcome measured by 5 point Glasgow Outcome Scale and dichotomised (1-3=unfavorable, 4-5=favorable). P-values were calculated by X^2 test. Figures in bold are the total number of samples successfully genotyped for the particular SNP. Success rates varied from 91.3% (TNF -238) to 55.7% (IL6 -174).

CLINICAL DATA

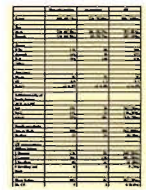


Illustration of the admission data available for this cohort of patients, related to possession of APOE ε4

DISCUSSION

Preliminary data analysis for the prospective cohort is illustrated. Each individual SNP has been examined for an association with 6-month outcome, measured using the Glasgow Outcome Scale. No SNP has reached significance at the 0.05 level, although the TNFα -308 and TGFβ -800 approach significance. Further analysis is proceeding to control for age, APOE genotype and initial CT findings.

After TBI TNFα is produced by microglia. It plays an important role in the initiation and control of the neuroinflammatory response that is provoked. The effects of TNF are generally neurotoxic. The -308 promoter SNP is associated with elevated TNF levels and the A allele may therefore be associated with increased neurotoxicity, which may be manifested as a poorer clinical outcome.

TGFβ limits the activation of microglia after TBI, and thus primarily exerts a neuroprotective effect. The -509 and -800 polymorphisms (which are in linkage disequilibrium) are associated with increased levels of TGFβ. It may therefore be expected that the -800 polymorphism would be associated with a better outcome after TBI due to a reduction in the degree of neuroinflammation. Our results seem to suggest this may hold true.

As indicated above further analysis to control for other factors is required before confirming these findings.

Analysis is also underway to detect associations between outcome and within-gene SNP haplotypes, and effects of gene-gene interaction.

Study of the retrospective cohort will enable us to detect much longer term outcome effects (mean 18 years post TBI). This cohort also has detailed neuropsychological outcome measures which will enable us to detect any influence of these cytokine genes on late cognitive decline.

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Genotyping was performed in the Human Genetics Division, University of Southampton, with help and advice from Professor I. Day.

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