Inflammation and cognition: the association between biomarker levels, their genetic determinants, and age-related cognitive decline.

Riccardo Emilio Marioni

Presented for the Degree of Doctor of Philosophy
University of Edinburgh
2010

Declaration

I declare that this thesis is my own composition and that it has not been submitted for any other degree or professional qualification. For the elements of the thesis that were a result of my work within a group of scientists, I declare that I have made a substantial contribution to this work. The data collection for the Aspirin for Asymptomatic Atherosclerosis (AAA) Trial, the Edinburgh Artery Study, and the 1936 Lothian Birth Cohort were all completed before the start of my studies. The only exceptions were the genotypic data from the AAA Trial and some of the genotypic data from the 1936 Lothian Birth Cohort. Data collection for the Edinburgh Type 2 Diabetes Study (ET2DS) began during the first year of my studies. Whilst I was not actively involved in this process, I did assist in marking the scripts from the cognitive test assessment. I was also involved in the data cleaning process and addition of the inflammatory biomarker and cognitive data to the main ET2DS database. I conducted all of the analyses in this thesis but accredit the Bayesian model for Mendelian randomisation to Professor Paul McKeigue. With his assistance, I developed and applied the model to the AAA Trial and ET2DS data.

Signed: Date:

Acknowledgements

I would like to acknowledge the Medical Research Council for funding my PhD studies. Without their financial support, none of this would have been possible. I must also thank the participants from the Aspirin for Asymptomatic Atherosclerosis Trial, the Edinburgh Type 2 Diabetes Study, the Edinburgh Artery Study, and the 1936 Lothian Birth Cohort.

I would also like to thank everyone who has helped me in the preparation and completion of my PhD thesis. In particular, my supervisors: Dr. Jackie Price, Professor Ian Deary, and Professor Gordon Murray. It has been an absolute pleasure to have worked with and to have been mentored by them for the past three years; I can't thank them enough for all their personal and professional support. I would also like to thank the many collaborators who have assisted me with the preparation, handling, and analyses of the data presented in this thesis: Professor Gordon Lowe, Dr. Ann Rumley, Professor Gerry Fowkes, Dr. Marlene Stewart, Dr. Snorri Björn Rafnsson, Professor Paul McKeigue, Dr. Rory Mitchell, Dr. Michelle Luciano, Dr. Lorna Houlihan, Dr. Alan Gow, Dr. Sarah Harris, Mrs. Christine Martin, Dr. Mark Strachan, and all those on the AAA Trial and ET2DS steering committees.

I am most grateful to all of my colleagues and friends in the Department of Public Health Sciences and the Department of Psychology. Their friendliness and willingness to help me in all aspects of my studies has been overwhelming. I would like to pay special thanks to: Dr. Snorri Björn Rafnsson, Dr. Michelle Luciano, Dr. Mike Allerhand, Dr. Niall Anderson, Dr. Pam Warner, Professor Robin Prescott, Mr. Robert Lee, Ms. Jennifer Bolton, Ms. Jennifer Ding, Ms. Julie MacMillan, Ms. Sarah McAllister, and Mrs. Rosa Bisset.

I must also thank all of my friends in Edinburgh. In particular, Pablo for his help with LaTeXformatting.

I would like to express my deepest thanks to my family and especially my brother, John. His guidance and advice means the world to me. However, the biggest thanks of all must go to my parents for their unconditional love and support.

I would like to dedicate this thesis to Mum, Dad, John, M, and T.

Abstract

Chronic inflammation and variations in blood flow have been implicated in the pathogenesis of cardiovascular disease. It is also possible that inflammatory and rheological processes are involved in the development of mild cognitive impairment and dementia, either through their association with vascular disease or via some other, more direct effect on the brain. Evidence is increasing for a causal relationship between Alzheimer's disease and inflammation, possibly related to inflammatory activation of microglia. Inflammatory processes may also be involved in the pathogenesis of cerebral small vessel disease, which in turn has been linked to cognitive impairment and dementia. There is also evidence showing that rheological factors affect cerebral blood flow. However, despite these findings, the associations between inflammatory and rheological markers and cognitive ability have not been extensively studied in large groups of ageing people.

The primary aim of this thesis was to test for associations between late-life levels of inflammatory and rheological markers (C-reactive protein (CRP), fibrinogen, tumor necrosis factor (TNF)-α, interleukin (IL)-6, plasma viscosity, and haematocrit) and cognitive ability. A genetic analysis was then performed to model single nucleotide polymorphisms (SNPs) in the genes encoding the markers against cognition in an attempt to determine the weight of evidence for a causal inflammation-cognition association.

Four studies were used to test these aims with the majority of the analysis being performed on the Aspirin for Asymptomatic Atherosclerosis (AAA) Trial (n = 3,350), and the Edinburgh Type 2 Diabetes Study (ET2DS) (n = 1,066). The Edinburgh Artery Study (n = 534), and the 1936 Lothian Birth Cohort (n = 1,091), were used as replication cohorts for the genetic analysis. All cohorts comprised community-dwelling, elderly citizens (aged around 70 years) living in central Scotland. With the exception of the ET2DS, all data used were for secondary analyses.

Cognitive ability was assessed in all studies using comprehensive batteries of neuropsychological tests that included a measure of crystallised intelligence in the form of a vocabulary test. As performance on such tests varies little across a lifespan, adjusting for these scores in the late-life models enabled the determination of estimated lifetime cognitive change. In the case of the 1936 Lothian Birth Cohort an actual age-11 IQ measure was available in addition to the cognitive follow-up scores recorded at age-70.

Linear regression showed small but significant associations between CRP, fibrinogen, and plasma viscosity, and cognition and estimated lifetime cognitive decline in the AAA Trial. Similar results were observed in the ET2DS for CRP, IL-6, and TNF-α. These associations tended to be of a magnitude whereby the markers explained 1% of the variance of the cognitive test scores. The cognitive domains most consistently associated with the markers were processing speed, and a data derived general intelligence factor.

A novel genetic analysis was then undertaken to model SNPs against cognitive ability and decline. Most of the results generated were null findings. However, strongly significant associations were found between the rs2227412 fibrinogen beta gene SNP and the cognitive test scores in the ET2DS. Furthermore, the genotype associated with the lowest cognitive scores was also related to higher levels of plasma fibrinogen.

Whilst replication of the association between the fibrinogen SNPs and cognition was not found across all cohorts, these results still indicate a potentially causal role for this haemostatic/inflammatory marker. To date, the majority of inflammation-cognition associations have focussed on the acute-phase protein CRP. The main outcomes from this thesis suggest that its close correlate, fibrinogen, is an equally, if not more important factor in the complex process of cognitive ageing.

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Chapter 1

Inflammation, cognition, Type 2 diabetes, and genetics.

1.1 Introduction

Decline in memory and other cognitive abilities is one of the most feared consequences of growing old. Given our generally ageing population, the public health consequences of such age-related cognitive decline are likely to increase over the coming years. It has been well established that by the time we observe clinical symptoms of cognitive deficits, much cerebral damage has already been done (Fillit & Butler 1997). There is thus an urgent need to identify preclinical markers of future cognitive decline in order to develop effective interventions aimed at reducing neurotoxicity before irreversible neural damage and dementia can occur. One potentially fruitful area in the search for such markers is the inflammatory system, which is thought to be involved in the development of Alzheimer's disease and may also be important in the development of vascular dementia (Jones 2001, Schmidt et al. 2002).

The main aims of this thesis were to use data from four large, prospective cohort studies based in central Scotland to test the associations between biomarkers of inflammation and rheology and cognitive ability. In addition to modelling the late-life biomarker levels against cognitive ability and decline, genetic analyses were used as a measure of causal inference.

This chapter reviews briefly some of the key background details that were required prior to conducting the main literature review and analysis. Specifically, a general overview of inflammation is provided, including some details of its immunological processes. A large section is then devoted to introducing cognitive ability, including general intelligence, and normal cognitive ageing and its

determinants. The cognition section closes with a brief description of both Mild Cognitive Impairment and dementia along with some of the neurological hallmarks associated with these conditions. The penultimate section of the chapter introduces Type 2 diabetes – using textbook information and current medical guidelines to define the disease, its diagnosis, and its risk factors, before two short literature review sections on its association with both inflammation and cognition. The chapter closes with an introductory section on genetics, which describes DNA structure and some of the types of genetic information commonly used for analyses in genetic epidemiology studies.

Chapters 2 to 4 then provide a detailed description of the specific biomarkers analysed in this thesis. Much of the analysis concentrates on the inflammatory biomarker, C-reactive protein (CRP), and the inflammatory/rheological biomarker, fibrinogen. Chapters 2 and 3 provide information on the history, production, structure, and genetic determinants of these respective markers. Furthermore, summaries of their associations with cognitive ability and cognitive decline were investigated by means of a literature review. A similar approach is taken in Chapter 4, which presents a concise review of the inflammatory markers tumor necrosis factor (TNF)-α and interleukin (IL)-6. Moreover, this chapter also introduces several markers of inflammation and cognition that were assessed as part of the genetic analysis.

A short section describing the main aims of the thesis is then presented before Chapters 5 to 7 introduce the studies and populations that were analysed. The two main studies, the Aspirin for Asymptomatic Atherosclerosis (AAA) Trial and the Edinburgh Type 2 Diabetes Study (ET2DS), are reviewed separately in Chapters 5 and 6, whilst the replication cohorts, the Edinburgh Artery Study (EAS) and the 1936 Lothian Birth Cohort (LBC1936), are reviewed together in Chapter 7.

The results of the biomarker-cognition analyses are provided in Chapter 8, with the results from the genetic analyses featuring in Chapter 9. A discussion of these findings is given in Chapter 10, which also includes a summary of the implications of this work and recommendations for future research.

1.2 Inflammation

Inflammation occurs as the initial response of the body's immune system to infection or irritation (Kumar et al. 2003). It attempts to remove an offending stimulus before instigating the healing process for the affected tissue. Inflamma-

tion can be divided into two categories: acute inflammation, which occurs in the short term (minutes, days, weeks), and chronic inflammation, which is ongoing and can last for months or even several years. The symptoms that characterise an acute inflammatory response are heat (calor), pain (dolor), redness (rubor), swelling (tumor), and/or loss of function (functio laesa). The first four of these were described by Celsus with the latter being attributed to Virchow (Hurley 1983).

At a microscopic level, inflammation involves a complicated sequence of events that includes:

- the dilation of arteries and capillaries, which results in increased permeability and blood flow,
- the flow of fluids, such as plasma proteins, from the circulatory system into the area of inflammation (exudation),
- a migration of white blood cells (leukocytes) into the inflammatory focus (Gallin et al. 1983, Kumar et al. 2003).

Immunologically, the inflammatory process is activated by the recognition of the material that is to be eliminated (the antigen). This can occur via specific or non-specific means. Specific recognition is mediated by antibodies or by receptors on white blood cells (T lymphocytes) whilst non-specific recognition relies on the identification of broken down (denatured) proteins by the complement pathway or by phagocytes (McGeer & McGeer 2002). During the non-specific process, a recognition component binds to the antigen, which initiates the production of pro-inflammatory substances (Kumar et al. 2003). These mediators result in the microscopic events described above although it is the job of phagocytes to actually destroy antigens through immune mechanisms (Gallin et al. 1983).

Whilst many immune processes are ongoing, resolution with the removal of antigens often occurs without displaying detectable signs of inflammation. If clinically apparent inflammation is present then this tends to indicate that:

- the immune system has encountered an unusually large amount of antigen,
- the antigen is presented in an unusual location,
- the antigen was difficult to digest (Gallin et al. 1983).

Inflammation and tissue injury at the immune level are characteristics of a wide variety of human diseases such as rheumatoid arthritis, chronic asthma, allergic reactions, and inflammatory bowel disease (Hurley 1983). The aetiology of

inflammation is complicated and studies have also implicated it with other health conditions and diseases including impaired cognitive function and dementia (Dik et al. 2005, Gunstad et al. 2006, Ravaglia et al. 2005, Schmidt et al. 2002, Schram et al. 2007, van Oijen et al. 2007, Yaffe et al. 2003). Whilst it has been peripheral levels of inflammatory markers that have been associated with these cognitive disorders, there are several pathways through which cytokines and inflammatory mediators can communicate directly with neurones in the brain (Strachan et al. 2008). This may result in the activation of cerebral endothelial cells and transportation processes across the blood-brain barrier (Konsman et al. 2002, Perry 2004). Moreover, macrophages in the brain are also capable of producing inflammatory cytokines (Strachan et al. 2008).

More details of the inflammatory response system and some of the individual proteins involved in these processes are discussed at length in Chapters 2 to 4.

1.3 Cognition

The neurobiology of cognitive ageing is a complicated process and understanding changes in cognition can be demanding for many reasons. One of the most notable challenges is the discrimination of normal ageing from neural pathologies (Whalley 2002). Given the increased risk of developing conditions such as Alzheimer's disease (AD), Parkinson's disease, diabetes, hypertension, and atherosclerosis with age, it is ever more likely that elderly adults will experience some form of neuropathologies (Hedden & Gabrieli 2004). Cognitive ageing research is also hampered somewhat by study design with longitudinal studies being expensive and time consuming to conduct. Only a handful of studies have followed up individuals from childhood or early adulthood into old-age although follow-up studies of elderly individuals ($>$ 60 years) are becoming more common place.

1.3.1 Defining 'intelligence'

Before compartmentalizing cognition into sub-units responsible for different functions, scientists attributed intellectual ability to a single 'general intelligence' component (Lezak 2004). This was postulated to increase during formative years with future tissue loss resulting from ageing, accident and disease. However, refinements to cognitive testing have facilitated greater precision and control over measurements of intellectual behaviour. Subsequently, cognitive ability is now recognised as a multi-factorial component that requires a combination of tests to target its specific domains. Nevertheless, there tends to be strong inter-

correlations between individual tests, reflecting the presence of a general intelligence factor (Deary & Batty 2007).

Two common approaches to defining intelligence and intellectual function are those of Lezak and Carroll. Lezak comes from a neuropsychological tradition, in which the aim is to devise tests for cognitive domains; Carroll comes from a psychometric tradition, in which the emphasis is to examine how tests of different domains are related to each other. The former approach tends to use case studies and case-control designs. The latter uses correlational designs with samples/cohorts to study individual differences in continua.

Lezak's approach

Lezak defined intellectual function into four major classes (Lezak 2004):

- receptive functions, which involve the acquisition, classification, integration, and processing of information.
- memory and learning for the obtainment and recall of information.
- thinking, which involves the organization and reorganization of information.
- expressive function, via which information is conveyed or acted upon.

Within these groupings it is possible to differentiate between functions that mediate verbal/symbolic information from those that involve data that cannot be described by words or symbols, such as complex visual or sound patterns.

Carroll's Three Stratum Theory

A compelling argument of intelligence is also presented in Carroll's Three Stratum Theory (Carroll 1993) whereby he identifies, via factor analyses, three layers representing narrow, broad, and general cognitive ability. The general ability stratum consists of the general intelligence factor with the broad stratum being separated into seven separate domains: crystallised intelligence, fluid intelligence, general memory and learning, broad visual perception, broad audio perception, broad retrieval ability, broad cognitive speediness and processing speed. The narrow stratum includes 69 abilities, each of which can be allocated into one of the seven categories within the broad stratum.

Within the seven broad categories of cognition, crystallised intelligence refers to retained knowledge that is accumulated over time. By contrast, fluid intelligence can be thought of as the information processing abilities, or the actual use of knowledge as opposed to its acquisition. Measures of fluid intelligence are less

robust to physiological adaptations such as ageing and physical damage to the brain than their crystallised counterparts (Deary & Batty 2007). For example, certain tests of vocabulary and verbal ability tend to remain preserved throughout the majority of a person's lifetime. This conservation can even remain during the initial stages of dementia whilst other, fluid abilities are showing significant signs of decline.

Central to intelligence capabilities are an individual's capacity to memorise and learn new information. Clinically, three types of memory can be defined: registration; short-term memory; and long-term memory (Lezak 2004). In the registration stage, information is held in a sensory store for a very brief period of one or two seconds. Despite its purported role defined above, Lezak notes that registration is "*neither strictly a memory function nor a perceptual function but rather a selecting process by which perceptions enter the memory system.*" The details that are processed during this short registration phase are then passed on to short-term memory, otherwise they decay rapidly.

Short-term memory encompasses the fixation of the information retained during the registry process. It behaves "*as a limited capacity store from which information is transferred to a more permanent store*" and "*as a limited capacity retrieval system*". This working or primary memory stage will retain information for between thirty seconds and two minutes unless it is reiterated via rehearsal. Rehearsal is defined as "*any repetitive mental process that serves to lengthen the duration of a memory trace.*"

The final segment of the memory system is that of long-term or secondary memory. Items committed to long term memory are consolidated as quickly as half-a-second after they enter short-term storage (Lezak 2004). Long-term memory can also be separated into recent and remote memories. These can span from memories amassed in the last few hours, days, weeks, or months, back to older memories from childhood. Moreover, in the majority of cases, long-term memories can be thought of as being a continuum with no definite cut-off between recent and remote events. This dichotomisation only becomes apparent and meaningful when investigating persons with problems of amnesia (Lezak 2004).

The category of cognitive speediness and information processing speed measures the ability to assimilate, process, and output new information. Theories suggesting processing speed as a measure of performance in simple and complex cognitive tasks have been proposed since the first days of cognitive testing (Deary et al. 2001). There is some evidence to support this with reaction time scores

being shown to correlate significantly with intelligence test scores (Deary 2000); the higher the cognitive function, the faster and less variable the reaction time. For the remaining broad categories, visual and audio perception defines the ability to interpret information from visible light and audible sounds to the eyes and ears, respectively. Finally, broad retrieval ability denotes "*a capacity to readily call up concepts, ideas, and names from long-term memory.* (Lezak 2004)"

Moving further 'downstream' beyond the third stratum yields the individual cognitive tests that are used to assess the cognitive domains identified within each stratum. Due to the complex nature of cognitive function it is very difficult to devise a test or group of tests to measure a single domain without tapping abilities from other areas. Nevertheless, classification of tests into general domains is still possible, although caution must be exercised when using results of individual tests to make inferences on the broader domains.

1.3.2 Cognitive change

Thus far just some of the difficulties in defining and measuring cognitive ability have been discussed. A further layer of complexity is added if one considers cognitive change and the identification of factors involved in cognitive decline. As already mentioned, one of the main difficulties in assessing cognitive decline is the discernment of normal ageing from pathological decline. This subsection addresses normal cognitive ageing and its determinants with Mild Cognitive Impairment and dementia being discussed in subsections 1.3.3 and 1.3.4.

Whilst performance on many cognitive tasks declines over time, the age at onset and the rate of decline can vary substantially across domains (Hedden & Gabrieli 2004). Much of the change in cognition may be attributed to a decline in the general factor although domain specific deficits can also be described (Whalley et al. 2004). Cross-sectional and longitudinal studies have both shown declination from early adulthood in the following domains: delayed recall of verbal information; working memory; short-term recall; and processing speed (Hedden & Gabrieli 2004). Several studies note processing speed to be the domain showing the most prominent changes across the adult lifespan (Hedden & Gabrieli 2004, Yankner et al. 2008). This may suggest why older persons require more time to absorb new information. It has also been hypothesised that specific candidate domains, such as processing speed, are the main focus of age-related changes and thus, mediate the effects of age on other domains (Whalley et al. 2004). However, despite these findings the evidence is far from conclusive with different patterns of decline in cognition being observed for different domains within different studies.

Estimated lifetime cognitive change

Although many areas of cognition decline over time, some abilities remain relatively stable throughout a lifetime's duration. Most notably these include well practised tasks or tasks involving knowledge. Long-term memory of life history, implicit memory, attention span, vocabulary, and semantic knowledge also show relative stability over time (Hedden & Gabrieli 2004, Yankner et al. 2008).

Given that performances on vocabulary-based tests tend not to vary, studies have used this information to help estimate lifetime cognitive change (Rafnsson et al. 2007). Scores from validated tests such as the Mill Hill Vocabulary Scale and the National Adult Reading Test can be used to estimate pre-morbid or prior cognitive ability (Deary et al. 2004). This is particularly useful in that very few studies have measures of cognition ranging from early adulthood to old age. It has been shown that late-life cognitive scores adjusted for a late-life vocabulary-based estimate of pre-morbid ability correlate highly with actual cognitive change measured across a lifetime (Crawford et al. 2001). The methodology applied by Rafnsson et al. to estimate cognitive change was to enter the vocabulary-based test scores as covariates in a regression model where late-life cognitive ability was the dependent variable. This technique was applied to the estimated lifetime cognitive change models in this thesis.

In addition to the stability of vocabulary-based measures of cognition, there is evidence to suggest that some processes, such as emotional stability, may improve with age (Yankner et al. 2008). The preservation of these facets may aid older persons to derive more efficient or effective strategies when performing tasks that younger persons rely on processing abilities for.

The change or lack of change in cognition across time has been assessed using both cross-sectional and longitudinal study designs. There are pros and cons to both methods with longitudinal studies having the obvious advantage of assessing change amongst the same group of individuals. However, they can also underestimate the actual associations due to practice effects and sample attrition, with the most severely impaired being more likely to be censored. The main problem involved in collecting cross-sectional data is the possibility of cohort differences between study groups.

Determinants of normal cognitive ageing

There are several factors that influence individual differences in normal cognitive ageing (Deary & Gow 2008, Hendrie et al. 2006). These include modifiable and non-modifiable determinants such as:

- genetic variation,
- brain white matter pathology and integrity,
- education,
- cardiovascular risk factors,
- depression,
- psychosocial factors (e.g. socio-economic status, social networks, stress),
- physical activity,
- chronic illness,
- lifestyle factors (e.g. diet).

1.3.3 Mild Cognitive Impairment

During the ageing process, trajectories of cognitive decline can differ markedly between individuals. Whilst some experience negligible change in cognitive function, others may deteriorate dramatically (Christensen 2001). Furthermore, many who go on to develop dementia will experience a transitional phase of decline prior to clinical diagnosis. This period of decline provides a window of opportunity for intervention and clinical treatment in the attempt to postpone or prevent further deterioration (Bischkopf et al. 2002).

A prominent concept in age-related cognitive decline is that of mild cognitive impairment (MCI). This accounts for the stage between normal ageing and dementia where a patient shows decreased performance on memory tasks but not sufficiently so as to be clinically diagnosed with dementia (Petersen et al. 1999). Although widely used as a diagnostic entity of sub-clinical cognitive impairment (Davis & Rockwood 2004), no unique MCI definition currently exists. The original proposal for MCI required the presentation of the following quartet of conditions:

- a memory complaint, preferably confirmed by an informant.
- an objective memory impairment for age.
- a relative preservation of other cognitive functions.
- no dementia. (Petersen et al. 1999)

In recent years, adaptations and refinements have been made to the criteria to include forms of mild dysfunction other than memory loss (Portet et al. 2006). The conversion rate of MCI into dementia is around 10-15% per year (Petersen et al. 1999) with the annual incidence of newly diagnosed cases of MCI being between 3-5% (Rusinek et al. 2003). Neurologically, the symptoms of MCI include an increased number of neurofibrillary tangles in an area of the brain associated with memory (the temporal lobe) (Hedden & Gabrieli 2004).

1.3.4 Dementia

At the opposite end of the cognitive spectrum from normal ageing is dementia. As previously mentioned, this state can be reached via declination in cognitive function in persons with MCI or by more direct means. The term dementia refers to a syndrome, not a single disease, and is characterised by chronic loss of cognitive function (van der Flier & Scheltens 2005). Over the next 40 years, the expected number of persons in the UK with dementia is expected to rise over two-fold from 700,000 to 1.7 million (Strachan et al. 2008).

The most common causes of dementia are Alzheimer's disease and through vascular complications (Vascular Dementia – VaD). Most commonly diagnosed in persons aged over 60, the neurological hallmarks of AD are amyloid plaques and neurofibrillary tangles. The former consist of an aggregation of insoluble β–amyloid proteins, which are thought initiate the deterioration of neuronal synapse function, composition, and function. Neurofibrillary tangles are described by Rosenzweig and Leiman (Rosenzweig & Leiman 1982) as "*abnormal whorls of filaments which formed a tangled array inside the cell*".

In comparison, VaD is a term for many different processes that result in dementia due to cerebral infarction, hypo-perfusion, or haemorrhage. These processes include:

- Large-vessel VaD.
- Small-vessel VaD.
- Ischaemic-hypoperfusive VaD.
- Haemorrhagic VaD.

The most common form of VaD results from cerebral small vessel disease, which is associated with the presence of lacunar infarcts (lesions caused by the occlusion of arteries) and white matter lesions (damage to the myelin) (Bruce

et al. 2003, Munshi et al. 2006). By contrast, large vessel cognitive impairment tends to be embolic with cerebral damage via numerous infarcts that are widely distributed throughout the brain, or via a single infarct in a critical area of the brain (Strachan et al. 2008). Ischaemic-hypoperfusive VaD occurs via reduced blood flow to the brain (Román 2004) whilst haemorrhagic VaD describes bleeds in the brain-ruptured blood vessels (Meyer et al. 2000).

Presently, several criteria can be used in the diagnosis of VaD (Graves 2004, Wetterling et al. 1996) although it is often not possible to distinguish between VaD and AD (van der Flier & Scheltens 2005). Complicating matters further, it is also possible for VaD and AD to co-exist in what is termed mixed dementia, and for both conditions to share underlying risk factors and neuropathological features (Ritchie & Lovestone 2002). Within normal ageing, a combination of oxidative stress, inflammation, and alterations to cerebral microvascular structure have been credited as the cause of cognitive impairment (Riddle et al. 2003) Although much of MCI and pathological ageing can also be attributed to these reactions, most healthy brains display a varying degree of neuropathological features that are typical of AD (Whalley et al. 2004).

In summary, the neuropathology of age-related cognitive decline is complicated and its biological basis remains largely unknown. Whilst current thinking is opposed to a widespread loss of neurons in favour of restricted loss to specific cortical regions (Rasmussen et al. 1996), the presence of neurofibrillary tangles and amyloid plaques in seemingly healthy brains makes clinical diagnoses of mental health complications extremely complex.

1.4 Diabetes

Diabetes Mellitus is a chronic metabolic disorder that is characterized by increased plasma glucose. There are two types of diabetes that can be diagnosed: insulin dependent (Type 1 diabetes), and non-insulin dependent (Type 2 diabetes). There are approximately 2.5 million people with diabetes in the UK and a further 500,000 with undiagnosed diabetes (Diabetes UK 2009). In the UK, Type 2 diabetes accounts for between 85–95% of all sufferers. Type 1 diabetes usually occurs before the age of 40 with Type 2 diabetes more likely to be present after this age (Diabetes UK 2009). However, it is becoming increasingly common for Type 2 diabetes to be diagnosed in people from as young as the age of seven. There is also an increased prevalence of Type 2 diabetes in some younger ethnic populations, for example South Asian and African Caribbean persons are

often diagnosed from 25 years upwards (Diabetes UK 2009). Moreover, by the year 2030 it has been projected that 366 million people worldwide (4.4% of the population) will have diabetes (Strachan et al. 2008).

Type 1 diabetes occurs via the destruction of beta cells in the pancreas (Williams & Pickup 1999). These cells are responsible for the production of insulin, an essential hormone in the metabolism of glucose. As a result, the body is forced to use its fat stores as a source of energy. Presently, the main treatment for Type 1 diabetes is the administration of insulin shots.

By contrast, Type 2 diabetes is characterised by high blood glucose levels resulting from insulin resistance (Williams & Pickup 1999). Insulin levels initially rise in an attempt to drive glucose into the cells. However, the pancreas ultimately struggles to keep up with the production and insulin levels fall. This leads to elevated blood glucose levels and symptoms of the condition.

1.4.1 Diagnosis

The following diagnostic criteria are described using information from the Second Edition of the Handbook of Diabetes (Williams & Pickup 1999) and from the World Health Organisation website[1].

Diabetes is diagnosed through the identification of chronic hyperglycemia. If a patient is presented with classic symptoms such as thirst, passing large volumes of urine (polyuria), or weight loss then a test of random blood glucose will provide an instant indication as to whether he/she has diabetes. If the patient's blood glucose level is slightly elevated or if there are no classical symptoms or signs then a formal test of oral glucose tolerance will be conducted. For the oral glucose tolerance test the subject is given a morning blood test after an overnight fast. After blood withdrawal, 75g of glucose is given by mouth before a further blood sample is taken two hours later. A diagnosis of diabetes is made if the fasting plasma glucose level is in excess of 7.0mmol/l or the value of the second test exceeds 11.1mmol/l. The international measurement criteria, which is set by the World Health Organisation also recognises an intermediate stage of abnormal plasma glucose levels, called impaired glucose tolerance (IGT). This is diagnosed when the fasting plasma glucose level is less than 7.0mmol/l and the two hour repeat measure is between 7.8–11.1mmol/l. Persons diagnosed with IGT are reviewed and retested periodically.

Once a diagnosis of diabetes has been made, the next stage is classification into Type 1 or Type 2 diabetes. It is more likely to be the former if the symptoms

[1]http://www.who.int/diabetes/en/

presented include weight loss and sustained ketosis (elevated levels of ketone bodies in the blood, which are used as an alternative energy source to glucose) although further tests can be carried out to aid a formal diagnosis.

1.4.2 Risk factors and management of diabetes

Whilst both types of diabetes are currently incurable, simple changes to diet and physical activity can help to control the effects of Type 2 diabetes (Diabetes UK 2009). Some of the strongest risk factors associated with the condition include obesity, old age, ethnicity, physical inactivity, previous gestational diabetes, and having one or more parent or sibling with Type 2 diabetes (Mayo Clinic Staff 2009).

As we get older, insulin production decreases due to age-related impairment of the pancreatic beta cells. Most persons that are diagnosed with Type 2 diabetes are over the age of 40 with the peak age of onset being around 60 years (Diabetes UK 2009, Williams & Pickup 1999). However, it is becoming increasingly more common for young adults and children to be diagnosed with diabetes (Diabetes UK 2009). This is mostly linked to increasing rates of obesity and decreased physical activity. The visceral fat that is present in truncal obesity is more metabolically active than peripheral fat and also circulates large quantities of non-esterified fatty acids (Williams & Pickup 1999). These acids have several properties that can lead to insulin resistance including inhibition of glucose uptake in muscles. The relationship between a lack of physical exercise and diabetes is thought to be due to exercise increasing insulin sensitivity and preventing obesity (Williams & Pickup 1999).

There is also variation in the prevalence of Type 2 diabetes by ethnicity (Diabetes UK 2009, Mayo Clinic Staff 2009, Williams & Pickup 1999). Higher rates are found in populations that come from a South Asian background. In the United States, African Americans, Mexican Americans, and Native Americans have particularly high incidence rates of the disease.

1.4.3 Association with cognition

There is longitudinal evidence to implicate Type 2 diabetes as a risk factor for age-related cognitive decline and dementia (Biessels et al. 2006, Cukierman et al. 2005, Strachan et al. 1997). Compared to a non-diabetic population, the risk of dementia is 1.5–2.5 times greater in persons with Type 2 diabetes (Price et al. 2008a). However, the exact mechanisms underpinning the association are unclear.

In addition to its relationship with hyperglycemia and hypoglycaemia, Type 2 diabetes is known to be a cause of microvascular disease, retinopathy, nephropathy, and macrovascular disease - including coronary heart disease and stroke (Starr & Convit 2007). Consequently, vascular dementia is common amongst older persons with Type 2 diabetes. Whilst it is likely that diabetes-related cognitive dysfunction has a multi-factorial pathogenesis, there is growing evidence to implicate a role for diabetes specific risk factors e.g. metabolic derangements and a range of circulating biomarkers and genetic risk factors (Price et al. 2008a).

The association between Type 2 diabetes and cognitive decline is well described and summarised concisely in a review paper by Kodl and Seaquist (Kodl & Seaquist 2008). They describe strong evidence for decreased cognitive performance in persons with Type 2 diabetes in the following domains: memory, psychomotor speed, executive function, verbal fluency, visual retention, attention, and complex motor functioning. Despite many studies examining the link between diabetes and cognitive function, there are inconsistencies in controlling for covariates. Age is almost always adjusted for although the same cannot be said for other covariates. Amongst the potential confounders and comorbidities, severe hypoglycemic events have been related to cognitive impairment in younger persons, although these tend to have less influence in ageing persons with diabetes (Kodl & Seaquist 2008). In addition to its association with other cardiovascular complications, those with diabetes are twice as likely to suffer from depression, which is similarly associated with impaired cognitive function. They are also at greater risk of developing vascular dementia and Alzheimer's disease (Kodl & Seaquist 2008).

One of the complications that precedes diabetes, impaired glucose tolerance (IGT) has been associated with poorer cognition across the domains of working memory, verbal declarative memory, and executive function (Messier 2005). These decrements were observed in older persons under fasting conditions but diminished after re-testing post consumption of a glucose solution. The relationship between IGT and cognitive dysfunction was also present but reduced in younger persons. Subsequently, it would seem natural to assume that progression from IGT to Type 2 diabetes would result in a concomitant change in cognition. However, this does not appear to be the case. Messier hypothesises that this may be due to confounding factors modulating the relationship (Messier 2005). As previously commented, Type 2 diabetes is a disease that often occurs in combination with other conditions leading to increased risk of cardio and cerebrovascular disease, which themselves are independent predictors of cognitive dysfunction.

Furthermore, short-term variation in blood glucose concentration may have a significant effect on cognitive function in those with diabetes. One study (of just 20 subjects) found an increase in blood glucose levels to 16.5mmol/l for one hour to have a negative impact in the working memory, attention, and mood (Sommerfield et al. 2004). In addition, a multi-centre randomised trial found strict glycemic control over a 26 week period in 145 persons with Type 2 diabetes to improve performance in working memory (Ryan et al. 2006). Finally, a one year follow-up study of 156 persons found elevated post-prandial glucose to associate significantly with poorer executive function and attention performance (Abbatecola et al. 2006). To observe significant associations over such short follow-up periods perhaps indicates that functional consequences of hyperglycemia e.g. altered cerebral blood flow, may cause cognitive dysfunction. Moreover, in a population of nearly 2,000 post-menopausal women, it was found that those with a HbA1c (glycated haemoglobin, which is a measure of glucose control over the past 6-8 weeks) of more than 7.0% had a 4-fold increase of developing mild cognitive impairment (Yaffe et al. 2006).

For those with good glycemic control who are under the age of 70, Type 2 diabetes has little impact upon cognitive function (Messier 2005). By contrast, those with poor glycemic control and underlying micro or macrovascular disease may be at risk of impaired cognition. Beyond the age threshold of 70 years, it is likely that accelerated cognitive decline is a result of Type 2 diabetes and its interaction with vascular dementia and Alzheimer's disease. The domains most affected in younger persons are processing speed and verbal episodic memory with extension to most domains beyond the age of 70. The severity of decline is noticed most for those with concurrent vascular disease (Messier 2005).

In terms of describing the mechanisms that correspond to the cognitive decline, one study forwards three potential pathways (Biessels et al. 2006). The first focuses on decline via ischemic vascular disease as Type 2 diabetes is closely involved in the pathology of both large and small blood vessels. Secondly, they propose the toxic effects of high glucose concentration, which is equivalent to the role of irregular glucose metabolism in other long-term complications of diabetes. Finally, it is possible that cognitive dysfunction is a result of affected insulin homeostasis in the brain. It appears that insulin, in accordance with insulin-degrading enzyme, modulates cognition, possibly through cerebral amyloid and tau metabolism.

1.4.4 Association with inflammation

In addition to its implication in the development of atherosclerosis, inflammation has been studied in relation to insulin resistance and Type 2 diabetes. Several studies have since reported that chronic, low-grade inflammation may induce insulin resistance and that it might be part of the pathway leading to diabetes. Furthermore, around 90% of persons with Type 2 diabetes show insulin resistance, which normally precedes the first symptoms of the disease (Sjohölm & Nyström 2006).

It is well recognised that cardiovascular disease is one of the main complications of insulin resistance and Type 2 diabetes. However, it may be possible that inflammation is not only a consequence of atherosclerosis but that it also plays an active role in the initiation of insulin resistance. Prospective studies have found elevated levels of acute-phase proteins (including C-reactive protein) to predict the development of insulin resistance and Type 2 diabetes (Nesto 2004).

One mechanism by which inflammation and insulin resistance are related is obesity (Barzilay & Freedland 2003). Persons with obesity and, in particular, truncal obesity tend to secrete an increased concentration of inflammatory cytokines and proteins. Some of these cytokines, such as TNF-α and IL-6 affect the sensitivity of insulin and its secretion. In addition, they also act on the liver, increasing the synthesis of other inflammatory proteins e.g. CRP and fibrinogen.

A second possible mechanism of association is during hyperglycaemia, which yields an increased concentration of glycation end products (Coppola et al. 2006). These activate the macrophages, increase oxidative stress, and thus up-regulate the synthesis of the IL-1, IL-6, and TNF-α, which in turn augments the production of CRP.

Conversely, it may be that chronic inflammation occurs as a by-product of the insulin resistance syndrome (Nesto 2004). Insulin is known to have acute anti-inflammatory properties that include the ability to suppress the synthesis of acute-phase proteins. Therefore, it is plausible that increased production of CRP and fibrinogen are a consequence of insulin resistance.

Whether inflammation lies on the causal pathway towards Type 2 diabetes is not yet clear although recent studies have developed methodologies to try and test this hypothesis. One such approach is Mendelian randomisation. Briefly, this models the exposure variable against the outcome whilst stratifying for genetic variants which control the synthesis of the exposure phenotype. As genetic information is determined at conception, this method helps to reduce for confounding effects that may arise from basic association studies. A detailed description of

Mendelian randomisation and the statistical methods used in its application is provided in Chapter 9.

One study that used this methodology investigated the association between CRP and insulin resistance and Type 2 diabetes (Brunner et al. 2008). Using genetic and physiological information from 4,674 men and women with Type 2 diabetes from the Whitehall Study (mean age 49 years), the authors tested the relationship between haplotype-stratified CRP levels against HOMA-IR (a measure of insulin resistance) and HbA1c. The findings from these analyses found it unlikely that CRP is causally associated with either of the factors. Similarly, a pooled analysis of *CRP* haplotypes and diabetes in the Whitehall Study and a replication cohort, the Northwick Park Heart Study II, showed no association between *CRP* variants and diabetes risk (Type 2 diabetes prevalence = 7.4%, n = 522). Finally, data from the Wellcome Trust Case Control Consortium also showed no association between *CRP* haplotypes and diabetes risk. In conclusion, the authors report that the CRP–diabetes relationship may be due to confounding from upstream effectors of inflammation as opposed to downstream markers. They suggest future investigations should examine cytokines at the start of the inflammatory cascade, for example IL-6 and TNF-α.

1.5 Genetics

Despite many epidemiological studies providing evidence for a relationship between a phenotype and disease outcome, it is often very difficult to determine if the association is causal. One of the most challenging aspects in such a situation is to account for all potential confounders and mediators within the model. Such complications can be partially resolved by a genetic epidemiology approach, whereby genetic variants can be modelled directly, or via an intermediate phenotype, against the outcome variable. The main advantage of considering DNA variation is that a person's germline is fixed at conception. This means that modelling with genetic data bypasses some of the problems of confounding. However, genetic association studies do require that the variant under investigation affects the intermediate phenotype or outcome variable. If the genetic variant is actually a proxy marker for the true causal variant or if there are multiple variants that affect the phenotype or outcome then it becomes much harder to annotate the causal mechanism and subsequently, develop interventions. The introductory material presented in the following subsections is common knowledge. Three textbooks were used to reference the majority of the content (Carey 2002, Fal-

coner & MacKay 1995, Plomin et al. 2000); individual references are included for descriptions of the more complex issues.

1.5.1 DNA

The genetic blueprint for life which regulates cell growth, division, and function is stored in a person's DNA. DNA is comprised of two long polymer strands that are made up of individual units called nucleotides. The genetic code is written using four nucleotides: adenine, cytosine, thymine and guanine (A, C, T, and G). These nucleotides form complementary base pairs (A–T and C–G) when the two polymer strands align, bond, and twist to give DNA its classic double helical shape.

Within eukaryotes DNA is organised into 46 entities known as chromosomes. There are 22 pairs of autosomal chromosomes and two sex chromosomes. Chromosomes are organised structures of DNA, sections of which are grouped together to form genes. Genes contain coding information for specific proteins, which subsequently correspond to a variety of biological traits.

When a gene is active a copy of it is made by a process called transcription. Transcription begins when the gene unwinds into two strands. Free RNA nucleotides then form complementary base pairs with one strand of DNA bases, and the mRNA strand is synthesised before it peels off from the DNA and moves from the cell nucleus into the cytoplasm. Translation of the mRNA into proteins then takes place in the cytoplasm. The mRNA strand attaches to a ribosome before tRNA molecules are used to read its coding triplets. Peptide bonds then form between the adjacent amino acids to form proteins.

The regions of the gene that are translated into proteins are known as exons with the non-translated regions referred to as introns. The number of and length of both introns and exons varies by gene. This main section of the gene is flanked at the front and back by the 5' and 3' untranslated regions (UTRs), respectively. Several roles in gene expression have been attributed to the UTRs, including mRNA stability, and translational efficiency (the rate of mRNA translation into proteins). In terms of location, genes are situated at fixed points on the chromosome called loci.

1.5.2 Genotypes, phenotypes, and haplotypes

As diploid organisms, humans have two copies of each gene in their somatic cells. Each copy of the gene is referred to as an allele and the combined information

from both alleles represents an individual's genotype. If the two copies of the gene are identical then the alleles will also be the same and the genotype is said to be homozygous. However, if there is allelic variation then the genotype will be heterozygous. The physically expressed form of the gene (the phenotype) is determined by the alleles and depending on the combination of alleles, recessive or dominant traits may be exhibited.

It can also be informative to consider groups of alleles on the same chromosome that are inherited together in blocks. The formal name for such a group is a haplotype – a contraction of the phrase 'haploid genotype'. Haplotypes are built using the details of consecutive alleles and are perhaps better markers of phenotypes than genotypes given that they reflect blocks of inherited information as opposed to single variants. Unfortunately, as humans are diploid organisms, haplotype inference is not guaranteed to be 100% accurate. Where a person is heterozygous for a genotype, it is not possible to determine for certain which allele should go with which haplotype. The alleles in this situation are described as being phase ambiguous (Spinka et al. 2005). In order to generate haplotypes, statistical methods such as the Expectation–Maximization (EM)–algorithm are used to impute haplotype frequencies at the population level. Computer software packages such as SimHap[2] can generate such haplotypes and then test for associations with outcome phenotypes, whilst incorporating the uncertainty around inferred haplotypes into the modelling procedure.

1.5.3 Genetic variation

There are numerous ways in which the DNA sequence of a gene can be altered. Depending on the location of the mutation and whether it alters the protein structure, genetic variants can have varying health effects. There are several different types of mutation that alter genetic structure such as small-scale and large-scale mutations.

Small-scale mutations incorporate alterations that affect a gene in one or a few nucleotides. These include:

- Single nucleotide polymorphisms (SNPs) - as the name would suggest, a SNP is DNA sequence variation whereby a single nucleotide in the genome varies between members of a species. This difference may result in a synonymous (silent) or non-synonymous (non-silent) mutation. A silent mutation is when there is a change in the DNA sequence but no change in the protein

[2]http://www.genepi.org.au/simhap.html

that is coded - hence there is no noticeable difference in the biological trait for which that particular gene is responsible for programming. By contrast, non-synonymous SNPs tend to be more dangerous in that the nucleotide alteration results in a different protein being coded, which could potentially cause serious damage.

- Insertions - this is where one or more nucleotides are added to the DNA. The normal causes of an insertion are transposons (sequences of DNA that are capable of moving within a cell) and errors during replication of repeating elements.

- Small deletions - this is when one or more nucleotides are removed from the DNA. They occur at random and generally tend to be irreversible.

Large-scale mutations can result from

- Amplifications - whereby duplicate copies are made of chromosomal regions, which increases the expression of the genes located in situ.

- Inversions and translocations - an inversion is where there is a reversal of the nucleotide information on a portion of the chromosome whereas translocation is the rearrangement of parts of between non-homologous chromosomes.

- Large deletions – this is when a deletion or recombination event causes the loss of an allele, resulting in a homozygotic site where before there were two alleles and either hetero or homozygosity.

1.5.4 Linkage, Linkage Disequilibrium, and TagSNPs

It is often of interest to consider allelic information from different loci on the same chromosome that are close enough together to be inherited as a unit. Loci that are inherited in such a manner are said to be linked. Therefore, knowing an allele at one point of the chromosome enables inference of the other alleles.

Furthermore, it is also possible for a non-random association of alleles that could potentially be located on different chromosomes. The strength of these associations can be quantified using a measurement of linkage disequilibrium (LD). There are several different statistics to consider when assessing LD, such as D' (a measure of the independence of the two alleles) and r^2 (how well one allele can predict another). In areas where there is evidence of high LD it is possible to infer information from across the region by typing a solitary SNP, called a tagSNP.

1.6 Summary

This chapter provided an introduction to the key fields reported in this thesis: inflammation, Type 2 diabetes, cognition, and genetics. An in-depth review of specific inflammatory biomarkers is provided in the following three chapters. This includes information on the function of the markers, the genes that encode the markers, the inter-relationship between the markers and the genetic variants, and the relationship between the marker levels, the genetic variants, and cognitive ability.

Chapter 2

C-reactive protein

2.1 Introduction

Building on the brief description of inflammation in Chapter 1, this chapter provides a detailed overview of the inflammatory biomarker C-reactive protein (CRP). It begins by charting the history of CRP, its molecular structure, and its function. This is followed by a literature review to determine the association between plasma CRP levels and cognitive ability. Finally, a summary of the *CRP* gene is presented with SNP variants being reported in relation to both plasma CRP levels and cognition function.

2.2 History

Discovered by Tillett and Francis in 1930, C-reactive protein (CRP) was found in the serum of patients with pneumococcal pneumonia (Tillett & Francis 1930). These patients' sera were found to be precipitating with a soluble extract, labelled fraction C, which was later identified as a polysaccharide of the cell wall of the pneumococcal bacteria. After resolution of the pneumonia, the precipitation was no longer found to occur between the sera and fraction C. However, in the instances where the pneumonia resulted in a fatal outcome, the precipitate could still be formed.

Future studies by Tillett and Francis showed similar test results for the following conditions: staphylococcus osteomyelitis, rheumatic fever, sub-acute bacterial endocarditis, and lung abscesses. However, negative test results were found for the precipitation reaction in patients suffering from viral infections, malaria, and tuberculosis (Ablij & Meinders 2002).

It was the work of Rachel Welsh in 1933 that helped to define the precipitation

phenomenon as a non-specific physiochemical reaction to bacterial infection as opposed to a specific antigen-antibody reaction. Eight years later in 1941, Avery and Abernethy established that the substance responsible for the precipitation reaction with fraction C was the protein CRP. (Gotschlich 1989)

2.3 Production

CRP is an acute-phase protein that is primarily synthesised and secreted into the circulation from the hepatocytes in the liver (Ablij & Meinders 2002) although studies have also shown that production can occur in adipocytes (Yeh 2005). An acute-phase protein is a protein whose plasma concentration increases or decreases by over 25% during an inflammatory attack (Kushner 1982). Healthy individuals generally have CRP levels less than 3mg/l (Pearson et al. 2003). These can increase as much as 1000-fold during acute inflammation (Ridker 2003) to a potential peak of around 400mg/l (Pepys & Berger 2001).

The mechanism that causes this proliferation of CRP is mostly driven by a rise in concentration of the cytokine interleukin (IL)-6 which is used to recruit CRP to the site of inflammation (Yudkin et al. 1999). Circulating levels of both interleukin (IL)-1β and tumor necrosis factor (TNF)-α have also been shown to regulate CRP mRNA transcription (Yudkin et al. 1999). Removal of CRP from the bloodstream is mono-exponential with a biological half-life of 19 hours (Koenig et al. 2003). As the half-life is unaffected by the relative level of CRP, the only significant determinant of plasma CRP is its rate of synthesis. This, in principal, justifies its clinical use to monitor inflammatory activity.

2.4 Structure and function

CRP is a protein from the pentraxin family with binding sites for ligands on each of the five sub-units of the molecule (Kuo et al. 2005). After binding with phosphocholine or a number of similar ligands such as damaged cell membranes, native or oxidised plasma lipoproteins, and various components of micro-organisms, the ligand-complexed CRP is recognised by the complement sub-component C1q (Ablij & Meinders 2002). This in turn leads to the formation of the C3 sub-component complex, and subsequently, to the activation of the human complement system – a biochemical cascade of the immune system that aids the removal of pathogens from the body (Feuerstein et al. 2003). Activation of the complement pathway enables the elimination of the ligand-CRP complex by causing it

to be engulfed (opsonized) and destroyed by phagocytes, such as macrophages.

CRP is also able to bind to receptors on antibody or immunoglobin (IgG) molecules, which themselves bind to many kinds of pathogens (Gewurz et al. 1995). IgG protects the body against viral and bacterial infection by complement activation. The effect of the CRP-IgG binding is to stimulate the action of phagocytic cells and to augment the phagocytosis of micro-organisms or damaged/dead host cell material.

It has also been hypothesised that CRP is involved in anti-inflammatory processes through a modified version of CRP (mCRP) (Ablij & Meinders 2002). The formation of mCRP occurs via the proteolysis of CRP, which dissociates into monomeric sub-units before structural change (denaturisation) leads to the creation of new molecules (Zouki et al. 2001). mCRP has the ability to bind to a low-affinity IgG receptor on the neutrophil (a type of white blood cell which removes pathogens via phagocytosis) (Zouki et al. 1997). This elicits the shedding of the adhesion molecule, L-selectin, from the neutrophil and prevents bonding to the endothelial cell. This process may provide an explanation as to why neutrophils are missing in atherosclerotic lesions (Rattazzi et al. 2003).

2.5 Measurement

Whilst CRP levels greater than 10mg/l are widely regarded as being associated with acute inflammation (Pearson et al. 2003), variation at lower levels of CRP is also of great interest. This is particularly the case when assessing chronic inflammatory conditions. Until the last 5-10 years, accurate and consistent measurement of low CRP levels was not possible. However, the introduction of high sensitivity CRP assays has resulted in a relatively inexpensive and reliable method to measure CRP levels from as low as 0.15mg/l (Yu & Rifai 2000). CRP samples are stable at room temperature and high sensitivity assays yield near identical results in both fresh and frozen plasma samples (Koenig 2005). Nonetheless, there is conflicting evidence on CRP measurement stability with some describing no diurnal variation and relatively stable results over a three month period (Li & Fang 2004). Another study found that 63% of CRP measurements in healthy adults fell into the same quartile when re-measured three months later (Ockene et al. 2001). By contrast, Riese et al. advocate re-sampling healthy persons with 'high' values (> 1.75mg/l for males, > 1.00mg/l for females not using oral contraceptives, and > 2.00mg/l for females using oral contraceptives) (Riese et al. 2002). Multiple measures of CRP are also strongly recommended when the par-

ticipants under investigation are suffering from cardiovascular disease (Koenig 2005, Stigant et al. 2005).

In my view, the crux of the single sample versus multiple samples debate surrounds the cardiovascular health of the participants. Li and Fang provide a clear and articulate argument to support the reliability of a single measure in healthy persons. Although it is possible that some participants may have an acute infection at the time of measurement, a large sample size should dominate these potential effects and provide an accurate and true reflection of CRP variability.

2.6 Factors influencing CRP levels

Plasma CRP levels can vary according to both modifiable and non-modifiable factors. Most commonly these include age, sex, BMI, smoking, and ethnicity. It has been well documented that CRP levels rise gradually throughout life in both men and women with females having slightly higher levels (Pankow et al. 2001), especially those receiving HRT (Eklund et al. 2005, Yu & Rifai 2000, Miller et al. 2005) or taking the contraceptive pill (Dreon et al. 2003, Döring et al. 2004).

Where studies stratify by ethnicity they hoped to reduce the heterogeneity across ethnic groups. There is far greater genetic variation in persons of African origin compared to those of European origin (Crawford et al. 2006b, Wang et al. 2006). Wang et al. reported 84 SNPs in the *CRP* gene, 37 of which had a minor allele frequency (MAF) $> 5\%$. Of these 37 SNPs one was Caucasian-unique, 15 were African-American unique with the remaining 21 SNPs common to both populations.

BMI and smoking status are common indicators of cardiovascular disease with higher levels of both strongly associated with raised CRP levels (Brull et al. 2003, Casas et al. 2006, D'Aiuto et al. 2005, Lau et al. 2005, Wang et al. 2006, Yu & Rifai 2000). According to one group, the association between BMI and CRP implies a state of low-grade systemic inflammation in overweight and obese persons (Visser et al. 1999). This association is quite substantial with Kathiresan et al. and Tchernof et al. finding 15% and 18% of CRP variation to be explained by BMI. In total, the portion of plasma CRP levels explained by non-genetic variables varies between 16% and 30% (Table 2.1). Some of the other common adjustable covariates included in these studies were alcohol intake, systolic and diastolic blood pressure, LDL/HDL/total cholesterol, and statin use.

Finally, recent studies of older people have found socioeconomic status (SES) in adult life to predict mid/late-life levels of inflammatory markers, including

Author	Adjustment Variables	% CRP Explained
Crawford et al. (2006b)	age, sex, ethnicity, smoking, education, CHD, BMI, total cholesterol, HDL cholesterol, triglycerides, HbA1c, diastolic BP	19%
Kathiresan et al. (2006a)	age, sex, BMI, HRT, smoking, total/HDL cholesterol ratio, hypertension, lipid lowering therapy, prevalent cardiovascular disease, triglycerides, systolic BP, diastolic BP	26%
Martínez-Calatrava et al. (2007)	age, sex, waist circumference, leptin level, IGT, smoking	16%
Miller et al. (2005)	age, BMI, smoking (PRINCE study)	21%
Miller et al. (2005)	age, BMI, smoking (PHS study)	20%
Pankow et al. (2001)	age, education, smoking, alcohol, HRT, obesity, diabetes	30% (women) 22% (men)
Tchernof et al. (2002)	BMI in post-menopausal women	18%

Table 2.1: Percentage of variation in plasma CRP levels explained by covariates.

CRP and fibrinogen (Pollitt et al. 2007, Tabassum et al. 2008). These associations were weakened when comparing the biomarker levels with childhood SES. Furthermore, adjustment for adult cardiovascular disease risk factors strongly attenuated the associations between CRP and early life SES. Nonetheless, both studies showed decreased SES to predict elevated marker levels. Specifically, the Atherosclerosis Risk in Communities (ARIC) study found a 17% increase in CRP level between the lowest and highest socioeconomic groups (n = 12,681) (Pollitt et al. 2007). These findings were more pronounced in whites than in blacks. Similarly consistent findings were also noted between fibrinogen and SES. The second study was the 1958 British Birth Cohort, which had measures on SES at birth, age 23, and age 42 in 5,951 persons (Tabassum et al. 2008). Comparing a cumulative score for SES with biomarker levels at age 44–45 the authors found significant associations with both fibrinogen and CRP ($p < 0.001$). Finally, one other study found adult but not childhood socioeconomic status to be linked to CRP levels of 1,484 persons from the Young Finns Study (Gimeno et al. 2007).

2.7 Association with disease

2.7.1 General

Although uncertainty surrounds the specific role of CRP in disease, it has consistently been associated with many conditions. High-sensitivity CRP is a strong predictor of vascular disease across a large cross-section of clinical populations that includes "*individuals without CVD, patients presenting with acute coronary syndromes, patients with stable angina or in the stable phase after MI, and patients with the metabolic syndrome, diabetes, or renal disease*" (Bassuk et al. 2004). In addition to being a marker and predictor of cardiovascular and inflammatory disease, CRP levels have also been associated with dementia (Schmidt et al. 2002) and a variety of cancers, including lung (Siemes et al. 2006) and colorectal (Erlinger et al. 2004). However, as already mentioned, it is unclear if CRP is actually functional in the development and progression of these conditions or if it is simply a marker of underlying inflammation.

2.7.2 Atherosclerosis

The presence of complement and CRP in atherosclerotic lesions via the oxidisation and phagocytosis of the LDL–CRP complex indicates a role for inflammation in atherosclerosis (Bhakdi et al. 2004). Furthermore, the continual production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1, by macrophages and T lymphocytes provides evidence that atherosclerosis is an inflammatory process (Ablij & Meinders 2002). These cytokines are released from atherosclerotic plaques into the blood, resulting in increased hepatic production of the inflammatory mediators CRP, serum amyloid P component, serum amyloid A, and fibrinogen. Of the markers, CRP is the quickest to react to an inflammatory stimulus, making it a good candidate to determine the inflammatory role in plaque formation (Ablij & Meinders 2002). Nevertheless, whether CRP plays a causal role in the inflammatory response surrounding atherosclerosis remains unresolved.

Immunohistochemical investigations have reported the presence of CRP in coronary arteries and its association with lipids within the artery walls (Zhang et al. 1999). Quantitative recombinant polymerase chain reaction (PCR) showed a 10-fold increase in CRP mRNA in athero-arterial tissue compared to normal arterial tissue (Yasojima et al. 2001). CRP has also been detected in atherosclerotic lesions from the earliest to the most advanced stages (Sun et al. 2005). Sun et al. conclude that the presence of CRP in lesions is derived from the circulation

instead of de novo synthesis by vascular cells due to the lack of CRP mRNA in atherosclerotic lesions and macrophages. However, a review study of CRP's role in vascular disease noted several novel cell types were able to produce CRP and complement within atherosclerotic plaques (Mazer & Rabbani 2004).

There is substantial evidence from in vitro laboratory studies of endothelial cells to indicate a pro-atherogenic role for CRP (Jialal et al. 2004). However, conflicting evidence from in vivo mouse models has failed to conclusively identify a causal relationship between CRP and vascular disease (Stenvinkel 2006). Where CRP has been shown not to be causally involved, its presence in atherosclerosis is hypothesised to be indirectly related to vascular damage with other inflammatory mediators being the main culprits (Stenvinkel 2006).

Inconsistent proof of a causal role is also described in a selection of epidemiological studies. There seems little doubt over the ability of CRP to predict future myocardial infarction and other vascular events, although recent studies question whether its predictive power is as strong as first thought (Sattar & Lowe 2006). Furthermore, there has been an increase in evidence questioning the projected causal function of CRP in atherogenesis. However, others note that CRP elevation precedes clinically symptomatic atherosclerosis and testing plasma CRP level is the only widely available clinical test that provides a tangible link between inflammation and atherosclerosis (Mazer & Rabbani 2004).

2.8 Association with cognition

In recent years, population-based epidemiological studies have been undertaken to ascertain the relationship between CRP levels and cognitive ability. A comprehensive search of the online medical databases PubMed[1], Embase, and Ovid Medline (through the University of Edinburgh library website[2]) was undertaken to identify large-scale, cross-sectional or longitudinal studies with extensive cognitive testing, that described the association between CRP levels and cognitive ability. Search terms were carefully selected to try and reduce the selection of studies that focussed on dementia diagnoses instead of milder forms of cognitive impairment. In addition to the database searches, when suitable papers were found, their references were also reviewed. Specifically, the terms used for the medline search were ('exp C-reactive protein' | 'C-reactive protein.mp' | 'CRP.mp') & ('exp cognition' | 'exp cognition disorders' | 'cognition.mp' | 'cognit$ impair$.mp' | 'cognitive de-

[1]http://www.ncbi.nlm.nih.gov/pubmed/

[2]http://www.lib.ed.ac.uk/resources/databases/findlita.shtml

cline.mp'). The search dates were set from 1950 to December 2008 and papers were limited to those written in English. Equivalent search terms/notation were used for the retrieval of papers from the PubMed and Embase databases.

During the search a single systematic review of the relationship between CRP and cognitive disorders was uncovered (Kuo et al. 2005). This paper assimilated information on CRP level and its association with stroke, cognitive disorders, and depression in the general population. Six studies were identified that described the association between CRP and cognitive impairment or dementia. However, all six of these papers were also retrieved in the main search.

Many of the studies identified in the literature search suffered from small sample sizes and/or a limited cognitive test battery. In addition, several studies considered a dementia or MCI diagnosis as opposed to a measure of late-life normal cognitive ageing. This is possibly due to dementia and MCI being more straightforward conditions to diagnose as there are formal criteria for their definitions.

2.8.1 Key papers

Of the papers reviewed, four included a comprehensive battery of cognitive tests administered to a large, population-based cohort over a substantial follow-up period (Alley et al. 2008, Dik et al. 2005, Schram et al. 2007, Weuve et al. 2006).

Chronologically, the oldest report was from 2005 where Dik et al. looked at inflammatory protein levels and cognitive decline in an elderly Dutch population. The study involved 998 participants from the Longitudinal Aging Study Amsterdam, mean age 74.6 years (s.d. 6.2). Cognition was assessed at both baseline and at 3 year follow-up on the domains of general cognition, memory, fluid intelligence, and information-processing speed. Serum measurements of four inflammatory markers were taken at baseline: CRP, α_1-antichymotrypsin (α_1-ACT), albumin, and IL-6.

The results of the study found increased levels of α_1-ACT to be associated with cognitive decline in general intelligence (measured using the Mini-Mental State Examination). No other significant associations were found with α_1-ACT or any of the other inflammatory markers and cognitive change. A secondary finding of the study was that carriers of the Apolipoprotein $\epsilon 4$ allele had lower CRP levels than non-carriers ($p < 0.001$).

A second Dutch study, this time by Schram et al., combined data from two independent, population-based cohort studies: the Rotterdam Study, and the Leiden 85-plus Study. The Rotterdam Study contained 3,874 participants of

mean age 72 who were followed up for an average of 4.6 years. The Leiden 85-plus Study contained 491 individuals all aged 85 who were followed up for 5 years. Both studies assessed global cognition, executive function, and memory. Linear regression was used to investigate the associations between inflammatory markers and cognitive function and decline.

The results from the Rotterdam study showed higher levels of CRP and IL-6 to be cross-sectionally associated with poorer global cognition and executive function (difference in cognitive test score per s.d. of CRP –0.03, 95% CI (–0.05, –0.02), $p < 0.001$ and –0.04, (–0.07, –0.02) , $p<0.001$; difference in cognitive test score per s.d. of IL-6 –0.08, 95% CI (–0.14, –0.02), $p = 0.009$ and –0.10, (–0.17, –0.03), $p = 0.008$). Associations of a similar magnitude were observed in the Leiden study although these were non-significant. However, when assessing cognitive decline, higher IL-6 levels were related to a steeper decline in memory in the Leiden group (annual decline per s.d. –0.123, (–0.233, –0.013), $p = 0.03$). The authors also looked at the effect of $APO\epsilon4$ on the associations and found that the presence of the $\epsilon4$ allele resulted in a significant association between IL-6 and decline in memory (Leiden only) and global cognition.

The penultimate study reviewed was a 2006 investigation by Weuve et al., which focussed on CRP and cognitive ability in older women. The cohort comprised 4,231 older participants of the Women's Health Study (age range 60–90) who had a CRP measurement taken between 1992 and 1996. From 1998 to 2000 a five test cognitive battery was administered to assess ability in the domains of general cognition, verbal memory, and category fluency. Regression modelling was used to compare mean cognitive test scores by quintile of CRP after adjustment for potential confounding variables. The results provided no indication for a difference in cognitive performance between the top and bottom CRP quintiles. For example, the adjusted mean difference between upper and lower quintile for a global cognitive score that combined results from all of the tests was 0.04, 95% CI (–0.02, 0.11), p for trend across quintiles = 0.38.

The most recent paper reviewed was from 2008 by Alley et al. Using growth curve modelling they investigated the association between IL-6 and CRP levels with cognitive change, which was assessed three times over a 7 year period. The population for the study consisted of 851 persons aged 70–79 at baseline who were participating in the MacArthur Study of Successful Aging. Cognition was assessed using five domain specific tests, a summary score of the five tests, and a general ability test. Scores were obtained at baseline and at a 3 year and 7 year follow-up. Cross-sectionally, the investigators found inverse relationships

between both inflammatory markers and cognition. However, upon adjustment for potential confounder variables, no significant effect was observed between either marker and baseline cognition or the rate of longitudinal cognitive change. Nonetheless, the authors did find an increased risk in incident decline in general ability for persons in the top tertile of IL-6 ($p < 0.05$).

Overall, these longitudinal data provide contrasting evidence to support the hypothesis that increased inflammation, measured by CRP, has a negative impact upon cognitive ability. Dik et al. suggest that their null findings may be due to the sensitivity of the assay used to measure the serum marker levels, or selective non-response and loss to follow-up of frail, elderly subjects. Their results may also have been affected by the follow-up period of 3 years, which is possibly too short a period in which to observe substantial cognitive decline. There may also have been evidence of a practice effect in their cognitive test results – something that might have affected the data for the study by Alley et al. In spite of the large sample size and battery of cognitive tests, Weuve et al. only focussed on CRP's ability to predict future cognitive ability (but not decline) in women. Therefore, I would be inclined to apply greatest weight to the findings of Schram et al., who reported some evidence for an association between elevated CRP levels and impaired cognitive performance but not cognitive decline. Finally, it should be noted that the effect sizes across all studies are modest. However, as Schram remarks, this does not imply that the involvement of systemic inflammation in the pathology of cognitive decline is modest as well.

The remainder of the papers identified in the systematic search are summarised below by study design type. An additional section is provided to describe the CRP and dementia papers that were uncovered despite the attempts to exclude them from the search.

2.8.2 Prospective cohort studies

Of the other studies reviewed, the main body of evidence points towards CRP being able to predict cognitive ability in both cross-sectional and longitudinal data. As with the results of the key papers, there is scant evidence to imply a predictive role for CRP in measures of cognitive change.

Five prospective cohort studies were retrieved in the literature search with all successfully relating CRP levels prospectively to follow-up cognition. These studies found baseline CRP to predict

- a 5 year follow-up association with a four point or greater decline on the

MMSE in 650 men and women (239 persons aged 75 years, 212 aged 80, and 199 aged 85): relative risk of decline for CRP > 5mg/l = 2.32, 95% CI (1.01, 5.46) (Tilvis et al. 2004).

- a 12 year follow up association with decreased memory capacity in 97 women, aged 60-70 years: standardised β = -0.842, 95% CI (-1.602, 0.083), p = 0.030 (Komulainen et al. 2007).

- a 6 year follow-up association with decrements in word learning in 92 men and women, mean age 55 years (s.d. 11), unstandardised regression coefficient β = -0.27, 95% CI (-0.45, -0.08), p < 0.05 (Teunissen et al. 2003).

- a 1 year follow-up association with attention-executive-psychomotor performance in 78 men and women, mean age 70.6 years (s.d. 7.4): standardised β = -0.22, p = 0.04 (Hoth et al. 2008).

- a 2 year follow-up association with a five point or greater decline on the modified MMSE in 3,031 men and women, mean age 77 years (s.d. 2.9): odds ratio for cognitive decline in highest versus lowest CRP tertile = 1.41, 95% CI (1.10, 1.79) (Yaffe et al. 2003).

Some criticisms that could be directed at the studies include measuring cognition using a single, generic test (Tilvis et al. 2004, Yaffe et al. 2003), having a short follow-up period (Yaffe et al. 2003) and performing analyses on very small samples (Komulainen et al. 2007, Teunissen et al. 2003). Specifically, Tilvis et al. assessed cognition using the MMSE, which is not a sensitive measure for subtle changes cognitive function. The studies by Komulainen and Teunissen, whilst having extensive cognitive testing and lengthy follow-ups, failed to have more than 100 subjects tested. This is going to affect the possibility of chance findings as well as the ability to generalise the results to other populations. Furthermore, it is confusing that both Yaffe and Tilvis split CRP levels into tertiles or high versus low. By taking such an approach, both studies are failing to fully use the continuous data collected, which subsequently is going to reduce the power to detect differences in cognition.

Nonetheless, the design of these four studies are far superior to that of Hoth et al. who cognitively assessed just 78 adults with cardiovascular disease at baseline and one year follow-up. Whilst they used a comprehensive 24 test battery, to re–test a relatively young cohort (age range 56–84) within one year is undoubtedly going to affect scores due to a practice effect. The primary analysis in the study was to create a composite z–score for five cognitive domains and to model the

baseline CRP levels against the change in z-scores over the one year period. They analysed the association between baseline CRP levels and change in z–scores for each domain after adjusting for age and baseline cognitive z–score. As the changes in z–scores (z–baseline – z–follow-up) are going to be highly correlated with the z–baseline scores, to adjust the model for the latter variable is highly questionable. Given the lack of consideration for appropriate statistical analyses, I would be inclined to interpret the findings from this study with caution.

2.8.3 Cross-sectional studies

In studies with a cross-sectional design, several significant associations between CRP and cognitive ability were found. Mangiafico et al. studied 164 persons with asymptomatic peripheral arterial disease (APAD) versus 164 healthy controls (Mangiafico et al. 2006). Both samples had a mean age of 70 years and contained 74% and 73% men, respectively. Linear regression results showed that those with APAD had significantly poorer cognitive test scores. Poorer cognition was also significantly predicted by CRP levels for patients with APAD (attention and verbal working memory: unstandardised β = –0.175, s.e. 0.046, p = 0.0002; attention and mental flexibility: β = 10.792, s.e. 2.946, p = 0.0003; visuo-constructive skills and visual memory: β = –0.337, s.e. 0.170, p = 0.0496). These findings support a hypothesis of CRP contributing to atherothrombotic lesions via complement activation, which subsequently leads to brain damage. However, the authors note that raised CRP levels may merely reflect the underlying inflammatory component of sub-clinical atherosclerotic cerebrovascular disease.

Another study investigated homocysteine and CRP levels with cognitive function in persons with cardiovascular disease (Gunstad et al. 2006). This study used an extensive 22 test battery to assess six major cognitive domains as well as obtaining MRI scans for 37 of its 126 participants. The gender distribution of the sample was 59% male and the mean age of participants was 69.2 years (s.d. 7.6). The results showed significant associations between CRP levels and several of the tests under each cognitive domain ($p < 0.05$). There were no associations between homocysteine and cognition or between either marker and white matter disease or whole brain volume from the MRI data.

The final study reviewed, used only the MMSE to assess cognition. The problem in using this test as a measure of cognitive ability is that its most common application is as a screening test for dementia. Subsequently, it exhibits a ceiling effect in healthy samples and is insensitive to small changes in cognition. Never-

theless, Ravaglia et al. investigated 540 well functioning, healthy, and cognitively normal Italian men and women (mean age 73 years (s.d. 6)) and found elevated CRP levels to be associated with lower MMSE scores (Ravaglia et al. 2005). In a similar approach to the studies of Yaffe and Tilvis, they split CRP into deciles and found the highest compared to the lowest (> 7mg/l) to have increased odds of 3.07, 95% CI (1.2, 7.9) for an MMSE score between 24–25 (out of 30).

2.8.4 CRP and dementia

The studies that investigated CRP levels with dementia gave mixed results. Schmidt, Arai, Engelhart, and Dimopoulos showed positive associations but van Oijen, and Haan found no relationship between the marker levels and dementia status (Arai et al. 2006, Dimopoulos et al. 2006, Engelhart et al. 2004, Haan et al. 2007, Schmidt et al. 2002, van Oijen et al. 2007). However, the Haan study did show $APO\epsilon 4$ stratified CRP levels to be associated with decreased dementia rates. Further, despite not finding an association between CRP and dementia (the odds ratio per s.d increase in CRP was 0.95, 95% CI (0.84 to 1.06)), van Oijen et al. did report an association between plasma fibrinogen levels and both vascular dementia and Alzheimer's disease. This study measured fibrinogen and CRP levels in 2,835 and 6,713 persons from the Rotterdam study and followed them up for mean of 5.7 years to obtain a dementia cohort of 395 cases. In their discussion, they argue that the association with fibrinogen but not CRP implies a haematological over an inflammatory mechanism for dementia – based on the fact that fibrinogen is a biomarker of both inflammation and haematosis.

In addition to the work by van Oijen, the study by Schmidt et al. is also of particular interest and importance – most notably due to its impressive study design and extensive follow-up period of 25 years. The study contained 1,050 Japanese-American men from the Honolulu-Asia Aging Study who were in their mid-fifties when they had CRP levels measured. Twenty five years later, these mid-life levels of CRP were found to be associated with dementia and all its major sub-types (VaD and AD). The odds ratio for dementia for those in the upper compared to lower quartile of CRP was 2.8, 95% CI (1.6, 5.1) with $p < 0.002$ for a trend across quartiles.

2.8.5 Summary

When considering the mixed results from the prospective cohort and cross-sectional studies, it appears that the time of measurement for CRP may be critical when

trying to associate it with impaired cognition. For example, CRP levels in late-life could potentially be affected by underlying vascular disease or other age-related medical conditions.

Nonetheless, there does appear to be some evidence linking CRP levels to both late life cognitive ability and dementia although the associations between CRP and late-life cognitive change are less well investigated. There is thus a need for large-scale prospective cohort studies, like that presented by Schram et al., with extensive cognitive testing and a long-term follow-up. This will help add to the growing literature for the cross-sectional and prospective associations. Well designed studies with multi-wave data will also help determine the relationship between baseline CRP levels and cognitive change, which will aid researchers in the quest to identify CRP as a causal or non-causal agent in the process of cognitive decline.

2.9 *CRP* gene

2.9.1 Location

CRP is located on the long arm of chromosome one (1q21-q23) and covers a 2.3kb area[3]. The gene is composed of two coding exons separated by a single intron and is flanked by a short (104bp) 5 prime untranslated region (5' UTR) and a longer (1.2kb) 3 prime untranslated region (3' UTR) (Miller et al. 2005).

2.9.2 *CRP* SNPs

The results from studies on the *CRP* gene provide indisputable evidence that it is polymorphic (Hage & Szalai 2007). One study systematically re-sequenced the *CRP* gene and identified as many as 40 different SNPs that contribute to the formation of up to 29 different haplotypes (Crawford et al. 2006b). High linkage disequilibrium between all known *CRP* SNPs has resulted in the usage of tagging SNPs to identify *CRP* haplotypes (Carlson et al. 2005, Hage & Szalai 2007, Stram 2004). In persons of European origin there are four common haplotypes describing the variation across the *CRP* gene, which can be identified using three tagging SNPs (Kardys et al. 2006). However, as noted in the review paper by Hage et al. many studies have established associations between CRP levels and both *CRP* haplotypes and disease risk but few have found a relationship between the *CRP* gene and disease risk. They conclude that as the *CRP* polymorphisms

[3]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr1:157948707-157950383

explain just a moderate proportion of serum CRP variation (∼10%) it would take extremely large studies to observe direct associations between SNPs/haplotypes and actual clinical events. In contrast to the *CRP* gene explaining only a modest amount of serum CRP levels, CRP levels are shown to be 30–50% heritable (Wang et al. 2006). This suggests a potential polygenic effect on the heritable aspect of CRP levels with multiple genes being responsible for its variation.

2.9.3 Association with CRP levels

The association between *CRP* SNPs and CRP levels has been extensively studied using polymorphisms from across the *CRP* gene. The majority of research has focussed primarily on five tagging polymorphisms; rs3091244 (a tri-allelic SNP located in the 5' UTR); rs1417938 (located in the first intron); rs1800947 (a synonymous SNP located in the second exon); and rs1205 and rs1130864 (located in the 3' UTR). The relationships between these SNPs and CRP levels have been largely consistent across different studies with conflicting results occurring in just a handful of investigations (Table 2.2).

Moving systematically across the genome, the first SNP under frequent investigation is the tri-allelic flanking SNP, rs3091244. Of the seven papers reviewed that studied this SNP, all found increased CRP levels in carriers of the T allele.

The next SNP along the genome is rs1417938, which is situated in the first and only intron. Its minor allele was associated with significantly lower CRP levels in three studies (Lange et al. 2006, Miller et al. 2005, Suk Danik et al. 2006), and with significantly higher CRP levels in two studies (Martínez-Calatrava et al. 2007, Suk et al. 2005). Another paper reported a significant association between rs1417938 and CRP levels but failed to state in which direction and with which allele the relationship was (Kathiresan et al. 2006a). Finally, a null finding was reported in the study by Crawford et al. (Crawford et al. 2006a)

The only SNP that was frequently investigated and that features in a coding region was rs1800947 in the second exon. Twelve papers were found comparing its allelic variants with CRP levels with eight showing an increase in CRP levels in carriers of the minor allele. Of the remaining papers, two failed to find a significant association (Crawford et al. 2006a, Morita et al. 2006), one found an inverse association with the minor allele (Balistreri et al. 2006), and as with rs1417938, Kathiresan et al. found a significant association but failed to report its direction.

The final two SNPs (rs1205 and rs1130864) are located in the 3' UTR and were reported in nine and eleven papers, respectively. Inverse associations were

described for the minor allele of rs1205 and positive associations for the minor allele of rs1130864 with CRP levels. None of the respective studies found associations in the opposite direction although some reported null associations (Morita et al. 2006, Paik et al. 2007, Wang et al. 2006). Furthermore, the Kathiresan paper once again identified both SNPs as being significant but failed to report the direction of the associations.

The study design and population characteristics varied across the individual studies and they have been summarised in Table 2.3. One criticism that can be directed at many of the studies is a small sample size. The number of participants ranged from 55 (D'Aiuto et al. 2005) to 7,159 (Crawford et al. 2006b) with six of the eighteen studies having a sample population of fewer than 1,000. These small sample studies make it difficult to generalise the results and increase the possibility of chance findings.

In the middle of 2008 a systematic review by Verzilli et al. provided a comprehensive report on the genetic variants that affect plasma CRP levels (Verzilli et al. 2008). They identified twenty-six studies of note (total n = 32,802), which had reviewed the associations of eight SNPs in the *CRP* gene (rs3093059, rs2794521, rs3091244, rs1417938, rs1800947, rs1130864, rs1205, and rs3093077) with CRP concentration. Although no SNP was typed in every study there were several partial overlaps.

Using a Bayesian regression model, they combined data from all sites and adjusted the associations for possible correlations with the remaining variants. This approach helps to extricate the effects at causal sites from the effects at the sites in LD with the causal variant(s). Of the SNPs investigated, rs1130864, rs1205, and rs3093077 held the strongest independent association with plasma CRP levels. One other SNP (rs1800947) was also deemed to be important although its value in the model varied by the prior selected.

	Common *CRP* SNPs				
Author and date	**rs3091244**	**rs1417938**	**rs1800947**	**rs1205**	**rs1130864**
Major allele	C (Tri-allelic SNP)	T	C	C	G
Minor allele	T and A	A	G	T	A
HapMap CEU Minor allele frequency		0.33	0.07	0.34	0.30
Zee & Ridker (2002)			+		
Brull et al. (2003)					+
Carlson et al. (2005)	+		+		
D'Aiuto et al. (2005)					+
Eklund et al. (2005)			+		
Miller et al. (2005)	+	-	+	-	+
Suk et al. (2005)		+	+		
Balistreri et al. (2006)			-		
Casas et al. (2006)					+
Crawford et al. (2006a)	+	NS	NS	-	
Kardys et al. (2006)				-	+
Kathiresan et al. (2006a)	+	?	?	?	?
Lange et al. (2006)		-	+	-	
Morita et al. (2006)	+		NS	NS	+
Suk Danik et al. (2006)	+	-	+	-	+
Wang et al. (2006)	+				NS
Martínez-Calatrava et al. (2007)		+	+	-	+
Paik et al. (2007)				NS	NS

Table 2.2: *CRP* gene polymorphisms that influence CRP levels. The associations reported are for the minor alleles with + implying increased CRP levels and - implying decreased CRP levels (NS = non-significant, ? = result not reported).

Table 2.3: Characteristics of studies investigating *CRP* gene polymorphisms in relation to CRP levels.

Author and date	**Study Design**	**n**	**Ethnicity**	**Sex (% Male)**	**Age (Years)**
Zee & Ridker (2002)	Case – Control	726 – 726	American	100	60.0 – 60.2
Brull et al. (2003)	Two Prospective Cohorts: British army recruits; CABG surgery group	250; 193	British	100 (79.8)	19.4 (62.7)
Carlson et al. (2005)	Prospective Cohort	3,790	52% African American, 48% European American	44.8	Range 18 – 30
D'Aiuto et al. (2005)	Prospective Cohort	55	British	52.7	48.3
Eklund et al. (2005)	Cross-sectional	165	Finnish	55.4	46.6M, 41.3F
Miller et al. (2005)	Three studies: WHS (RCT); PRINCE (Prospective Cohort); PHS (Case – Control)	358–359; 1,110; 640–640	American	0; 72.3; 100	54.81–51.0; Not stated; 60.9–60.6
Suk et al. (2005)	Cross-sectional	2,397	American	70.4	62.2
Balistreri et al. (2006)	Case – Control	106 – 120	Sicilian	100	41 – 39
Casas et al. (2006)	Combination of six studies	4,659	British	100	Not stated
Crawford et al. (2006a)	Cross-sectional	7,159	American	48.1	40.8
Kardys et al. (2006)	Prospective Cohort	5,972	Dutch	41.0	68.9
Kathiresan et al. (2006a)	Cross-sectional	3,301	American	47.0	61.0
Lange et al. (2006)	Prospective Cohort	3,941; 700	European American; African American	40.7; 36.0	72.6; 72.7
Morita et al. (2006)	Cross-sectional	315	Japanese	48.9	77.9
Suk Danik et al. (2006)	Cross-sectional	1,847	European American	79.0	58.0

Continued on Next Page...

Author and date	**Study Design**	**n**	**Ethnicity**	**Sex (% Male)**	**Age (Years)**
Wang et al. (2006)	Cross-sectional	1,296	American	48.5	54.7
Martínez-Calatrava et al. (2007)	Cross-sectional	844	Spanish	46.7	55.0
Paik et al. (2007)	Cross-sectional	677	Korean	100	46.0

2.9.4 Association with cardiovascular disease

As mentioned previously, raised CRP levels have been implicated as risk factors for many different cardiovascular complications. Although there is evidence connecting *CRP* polymorphisms with CRP levels, few studies have positively associated *CRP* gene variants with cardiovascular risk. Most notably, data from prospective cohort studies such as the Physicians Health Study, the Framingham Heart Study, the NHLBI Family Heart Study, and the Rotterdam Study have found no associations between *CRP* genotype, CRP levels, and MI or stroke (Kardys et al. 2006, Miller et al. 2005, Kathiresan et al. 2006a, Wang et al. 2006).

However, one study did find a genetic link between *CRP* and cardiovascular events in two different ethnic populations aged ≥ 65 (Lange et al. 2006). In a study of 3,941 white subjects, the rs1417938 SNP was found to significantly predict stroke and cardiovascular mortality (hazard ratios 1.40, 95% CI (1.06, 1.87), 1.40 (1.10, 1.90), respectively). In 700 black participants, the rs3093058 polymorphism was a significant predictor of increased risk of MI and stroke (hazard ratios 4.08, 95% CI (1.58, 10.53), 3.64 (1.41, 9.39), respectively).

Other studies that noted associations between *CRP* genotype and disease status showed a history of acute MI to be linked to the C allele of the rs1800947 SNP (Balistreri et al. 2006). One study also found an association between the same SNP and atherosclerosis, as presented by a measurement of pulse wave velocity (Morita et al. 2006). A weak association was found with the rs1130864 SNP and non-fatal MI with an odds ratio for the TT genotype and an event being 1.25, 95% CI (1.09, 1.43) using records from over 4,000 males from six separate European studies (Casas et al. 2006). Furthermore, some researchers found SNPs that were not associated with CRP levels but were directly related to disease status. A 2005 study by Chen et al. showed the A allele of rs2794521 to be linked with coronary heart disease in a Han Chinese population (Chen et al. 2005). This finding was confirmed in the Physicians' Health Study where the SNP variant was related to a decreased risk of MI (Miller et al. 2005). Finally, the functional, tri-allelic rs3091244 SNP has been linked to both CRP levels and increased coronary heart disease in 7,159 American participants of the NHANES-III, Third National Health and Nutritional Examination Study (Crawford et al. 2006a).

These findings add to the evidence for CRP being both a marker of and active participant in cardiovascular disease. It is therefore possible that *CRP* genotypes influence CRP levels, which then affect the onset and progression of cardiovascular events.

2.9.5 Association with cognition

To my knowledge there are not currently (as of 25^{th} March 2009) any studies of *CRP* genetic variants and non-pathological cognitive ability or decline. However, studies have looked at the relationship between *CRP* haplotypes and dementia (van Oijen et al. 2007), and between *CRP* SNPs and six week cognitive decline post cardiac surgery (Mathew et al. 2007).

The study by Van Oijen et al. tested the association between three *CRP* SNPs (rs1205, rs1130864, and rs3093068), their haplotypes, and incident dementia in the Rotterdam Study population. During a mean follow-up of 9.2 years, 607 cases of incident dementia were identified from the baseline population of 5,972. The mean age of the sample at baseline was 68.9 (s.d. 8.7). Results from Cox proportional hazard models showed the T allele of rs1205 to associate with both lower plasma CRP levels and lower risk of dementia (hazard ratio 0.73, 95% CI (0.53, 0.98)) and AD (hazard ratio 0.73, 95% CI (0.53, 0.98)).

The second of the studies monitored cognitive ability in 513 persons pre and post coronary artery bypass graft (CABG) surgery and modelled cognitive decline against several inflammatory marker SNPs (including two *CRP* SNPs: rs1205 and rs1800947). Cognition was assessed the day before surgery and six weeks after surgery using a five test battery. Information from the five tests was combined to derive four underlying traits: verbal memory and language comprehension (short-term and delayed); attention, psychomotor processing speed, and concentration; abstraction and visuo-spatial orientation; and figural memory. Cognitive decline was defined as a one standard deviation or greater decrease in performance on at least one of the domains. The results of the logistic regression analysis showed the minor allele of rs1800947 to associate with a reduction in cognitive deficit in a European-American population: odds ratio 0.37, 95% CI (0.16, 0.78), $p = 0.013$.

2.10 Summary

There is evidence to associate raised plasma CRP levels with cardiovascular disease and cognitive dysfunction. Findings for the latter condition varied, particularly in relation to CRP's association with cognitive decline. Nonetheless, there is sufficient support for a small but significant association between CRP and late-life cognitive ability. In addition, several studies have related SNP variants within the *CRP* gene to raised CRP levels. As of yet, no studies have looked directly at *CRP* SNP variants and cognitive function.

Chapter 3

Fibrinogen

3.1 Introduction

Similar to the previous chapter on CRP, this chapter provides a summary of the inflammatory/haemostatic biomarker fibrinogen. A short section describes the history of fibrinogen, its structure and its function, before a longer review reports its association with cognitive ability. Brief mentions are also given to the haemostatic markers plasma viscosity and haematocrit. The chapter closes with a description of the three fibrinogen genes that encode the biomarker, including a review of the association of their SNP variants with plasma fibrinogen levels and cognitive ability.

3.2 History

The study of haematology has been documented from the year 400 BC and is a topic that has even featured in the works of the Philosophers Plato and Aristotle (Doyle & Addis 2006). In his Timaeus, Plato hypothesised that during the cooling of blood, fibres in the blood were active in the clotting process. Further, Aristotle believed that these fibres were solids composed of earth and that their removal from the blood inhibited clotting.

It wasn't until the 1770s and the work of William Hewson that major developments were made in understanding the clotting process (Doyle et al. 2006). Hewson showed the source of the fibres to be the coagulable lymph, now referred to as blood plasma. The use of the term fibrin to describe the clotting fibres was first presented by Jean-Antoine Chaptal towards the end of the 18th century (Doyle & Addis 2006). His theories centred on the belief that a continual removal of fibrin, possibly for nutritional requirements, kept the blood in a fluid state.

In the latter half of following century, the production of fibrin was described in multiple studies as resulting from a precursor molecule as opposed to being a constituent of blood. Buchanan and Schmidt then managed to identify thrombin as the agent which converted this molecule, fibrinogen, to fibrin (Doyle & Addis 2006).

Today, fibrinogen levels reflect the thrombotic potential of blood; as a haematological factor it has been of interest to those studying cognitive decline since such factors are related to cerebrovascular disease, which in turn has been linked with cognitive function (Kritchevsky et al. 2005).

3.3 Production

As with CRP, production and synthesis of fibrinogen mainly occurs through hepatocytes in the liver although production is also possible in megakaryocytes (Pulanić & Rudan 2005). These bone marrow cells are mainly responsible for the production of blood platelets, which are requisite for normal blood clotting (Kaushansky 2008). Plasma levels of fibrinogen are less prone to acute changes than CRP. A normal range is defined as being between 150-450mg/l (Kamath & Lip 2003) with levels above this associated with an increased risk of cardiovascular disease (Banks et al. 2006). The production and synthesis of fibrinogen is controlled by a host of cytokines and other molecules. Notably, IL-1, IL-6, and TNF-α have been shown to stimulate fibrinogen production whilst IL-4, IL-10, IL-13, and high plasma albumin suppress its synthesis (Pulanić & Rudan 2005). Fibrinogen has a biological half-life of around 100 hours (Kamath & Lip 2003).

3.4 Structure and function

Fibrinogen is a hexamer that consists of two symmetric half molecules that contain one of each of the alpha, beta, and gamma polypeptide chains (Kant et al. 1985). Through the action of the enzyme, thrombin, it is converted into the insoluble protein, fibrin (Reinhart 2003). The primary function of fibrin is to form an interlacing, mesh-type network that helps to reduce bleeding around a wound site in conjunction with the bone marrow cell-derived platelets. In addition to its vital function as a clotting factor in the blood coagulation system, fibrinogen is involved in numerous pathophysiological processes such as inflammation, atherogenesis, and thrombogenesis (Pulanić & Rudan 2005). The exact means by which fibrinogen is implicated in these often related processes is unclear although

a variety of mechanisms have been postulated regarding thrombogenesis. These include fibrinogen infiltration of the cell wall, haemorrhaging due to increased blood viscosity, and increased platelet aggregation (Kamath & Lip 2003).

Fibrinogen is also the main determinant of plasma viscosity. Together with red blood cell deformation, red blood cell aggregation, and the volume fraction of red blood cells (haematocrit) these comprise blood viscosity – the intrinsic resistance of blood to flow in vessels (Lowe et al. 1997). Moreover, increased viscosity may lead to impaired microcirculatory flow, endothelial damage and thrombosis predisposition (Koenig 2003).

3.5 Factors influencing fibrinogen levels

Showing a similar pattern to CRP, fibrinogen levels are influenced by several physiological, pathological, and lifestyle factors (Fibrinogen Studies Collaboration 2007, Kamath & Lip 2003, Lowe et al. 2004, Pulanić & Rudan 2005). Of the non-modifiable factors, increased age and female gender, particularly during post-menopausal years, are again linked with increased marker levels. Women using oral contraceptive medication are reported to have further raised levels.

Several studies have investigated variability in fibrinogen levels across different ethnic groups. This information has been summarised by Heinrich and Assmann, who comment that lower fibrinogen levels are found in Asian persons and higher levels in African Americans and Native Americans compared to those in white populations (Heinrich & Assmann 1995). Whilst raised levels are generally associated with increased risk of cardiovascular complications, two ethnic groups for whom this does not apply are rural Africans and Greenland Eskimos.

Factors that are positively associated with fibrinogen levels include a selection of common cardiovascular risk factors: BMI, waist-hip ratio, diabetes, LDL-cholesterol, and smoking (Kamath & Lip 2003, Lowe et al. 2004, Pulanić & Rudan 2005). Of these, smoking appears to be the strongest determinant of plasma fibrinogen levels. It has been estimated that half of smoking related cardiovascular damage is meditated through proliferation of fibrinogen levels (Heinrich & Assmann 1995, Pulanić & Rudan 2005). Whilst there is a dose-dependent response between the number of cigarettes smoked and age-adjusted fibrinogen levels, the cessation of smoking results in a rapid reduction of fibrinogen levels (Heinrich & Assmann 1995). However, it can take up to ten years or more for the levels to return to those of a healthy individual who has never smoked (Pulanić & Rudan 2005).

Some of the factors inversely associated with fibrinogen levels include HDL-cholesterol, exercise, alcohol consumption, and a host of pharmacological interventions (Kamath & Lip 2003, Pulanić & Rudan 2005, Reinhart 2003). The most contentious of these associations is probably for alcohol intake. Several studies have shown linear associations with fibrinogen levels, even at the stage where alcohol intake is damaging liver cells. By contrast, other studies have reported U-shaped associations or sometimes no relationship at all.

3.6 Association with disease

3.6.1 General

Similar to the associations found with CRP, raised fibrinogen levels have been implicated in several physiological complications. Most commonly, it has been linked to coronary heart disease, MI, and major cardiovascular diseases (Kamath & Lip 2003, Pulanić & Rudan 2005, Reinhart 2003, Welsh et al. 2008) although studies have also reported increased levels to be associated with Type 1 and Type 2 diabetes (Pulanić & Rudan 2005). Furthermore, there is evidence to implicate elevated fibrinogen levels in persons suffering from both vascular and non-vascular dementia (van Oijen et al. 2005). However, despite the discovery of positive associations with many diseases, the mechanisms driving these relationships have not been fully determined. Several possibilities have been postulated, particularly with regards to atherosclerosis and these will be discussed in more detail below.

3.6.2 Atherosclerosis

As described in the corresponding section of the CRP review, there is substantial evidence linking inflammatory processes with atherosclerosis. However, in contrast with CRP, there is more support to causally implicate fibrinogen in both atherogenesis and thrombogenesis.

In a review paper of fibrinogen, its biochemistry, epidemiology, and determinants, Kamath and Lip comment that there can be little doubt over fibrin deposits initiating and being an active factor in the development of atherosclerotic plaques (Kamath & Lip 2003). Given the close relationship between atherogenesis and thrombogenesis it is likely that fibrinogen, a key component in the latter process, will also be active in the formation of atherosclerotic lesions. Heinrich and Assmann add support to this claim, noting that it has been known for many years that thrombosis contributes to atherogenesis (Heinrich & Assmann 1995).

To help understand the role of fibrinogen in atherogenesis, Heinrich and Assmann suggest a cyclical process to describe atherothrombosis formation. Initially, they propose a mild acute-phase reaction in response to vessel-wall alteration resulting from a chronic stimulus. This yields an increase in fibrinogen levels which in turn leads to increased fibrin deposits, pushing the haemostatic balance towards thrombogenesis.

As fibrin is an adsorptive surface for LDL cholesterol, its breakdown during fibrinolysis leads to lipid aggregation and plaque growth. Eventual fissure of the plaque results in an inflammatory response and a return to the start of the atherothrombotic cycle. Subsequently, Kamath and Lip propose fibrinogen to be a more useful acute-phase protein to measure than CRP due to its more specific relationship with vascular disease. In addition to being a strong predictor of cardiovascular events in persons with recognized atherosclerotic vascular disease, fibrinogen levels are also elevated prior to the onset of stroke and acute myocardial infarction in patients with transient ischemic attack, and chronic stable angina pectoris, respectively. This implies that raised fibrinogen levels are an independent risk factor for acute cardiovascular and cerebrovascular events.

The epidemiological evidence relating plasma fibrinogen levels to cardiovascular events is best summarised in a comprehensive meta-analysis from 2005 (Fibrinogen Studies Collaboration 2005). A previous meta-analysis of 18 prospective, long-term studies showed an increased risk ratio for coronary heart disease of 1.8, 95% CI (1.6, 2.0) per 1g/l increase in plasma fibrinogen levels (Danesh et al. 1998). However, the nature of the data collected prevented a thorough review of the independent associations between fibrinogen, CHD and other vascular or non-vascular outcomes.

By contrast, the Fibrinogen Studies Collaboration report included information on 154,211 persons from 31 prospective studies each with a 1 year minimum of follow-up. The summary statistics for the data reported a total of 6,944 non-fatal myocardial infarction or strokes and 13,210 deaths across all cohorts. The overall findings of the meta-analysis were presented as hazard ratios for disease outcome per 1g/l increase in fibrinogen. The age and sex-adjusted ratios were 2.42, 95% CI (2.24, 2.60) for CHD, 2.06 (1.83, 2.33) for stroke, 2.76 (2.28, 3.35) for other vascular mortality, and 2.03 (1.90, 2.18) for non-vascular mortality. The ratios for CHD and stroke were reduced to around 1.8 after adjustment for several cardiovascular risk factors.

In summary, there is compelling epidemiological evidence for an association between increased fibrinogen levels and cardiovascular disease. The literature also

suggests several mechanisms through which fibrinogen might be causally involved in atherosclerosis.

3.7 Association with cognition

After uncovering a substantial body of evidence for an association with atherosclerosis and cardiovascular disease, a database search was conducted to identify papers looking at the relationship between plasma fibrinogen levels and cognitive ability. The search strategy was very similar to that used in Section 2.8 for the report on CRP and cognition. Briefly, terms were included to incorporate fibrinogen, fibrin and other similar derivatives along with cognitive ability and decline but not dementia. The list of Medline search terms read ('exp Fibrinogen' | 'fibrinogen.mp' | 'Fibrin') & ('exp cognition' | 'exp cognition disorders' | 'cognition.mp' | 'cognit$ impair$.mp' | 'cognitive decline.mp'). The search dates were again set from 1950 to December 2008 and papers were limited to those written in English. Equivalent terms were used in the search of the PubMed and Embase databases. Of the reviewed studies, the predominant approach taken was that of a cross-sectional design with just a solitary large-scale, long-term follow-up paper with a large battery of cognitive testing being identified. To my knowledge, there are no systematic reviews currently available summarising the relationship between fibrinogen and cognition.

3.7.1 Key papers

The single key paper identified followed up 452 people from the Edinburgh Artery Study over a period of 16 years (Rafnsson et al. 2007). Cognitive information was obtained using a battery of four tests that assessed the domains of verbal declarative memory (Logical Memory Test – LM), nonverbal reasoning (Raven's Standard Progressive Matrices – RAVENS), executive function (Verbal Fluency Test – VFT), and information processing speed (Digit Symbol Test – DST). A general cognitive factor, g, was derived by taking the standardised scores from the first unrotated component of a principal components analysis. Biomarker information including plasma fibrinogen and IL-6 levels was collected at baseline whilst cognitive measures were assessed at both 11 year and 16 year follow-up.

In an age and sex-adjusted linear regression analysis, fibrinogen levels associated inversely with two of the four 16 year follow-up cognitive tests (VFT and DST), and also with g. Further adjustments were made to control for baseline cognitive scores and for peak cognitive ability, measured using the National

Adult Reading Test (NART). These resulted in no significant associations except for RAVENS (baseline adjusted) and LM (NART adjusted). IL-6 associated significantly with RAVENS and g for both the unadjusted and adjusted models. Age and sex-adjusted associations were also found with DST and VFT with the former remaining significant after adjustment for NART scores.

These results suggest that late-life inflammatory biomarker levels are associated with late-life cognitive ability and to a lesser degree with late-life cognitive change and estimated lifetime cognitive decline. The short length of the cognitive follow-up may have reduced the potential effect sizes for cognitive change due to practice effects and/or too short a window in which to observe change - even in a elderly cohort who were a mean age of 73.1 years at baseline cognitive testing.

Rafnsson et al. postulate several mechanisms via which fibrinogen may be involved in the process of cognitive decline. These include an increase in plasma viscosity leading to reduced cerebral blood flow. In their study, the fibrinogen–cognition association was retained after adjustment for prevalent and incident CVD, suggesting a relationship independent of vascular complications. However, they noted that the association may be mediated by cerebral lesions, which have been shown to associate with both fibrinogen levels and cognitive decline (Knuiman et al. 2001, Schneider et al. 2004, 2003). Furthermore, fibrinogen levels were shown to correlate strongly with D-dimer levels (a degradation product of fibrinolysis that is used to help diagnose thromboses) even though the latter didn't associate significantly with cognition. This implies a role for fibrinogen over and above its coagulant properties in cognitive decline. Nonetheless, an independent study showed a strongly significant association between D-dimer levels and cognitive decline over a 4 year period in 1,723 elderly people (mean age 77.6 years (s.d. 5.4)) (Wilson et al. 2003).

3.7.2 Cross-sectional studies

Of the other studies reviewed all were cross-sectional in design with date of publication ranging between 1997 and 2008 (Table 3.1). The majority of evidence from these investigations points towards no association between fibrinogen and cognition. However, these conclusions need to be interpreted with caution as there are several limitations that may be influencing the relationships reported. In accordance with the studies describing the associations between CRP and cognition, these are centred around sample size concerns and limited or unsuitable cognitive testing.

Sample size ranged from 270 to 15,800 although only two studies assessed more

than 1,000 persons. Moreover, none of the smaller studies report the effect sizes they were expecting or were powered to detect. It is clear from the prospective studies on CRP and cognition that the associations discovered for inflammatory markers are likely to be small, accounting for only a small proportion of the variation in cognition. Subsequently, it is of critical importance for authors to declare what effect sizes they are expecting and are able to detect.

The largest of the studies sampled 15,800 middle-aged-to-elderly persons from four separate US communities (Cerhan et al. 1998). Cognition was assessed using tests of memory, processing speed, and executive function. Fibrinogen measures were taken on all participants but were split into quintiles for the analysis. ANCOVA adjusting for age, education, occupation, race, and study trial centre found significant differences between fibrinogen quintiles and both memory and processing speed scores ($p < 0.05$ and $p < 0.005$). In both situations, the upper quintiles corresponded to poorer cognitive scores.

With regards to the rest of the literature, many studies used a single, generic test to assess late-life cognitive ability. Two studies used the MMSE for their analysis (Fischer et al. 2006, Kostka et al. 2008). The flaws in taking this approach have been discussed at length previously. Another study used the Alice Heim (AH)-4 Test to assess general ability and the choice reaction time to measure decision speed (Elwood et al. 2001). Although a single test, the AH-4 provides a fairly extensive evaluation of general ability with 65 test items of increasing difficulty that alternate between mathematical and verbal reasoning. In their report, Elwood et al. found no consistent trends to associate higher fibrinogen levels with poorer performance although there is evidence to relate levels in the highest quartile with slower reaction times. Significant results were presented for the associations between plasma viscosity and AH4 and reaction time scores (age and social class-adjusted regression p-values 0.001 and 0.003, respectively). Given that fibrinogen is the main constituent of plasma viscosity this remains consistent with the hypothesis of cerebral blood flow being directly linked to cognitive function rather than through atherosclerosis or other disease processes. A significant U-shaped relationship was also found between haematocrit and age and social class-adjusted reaction times ($p < 0.05$).

There was no strong evidence for an association between fibrinogen and cognition in the papers presented by Kilander et al. and Stewart et al. Despite this, the latter study found an inverse relationship between cognitive ability and the quintile with the lowest fibrinogen levels. However, no obvious pattern of results was present for those in the upper quintiles.

3.7.3 Summary

In conclusion, there have been very few comprehensive studies investigating the associations between fibrinogen and late-life cognition. The literature available suggests a similar pattern of results to those of CRP and cognition i.e. small but significant associations between higher marker levels and decreased cognitive ability. There is also a need to explore and understand the role of fibrinogen and the mechanisms through which it may relate to cognition. The arguments reviewed indicate a potential association through decreased blood flow and increased plasma viscosity that is independent of atherosclerotic and inflammatory processes.

Table 3.1: Characteristics of studies investigating plasma fibrinogen levels in relation to cognitive ability.

Author and date	**n**	**Ethnicity**	**Sex (% Male)**	**Age (Years)**	**Cognitive Outcome**	**Exposure**	**Results**
Kilander et al. (1997)	540	Swedish	100	72.2	13 test battery, standardised and aggregated to give a g-factor	Vascular risk factors and education	Smoking and obesity inversely related to both education and cognition. No association between fibrinogen and cognition.
Cerhan et al. (1998)	13,913	American	45.0	Range 45 – 64	Delayed word recall, Digit symbol subtest, Word fluency test	Multiple demographic and physiological variables including plasma fibrinogen	Delayed word recall and digit symbol subtest correlated significantly and negatively with plasma fibrinogen levels.
Stewart et al. (2006)	278	Caribbean born (living in UK)	55.4	72.2	11 test battery. MCI defined as $\geq$ 6 test scores $<$ 30th %ile. Severe CI defined as $\geq$ 6 test scores $<$ 10th %ile	Cardiovascular risk factors	Differences between vascular risk factors and cognition by education. Fibrinogen inversely associated with education. Suggested inverse relationship between fibrinogen and cognition.
Fischer et al. (2006)	606	Austrian	40.8	75.8	MMSE	Vascular risk factors	No association between exposures and outcome except for smoking.

Continued on Next Page...

Author and date	**n**	**Ethnicity**	**Sex (% Male)**	**Age (Years)**	**Cognitive Outcome**	**Exposure**	**Results**
Elwood et al. (2001)	2,154	Welsh	100	Range 55–69	Alice Heim-4 and Choice Reaction Time	Rheological and haematologic biomarkers	Haematocrit and plasma viscosity associated with impaired cognitive function. No association with fibrinogen.
Kostka et al. (2008)	270	Polish	39.6	70.0	MMSE	Fibrinogen	No significant correlation between fibrinogen and MMSE

3.8 Fibrinogen genes

3.8.1 Location

Fibrinogen is encoded by three genes that represent its different polypeptide chains: fibrinogen alpha (*FGA*)[1], fibrinogen beta (*FGB*)[2], and fibrinogen gamma (*FGG*)[3]. The genes are located on the long arm of chromosome four (4q23-q33) (Kant et al. 1985) and cover a 50kb area (Carty et al. 2008). The *FGG* gene contains 10 exons and is aligned 10kb upstream from the *FGA* which contains 6 exons (Uitte de Willige et al. 2005). The *FGB* gene is located downstream from the *FGA* gene and contains 8 exons (Tuddenham & Cooper 1994).

3.8.2 Fibrinogen SNPs

All three fibrinogen encoding genes have been investigated with respect to polymorphisms that may explain fibrinogen levels or disease status. The function of these genes varies with the alpha and gamma chains believed to affect clot structure with the beta gene being involved in fibrinogen synthesis (Lim et al. 2003). Due to evidence from in vitro research implicating *FGB* polymorphisms in fibrinogen production, it has been the most extensively studied of the trio of genes. Studies have also shown fibrinogen synthesis to be affected by the other two chains yet, despite this fact, variants in the promoter regions of the *FGA* and *FGG* genes are infrequently investigated (de Maat 2005). In accordance with results from genetic epidemiology studies of the *CRP* gene, SNPs across the three fibrinogen genes have been significantly associated with fibrinogen levels but to a much lesser degree with disease outcomes.

Variation in fibrinogen levels has been reported to be between 20-51% heritable (Pulanić & Rudan 2005) yet only a small fraction of this variance can be explained by the fibrinogen genes. Indeed, a genome wide association study for variants affecting fibrinogen levels failed to identify any significant polymorphisms within the fibrinogen encoding genes (Yang et al. 2003). The highest LOD scores (base 10 logarithm of the odds) for the scan were found on chromosomes two and ten but no specific genes could be established on either segment. Twin studies have also shown environmental effects to have greater influence on plasma fibrinogen levels compared to genetic variation (Kamath & Lip 2003).

[1]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr4:155725879-155731347
[2]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr4:155703582-155711688
[3]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr4:155744736-155753352

3.8.3 Association with plasma fibrinogen levels

The association between fibrinogen SNPs and fibrinogen levels has been studied using polymorphisms from across all three fibrinogen genes. To date, research has focussed primarily on variants in the beta chain due to its role in fibrinogen synthesis. Studies have attributed 4.2% and between 5-9% of plasma fibrinogen variation to the alpha and beta genes, respectively (Humphries et al. 1987, 1992).

One of first SNPs to be identified in the beta gene was the Bcl-I (or 6638ins28) polymorphism (Humphries et al. 1987). This variant was discovered to be in complete linkage disequilibrium with the -455G/A SNP (rs1800790) and has gone on to be one of the most widely studied fibrinogen SNPs. Consistent reports from multiple studies have associated it with fibrinogen levels – the most commonly quoted effect size is a 7-10% increase for carriers of the A allele (approximately 20% of the population) (Pulanić & Rudan 2005). Although it is a non-coding polymorphism, some authors propose -455G/A to be functional due to its proximal location to other cytokines which have been associated with fibrinogen levels (de Maat 2001). A study that investigated fourteen SNPs from across the beta gene in 1,811 participants from the Framingham Heart Study found nine to associate significantly with plasma fibrinogen levels (Kathiresan et al. 2006b). In order to account for correlations and linkage between them, a stepwise regression was performed to identify the SNPs of most importance. This approach identified the -455G/A polymorphism as the single most important predictor of fibrinogen levels. In accordance with the figures from the review studies, the A allele was associated with increased plasma levels, explaining around 1% of the variation. In a separate study of fibrinogen SNPs with fibrinogen levels, just under 2% of the total fibrinogen variation was explained by a collection of 22 polymorphisms from across the three fibrinogen genes (Reiner et al. 2006).

In addition to the -455G/A SNP two of the most commonly reported non-coding polymorphisms are the -148C/T (rs1800787) and the -854G/A (rs1800791) variants (de Maat 2001, Kamath & Lip 2003, Koenig & Ernst 1992). Both are located in the promoter region of the beta gene, which is the section towards the 5' UTR that initialises the transcription process. There is also a suggestion that -148C/T is functional due to its location being very close to the IL-6 responsive element (de Maat 2001).

Two coding polymorphisms have also been identified and described in detail (Scott et al. 2004). The first is the Thr312Ala variant which is located in the alpha chain and is important in the interaction with Factor XIII, which determines the stiffness of the blood clot. The second variant is in the beta chain and is labelled

Arg448Lys. This polymorphism is involved in clot permeability by determining the tightness and fineness of the clot structure.

Given the relatively large size of the fibrinogen genes and the high LD between many of the SNPs, some studies have taken a haplotype or tagSNP approach for their analysis. One such study typed 44 SNPs across the three genes and found three main haplotype blocks, each of which could be represented by 4-6 common haplotypes (Kathiresan et al. 2006b). In total, 16 tagSNPs were required to predict all haplotypes with a frequency above 5%. The association of the haplotypes with fibrinogen levels matched the findings for the individual SNPs; haplotypes with the -455G/A and -148C/T allele resulted in increased levels whilst those with the -854G/A allele resulted in decreased levels.

One study notes that despite fibrinogen heritability being relatively high, the overall explanatory contribution of individual SNPs towards intermediate phenotypes such as plasma fibrinogen level is typically small (Scott et al. 2004). This may, in part explain the inconsistent findings between genetic variants and clinical outcomes. Pulanić and Rudan reaffirm this in the specific context of the beta gene, noting the modest effect sizes and difficulties in replicating positive associations. They suggest a shift in focus towards gene-environment interactions to attain a more precise measure of plasma fibrinogen levels.

3.8.4 Association with cardiovascular disease

There is a substantial body of evidence associating fibrinogen levels with cardiovascular disease. In the previous subsection genetic variants of the three fibrinogen genes were reviewed in relation to fibrinogen levels. In this subsection the relationships between fibrinogen SNPs and cardiovascular disease outcomes are explored.

On the whole, the evidence for associations with cardiovascular disease favours null findings. As with the studies of SNPs and fibrinogen levels, the main SNP reported was the -455G/A variant. The review studies reporting a positive association with disease outcome described significant findings with the development of coronary artery disease (CAD) in persons with Type 2 diabetes, with the progression of atheroma, CAD, and the development of cerebral infarcts for carriers of the A allele (Scott et al. 2004). Although this highlights numerous significant associations, these findings are by no means replicated across all studies. A review study also noted an association between this SNP and risk of CHD – odds ratio 1.77, 95% CI (1.08, 2.90). However, in contrast with the other studies this increased risk was for carriers of the major G allele (Koenig & Ernst 1992).

The other polymorphisms associated with disease status included the Bcl-I variant with CAD (Scott et al. 2004), CHD (Koenig & Ernst 1992), and MI (de Maat 2005) but in the latter case, only in the additional presence of a second risk factor such as Helicobacter Pylori infection. The two coding polymorphisms introduced previously were significantly associated with post-stroke mortality in subjects with atrial fibrillation, pulmonary embolism in DVT sufferers (Thr312Ala), and with some forms of macrovascular disease (Arg448Lys) (Scott et al. 2004). However, it must be noted that in the case of the Arg448Lys associations, positive findings were not observed in all of the reviewed studies.

Haplotype associations with CVD were also investigated, again with mixed results. A three SNP haplotype including the -455G/A and -854G/A variants, associated the minor allele haplotype with lower mortality in patients with sepsis (Manocha et al. 2007). This haplotype was also associated with increased fibrinogen levels, implying a beneficial impact of high fibrinogen in persons with sepsis. Other haplotype studies associated fibrinogen gene variation with ischemic stroke in men (a twelve SNP haplotype covering all three genes) (Carty et al. 2008), MI (a two SNP alpha-gamma haplotype) (van Oijen et al. 2008), and small vessel disease (an eight SNP alpha-gamma haplotype) (Mannila et al. 2007). Two of these papers failed to find associations with individual SNPs emphasising that a haplotype approach to genetic epidemiology may yield increased power to detect associations. However, a large-scale study of 1,811 Framingham Heart Study participants by revealed no relationship between either SNPs or haplotypes with CVD (Kathiresan et al. 2006b).

To summarise, in accordance with the studies investigating *CRP* SNPs with CVD, there is inconclusive evidence as to the relationship between fibrinogen SNPs, fibrinogen levels, and CVD. These inconsistent findings suggest the need for further large-scale epidemiological studies to help decipher the exact role of fibrinogen in cardiovascular disease outcomes.

3.8.5 Association with cognition

There have been very few studies conducted to test the association between fibrinogen SNPs and cognitive ability. A literature review found no directly relevant papers on this topic. However, some studies have investigated the association between fibrinogen SNPs/haplotypes and cerebral small vessel disease (Martiskainen et al. 2003, van Oijen et al. 2008).

The largest and most comprehensive of these studies was by Van Oijen et al. who used logistic regression models to analyse the association between fib-

rinogen levels and haplotypes with silent brain infarcts and white matter lesions (van Oijen et al. 2008). The cohort for the analysis was 1,077 persons (mean age 72) from the Rotterdam Study undergoing cerebral MRI. Haplotypes were generated using seven tagging SNPs from the *FGA* and *FGG* genes. These haplotypes explained the common variation in the two genes. In addition, they also genotyped the functional 2148C/T polymorphism (rs1800787) in the *FGB* gene that has been shown to directly affect *FGB* promoter activity (Verschuur et al. 2005). The results of the study found small but significant associations between two of the *FGA-FGG* haplotypes and the presence of silent brain infarcts and periventricular white matter lesions on brain MRI. Conversely, no associations were observed between the *FGB* promoter SNP or plasma fibrinogen levels and the MRI output. There were also null findings between the *FGA-FGG* haplotypes and plasma fibrinogen levels. These findings provide evidence for a relationship between the common variants in the fibrinogen genes and cerebral small vessel disease. The authors propose structure of the fibrin clots as opposed to plasma fibrinogen levels as a potential causal factor in the pathogenesis of cerebral small vessel disease.

3.9 Summary

This chapter has presented extensive background details on plasma fibrinogen and the three genes that encode it. Despite a lack of studies investigating fibrinogen levels with cognitive ability, there does seem to be a degree of evidence pointing in the direction of small but consistently inverse associations. There is currently a lack of papers focussing on the relationship between the genes that encode fibrinogen and cognitive ability, although associations have been found between some SNPs and cerebral small vessel disease.

Chapter 4

Other inflammatory biomarkers and inflammatory marker genes

4.1 Introduction

In addition to CRP and fibrinogen, several other inflammatory markers and marker genes have been posited as predictors of cognitive ability. In this short chapter, the relationship between plasma levels and SNP variants of interleukin-6 and tumor necrosis factor-α and cognition are discussed. A short summary is also presented for four other genes related to inflammatory pathways and cognitive ability: *TCF-1*, *F13A1*, *IL-1*, and *APO*ϵ.

4.2 Tumor Necrosis Factor-α and Interleukin-6

During inflammation the levels of CRP and fibrinogen are regulated by several factors including the cytokines tumor necrosis factor-α and interleukin-6 (Ablij & Meinders 2002). These inflammatory cytokines have also been studied independently with regard to their associations with cognitive ability, cardiovascular disease, and inflammatory disease. TNF-α and IL-6 are released by white blood cells in response to an inflammatory stimulus, resulting in increased synthesis of acute-phase proteins in the liver (Gallin et al. 1983). This pro-inflammatory response is vital in stimulating the immune system and thus, in the removal of pathogens and post-infection healing.

4.3 Production and function

4.3.1 Tumor Necrosis Factor-α

TNF-α was first named in 1975 because of its ability to destroy malignant tumor tissue in mice (Carswell et al. 1975). In modern times it is recognised primarily as a regulator of the immune system. The main production of TNF-α takes place in macrophages although synthesis occurs in several other cells, including glial cells in the brain (Frei et al. 1987). Whilst it is vital in maintaining immunity, an excess concentration of TNF-α can cause a deleterious effect on neurons (Allan & Rothwell 2001, Viviani et al. 2004). In addition, as TNF-α is a promoter of the inflammatory response, it is a key factor in chronic inflammatory diseases such as rheumatoid arthritis (Feldmann et al. 1996). Functionally, TNF-α is often found to operate in conjunction with interleukin-1 and interleukin-6. The *TNF-α* gene covers approximately a 3kb region and is located on the short arm of chromosome six (6p21.3)[1]. It contains four exons with the last one responsible for coding more than 80% of the secreted protein (Nedwin et al. 1985).

4.3.2 Interleukin-6

IL-6 is a cytokine that can induce both a pro and anti-inflammatory response. It is produced by macrophages and T cell lymphocytes and its presence helps stimulate the production of a large number of liver-based acute-phase proteins (Pradhan et al. 2001). The production of IL-6 is part of the immune response to harmful stimuli such as infections, burns, and trauma. Its primary pro-inflammatory functions include activation of the acute-phase response, and the growth and differentiation of B and T cells (Maggio et al. 2006). The anti-inflammatory properties of IL-6 are mediated through its ability to inhibit the production of pro-inflammatory cytokines such as TNF-α (Maggio et al. 2006). The *IL-6* gene is located on the short arm of chromosome seven (7p21)[2] and contains six exons (Li et al. 2004)

[1] http://genome.ucsc.edu/cgi-bin/hgTracks?org=Human&position=chr6:31651314-31654092

[2] http://genome.ucsc.edu/cgi-bin/hgTracks?org=Human&position=chr7:22732028-22738091

4.4 Association with cognition

4.4.1 Prospective evidence

Several studies have investigated the relationship of plasma IL-6 and TNF-α levels with cognition. Of the prospective evidence reviewed, three studies were in relation to IL-6 with one also investigating TNF-α. Chronologically, the oldest of these was from 2003 where the authors studied 3,031 African-American and white men and women (mean age 74 years) from the Health, Aging, and Body Composition Study (Yaffe et al. 2003). Serum levels of three inflammatory markers (IL-6, TNF-α, and CRP) were measured and modelled against two year cognitive change in scores on the modified MMSE (3MS). After age-adjustment the odds ratios for cognitive decline (a 5 point or greater decline in 3MS score) in the highest versus lowest tertile of marker level were significant for IL-6 (odds ratio 1.34, 95% CI (1.06, 1.69)) but not for TNF-α (odds ratio 1.12, 95% CI (0.88, 1.43)). Further adjustment for other demographic variables and comorbidities rendered the associations non-significant although the magnitude of the point estimates remained similar. The results for CRP have been reported in Section 2.8.2. Also discussed previously, the limitations of this study surround the short follow-up period and the use of a single, general ability measure to assess cognition.

The second paper reviewed was a 2007 report that studied 290 persons aged 55-75 years over a three year period (Jordanova et al. 2007). Baseline levels of the inflammatory markers, IL-6, CRP, and serum amyloid A were measured and cognition was assessed at both time-points with decline in individual tests and a composite outcome used as indicators of cognitive change. Upon adjustment for potential confounding factors, raised IL-6 levels but not CRP levels were associated with cognitive decline (odds ratio for decline in g for high versus low IL-6 quartile was 2.9, 95% CI (1.1, 7.5)). Strong associations were also found between IL-6 levels and decline in orientation, immediate verbal recall tasks, and to a lesser degree with delayed recall and psychomotor speed. Although this paper used an extensive cognitive battery and assessed multiple inflammatory markers, as it focused solely on Afro-Caribbean persons it is difficult to generalise these findings to a wider population. Further, splitting the marker levels into quartiles and then comparing top and bottom categories is going to compromise the power to detect an effect in what is already a relatively small and heterogeneous (by age) sample.

The most recent paper reviewed was from 2008 (Alley et al. 2008). This paper was reviewed as part of the literature search for CRP. To recap, growth curve

modelling showed there to be little evidence for an association between IL-6 and CRP levels with cognitive change, which was assessed three times over a seven year period in 851 persons aged 70-79 years at baseline who were participating in the MacArthur Study of Successful Aging.

Of the three papers, this study presents the most rigorous argument for the evidence of a longitudinal association between IL-6 and cognition. Due to its reasonably large sample size and comprehensive assessment of cognitive ability at three separate waves I would provide greater weight to their null findings than to the positive results reported by Jordanova and Yaffe.

4.4.2 Cross-sectional evidence

As with the longitudinal studies, three cross-sectional studies were found that investigated the association between IL-6 and TNF-α levels and cognitive ability. The most relevant of these was a report by van Exel et al. who measured a ratio of the pro-inflammatory cytokine TNF-α by the anti-inflammatory cytokine IL-10 in 599 members of the Leiden 85-Plus Study (van Exel et al. 2003). An indication of atherosclerosis was assessed using self-reported CVD and an electrocardiogram (a graphical recording of the cardiac cycle) to report MI or myocardial ischemia. Cognition was measured using the MMSE and a four test cognitive battery; a dementia diagnosis was also made where appropriate. A linear regression analysis found the inflammatory ratio to associate inversely with all of the measures of cognition, including dementia, but only in those with cardiovascular disease. The associations remained after adjustment for confounders such as sex, education, non-steroidal anti-inflammatory drug (NSAID) use, total cholesterol, HbA1c, history of hypertension, and history of stroke. As to a mechanism, the authors hypothesise that atherosclerosis causes a pro-inflammatory response, which in turn leads to neurodegeneration, cognitive decline, and dementia.

Of the two other studies reviewed, the paper by Wright et al. found an inverse association between IL-6 levels and MMSE scores in 269 stroke free participants in the Northern Manhattan Study (mean age 66.7 years (s.d. 8.4)) (Wright et al. 2006). However, this was a small study on a racially diverse population who were assessed with a single measure of cognitive ability. The final paper analysed IL-6 levels in relation to cognitive function in a group of 500 middle aged persons (mean age 44.6 years (s.d. 6.7)) (Marsland et al. 2006). This study used an extensive battery from the Wechsler Memory Scale to assess memory and two individual tests to assess executive function. Despite the cohort being much younger than the others reported, an inverse relationship was found between IL-6

and cognition, which was independent of a number of health factors such as BMI, smoking and hypertension. These findings raise the possibility that IL-6 may represent a biomarker for risk of future cognitive decline.

In summary, there is mixed evidence from both cross-sectional and longitudinal studies as to the relationship between IL-6 and TNF-α with cognitive ability and cognitive decline. Whilst some studies have used an extensive battery of tests to measure cognition, and had a reasonably long follow-up period, the number of participants in the studies has been quite small. As with the CRP/fibrinogen associations with cognition, there is a need for large-scale prospective cohort studies that can confirm or deny the existence of these small effects.

4.5 Association between SNPs and cognition

Further to the studies analysing TNF-α and IL-6 levels with cognitive ability, a study by Bernhard Baune investigated the relationship between SNPs of the *TNF-α*, *IL-6* and *IL-1β* genes with cognition (Baune et al. 2008). Using a relatively small study of 369 elderly participants of the Memory and Morbidity in Augsburg Elderly (MEMO)-study, they assessed the relationship between a single SNP from each of the three genes with measures of memory, processing speed, and motor function.

The SNPs selected for the analysis were rs1800269 (*TNF-α*) due to previous associations with dementia in centenarians (Bruunsgaard et al. 2004), rs1800796, (*IL-6*) which has been associated with detrimental effects on cognitive development in children (Harding et al. 2005), and rs16944 (*IL-1β*). Linear regression models yielded significant associations between the *IL-1β* SNP and memory performance ($p = 0.003$) and between the *TNF-α* SNP and processing speed ($p = 0.004$) after adjustment for age, sex, education, and MMSE score. In the case of the *IL-1β* SNP, the CC genotype was consistently but not significantly associated with increased performance across the other cognitive domains whereas the GA/AA genotypes for the *TNF-α* SNP were associated with poorer performance on the two tests where significance was not attained. Whilst it is possible that this implies a domain specific effect for the SNP it is also plausible that the result is a false positive. Given the small sample size for the analysis and limited number of SNPs typed in each gene, additional follow-up studies are required to replicate the findings.

4.6 Additional inflammation/cognition genes

4.6.1 Transcription Factor Gene 1

Located on chromosome twelve (12q24.31)[3] Transcription factor gene-1 (*TCF-1*) - also known as *HNF1A* - encodes the transcription factor hepatocyte nuclear factor (HNF)-1α (Reiner et al. 2008a). The Hepatocyte Nuclear Factor (*HNF*) genes regulate the transcription of proteins involved with blood clotting factors, enzymes, and transporters involved with glucose, cholesterol, fatty acid transport and metabolism (Armendariz & Krauss 2009). HNF-1α is expressed predominantly in the liver and has been associated with transactivation (increased gene expression triggered by endogenous cellular or viral proteins) of CRP (Toniatti et al. 1990).

The relationship between *TCF-1* genetic variants and CRP levels has been described in detail (Reiner et al. 2008a). By combining data from the Cardiovascular Health Study (CHS) (n = 4,333) and the Pharmacogenomics and Risk of Cardiovascular Disease (PARC) (n = 909 + 773), Bayes factors were computed to identify SNPs with the strongest evidence of association with CRP levels. The PARC study included data from two separate groups of of late-life men and women (mean age 62 years (s.d. 14) and 63 years (s.d. 13), respectively). The characteristics of these groups were similar with 32% and 25% of the samples being female and with mean CRP levels being 0.89 mg/l (s.d. 1.52) and 0.69 mg/l (s.d. 1.27). By contrast, the CHS demographics differed substantially (mean age 73 years (s.d. 6), 57% female, mean CRP 3.5 mg/l (s.d. 5.9)).

In the first stage of analysis, SNPs from a variety of genes were modelled against CRP levels in both studies. This identified strong predictors in the *CRP* and *APOϵ* genes. Modest Bayes factors were observed for variants in the *TCF-1* gene across both studies. Given the previous reporting of *CRP*/*APOϵ* variants and CRP levels, Reiner and colleagues decided to focus their investigation on variations in the *TCF-1* gene. Combining the SNP information from the two studies with information on multimarker LD from HapMap, they assessed the strength of evidence for phenotypic association with typed and untyped SNPs in the *TCF-1* region. Of the 193 SNPs in this region, the strongest evidence of an association was with a cluster of five untyped SNPs in perfect LD (rs7310409, rs2393775, rs7979473, rs2393791, and rs7979478).

Whilst these studies provide convincing evidence for a link between the *TCF-1*

[3]http://genome.ucsc.edu/cgi-bin/hgTracks?org=Human&position=chr12:119900932-119924695

gene and CRP levels, the heterogeneity between the two populations in Reiner's paper could be a cause for concern. However, as age, gender, BMI, and smoking are adjusted for in the analyses, it would appear that the major influences on CRP have been accounted for.

A 2005 study by Soria et al. conducted a genome-wide scan on 21 Spanish families to search for determinants of plasma fibrinogen levels (Soria et al. 2005). The study participants were part of the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project and included 398 persons belonging to pedigrees of 3-5 generations. The age of participants ranged from < 1 to 88 years. In an age and sex-adjusted analysis, fibrinogen levels were significantly associated with three of the ten *TCF-1* SNPs included in the analysis: rs1169292, rs1169301, and rs2464196. However, no associations were found between any of the *TCF-1* haplotypes that were generated, leading the authors to conclude it was likely that the functional variant was not typed in the analysis. One problem with the SNP analysis was the conclusion of a positive association at the nominal significance level of 0.05. Had a correction for multiple testing been applied then the *TCF-1*–fibrinogen association would have been null. Secondly, the authors failed to investigate the LD structure of the SNPs in question. It is therefore possible that the three positive SNP associations are actually being tagged by each other and that the multiple positive findings are simply a replication.

4.6.2 Coagulation factor XIII, A1 polypeptide

The *F13A1* or Coagulation factor XIII, A1 polypeptide gene is located on chromosome six (6p25.3-p24.3)[4] and encodes coagulation factor XIII. After the conversion of fibrinogen into fibrin via the enzyme thrombin, factor XIII stabilises the newly formed mesh of fibrin by cross-linking the molecules. This process results in the formation of clots.

A pair of studies yielded similar, null associations between the rs5985, *F13A1* variant and fibrinogen levels (Mannila et al. 2006, 2007). However, as the primary aim of both studies was to consider the association between SNPs and fibrinogen levels in MI patients, this reduces the ability to generalise the results to healthy subjects.

The first study comprised 327 Swedish persons who were survivors of a first MI, and 60 controls (selected by rs1049636 genotype - a *FGG* SNP that has been associated with altered fibrin clot structure). Significant epistasis (gene-gene

[4]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr6:6089310-6265923

interaction) on plasma fibrinogen concentration was detected between rs2070011 (*FGA*) and rs5985.

In the second study, 1,213 men and women diagnosed with an MI were compared with 1,561 healthy controls of similar age, sex and area of residence. Plasma fibrinogen and IL-6 levels were assessed for differences by fibrinogen SNPs and haplotypes, and the *F13A1* rs5985 SNP. Concordant with their previous findings, the *F13A1* SNP did not appear to exert any influence upon plasma fibrinogen concentration.

4.6.3 Interleukin-1

One of the first cytokines to be discovered, interleukin-1 is composed of two distinct proteins IL-1α and IL-1β. Both proteins are coded for on chromosome two (α 2q12-q21, β 2q13-q21)[5,6] and are pro-inflammatory components of the immune system. Functionally, IL-1 facilitates the movement of leukocytes to sites of infection in addition to regulating the hypothalamus, which raises body temperature (Dinarello 1994). These processes complement the immune system and help the body to combat pathogens and fight infection.

Several studies have investigated the relationship between plasma levels of IL-1, its genetic variants and cognition but with mixed results (Holmes et al. 2003, Trompet et al. 2008, van den Biggelaar et al. 2007). Van den Biggelaar et al. investigated whether inflammation precedes depressive symptoms and cognitive decline in 267 persons, aged 85 years from the Leiden 85-Plus study. Participants were prospectively followed up for five years until age 90 when they were assessed for depressive symptoms (Geriatric Depression Scale) and cognitive ability (MMSE). Whilst higher levels of IL-1β were associated with an increase in depressive symptoms, no associations were observed with cognitive dysfunction. Moving from biomarker levels to genetic markers, Trompet et al. investigated several variants in the interleukin-1 beta-converting enzyme (*IL-1βC*) gene – a gene that is likely to influence plasma IL-1β levels. Cross-sectional and longitudinal associations were observed for individual SNPs and haplotypes in 5,804 persons aged 75 years (s.d. 3) from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Some of the genetic variants in the *IL-1βC* gene that associated with better performance on cognitive function also associated with lower IL-1β plasma levels. This was particularly the case for the cognitive measure of executive function, which was assessed using the Stroop Test and the Letter-

[5]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr2:113247963-113259442
[6]http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr2:113303808-113309674

Digit Coding Test. Cognition was assessed at six separate time-points during the study: before randomization, at baseline, after 9, 18 and 30 months, and at the end of the study (36–48 months). Linear regression was used in the cross-sectional analyses with mixed effects models being used to analyse the multi-wave data.

Polymorphisms from the *IL-1* genes have also been associated with systemic levels of inflammatory markers including CRP and IL-6 (Reiner et al. 2008b) and with central obesity and metabolic syndrome in a population with CHD (Carter et al. 2008a).

4.6.4 Apolipoprotein ϵ

One further gene that was investigated during the analysis for this thesis was Apolipoprotein ϵ ($APO\epsilon$). The $APO\epsilon$ gene is located on chromosome nineteen (19q13.2)[7] and has three alleles - $\epsilon 2$, $\epsilon 3$, $\epsilon 4$. By genotyping two SNPs in the region (rs429358 and rs7412) and generating haplotypes, allele status can be determined (Seripa et al. 2007). In particular, the TT haplotype generates the $\epsilon 2$ allele, the TC haplotype generates the $\epsilon 3$ allele, and the CC haplotype generates the $\epsilon 4$ allele. The frequency of these three alleles in the general population is approximately 0.08, 0.78, and 0.14, respectively (Rebeck et al. 1993).

As the major lipid carrying protein of the central nervous system, $APO\epsilon$ is critical for the management of cholesterol transportation in the repair, growth, and maintenance of myelin and neuronal membranes during development or after injury (Seripa et al. 2007). There is also evidence that it is a potent scavenger of soluble amyloid and therefore, may play a role in the deposition of amyloid plaques, which are a characteristic of an AD brain.

$APO\epsilon$ has been studied extensively in relation to cognitive ability and is, to date, one of only a few genes to be consistently associated with this trait (Bertram & Tanzi 2008). Of the various $APO\epsilon$ alleles, carriers of the $\epsilon 4$ allele are at greater risk of developing dementia.

Moreover, CRP levels have been shown to vary by $APO\epsilon$ genotype. Several studies have found that the high risk allele for dementia ($\epsilon 4$) was associated with lower levels of CRP (Grönroos et al. 2008, Judson et al. 2004, Kahri et al. 2006, März et al. 2004). This finding is somewhat counter-intuitive given the independent associations between both elevated CRP levels and $APO\epsilon 4$ status with impaired cognition. The study by März et al. also looked at the association between plasma fibrinogen levels and $APO\epsilon$ status but found no evidence for a relationship.

[7] http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr19:50100879-50104490

4.7 Summary

This chapter provided a brief introduction to the biomarkers IL-6 and TNF-α. The cross-sectional and prospective evidence for associations with cognitive ability and cognitive decline is mixed. Whilst the evidence for an association is slightly stronger for IL-6, the number of well-designed, large studies has been few. As with fibrinogen and CRP, the potential mechanisms through which IL-6 and TNF-α operate in relation to cognition are currently unclear. In addition to the biomarker-cognition studies, one group studied a single SNP from each of the *IL-6* and *TNF-α* genes and found evidence for significant associations with late-life cognition. Finally, this chapter also introduced several other genes (*IL-1*, *TCF-1*, *F13A1*, and *APOϵ*) that have been related to inflammation and cognitive ability. Brief descriptions were provided for the structure and function of these genes before they were related to CRP levels (*TCF-1* and *IL-1*), fibrinogen levels (*F13A1*), IL-6 levels (*IL-1*), and cognitive ability (*IL-1* and *APOϵ*).

Literature summary and study aims

Determining the relationship between individual components of the inflammatory and rheological systems and cognitive decline is important in order to inform the development of targeted preventive interventions. However, data from large-scale epidemiological studies with adequate cognitive testing and reasonable length of follow-up are scarce. Although a number of studies have explored the association between the inflammatory marker C-reactive protein (CRP) and cognitive decline, few have been of sufficient size and methodological rigour and even these gave contrasting results (Alley et al. 2008, Dik et al. 2005, Schram et al. 2007, Weuve et al. 2006). A single, relatively small study on plasma fibrinogen and cognitive decline found an association with 5–year change in non-verbal reasoning scores and estimated lifetime decline of memory scores, but not with decline in general intelligence, executive function or processing speed (Rafnsson et al. 2007). A study on the association between human cognition and blood rheology found significant associations between plasma viscosity and haematocrit and tests of general intelligence and decision speed. However, findings were confined to men and did not address the issue of cognitive decline subsequent to measurement of these rheological factors (Elwood et al. 2001). Finally, there have been very few large-scale, long term follow-up studies to associate levels of the upstream inflammatory markers IL-6 and TNF-α with cognitive function.

In view of these limited findings, the hypothesis that higher levels of the inflammatory markers CRP and fibrinogen and of the rheological factors plasma viscosity and haematocrit are associated with cognitive decline was investigated using data from the Aspirin for Asymptomatic Atherosclerosis (AAA) Trial. This cohort had the advantage not only of size (over 2,000 subjects cognitively tested), availability of detailed cognitive testing within a range of cognitive domains and 5-years of follow-up, but was also free of clinical cardiovascular disease at baseline when inflammatory and rheological markers were measured. Given that CRP

levels in particular may be affected by atherosclerosis (a strong risk factor for cognitive decline), this helps to reduce the chance that any observed associations between inflammatory marker and cognitive decline were confounded by prevalent vascular disease at baseline.

Furthermore, a similar hypothesis was then tested using cross-sectional data from the Edinburgh Type 2 Diabetes Study (ET2DS). Specifically, the aims were to determine the associations between the downstream inflammatory markers CRP and fibrinogen, the upstream inflammatory markers IL-6 and TNF-α, and a general inflammation factor, (derived using information from the four markers) and cognition. As persons with Type 2 diabetes are at greater risk of both cognitive impairment and dementia, and given that they also decline at a faster rate, this population should provide an accelerated model of cognitive decline compared to the AAA Trial population.

To determine the mechanisms of the potential relationships, a genetic association analysis was then performed using SNP information from the *CRP*, *FGA*, and *FGB* genes in the AAA Trial, the ET2DS, and two replication cohorts, the Edinburgh Artery Study (EAS), and the 1936 Lothian Birth Cohort (LBC1936). Several SNPs from the genes described in Chapter 4 were also investigated in relation to cognitive ability. The following chapters describe in detail the study methodology of these cohorts, the statistical analysis of their data, the results generated from the analyses, and their implications.

Chapter 5

The Aspirin for Asymptomatic Atherosclerosis (AAA) Trial

5.1 Introduction

This chapter describes the baseline methodology and follow-up procedures employed by the Aspirin for Asymptomatic Atherosclerosis (AAA) Trial, a randomised controlled trial of aspirin on the prevention of cardiovascular disease in an asymptomatic atherosclerotic population. Moreover, the chapter provides information on the AAA Trial study population, the procedures of recruitment at baseline, and the layout of the baseline and follow-up clinical examinations. In addition, the cognitive function testing that was carried out in 1999/2001 and 2003/2006 is discussed in depth. The chapter draws to a close by describing the methods of data storage, variable manipulation and transformation in preparation for the main analyses. Much of the descriptive information presented in this chapter is referenced from the AAA Trial grant application, in addition to previously published papers that analysed data from the Trial (Price et al. 2006, 2008c, Stewart et al. 2006).

5.2 The AAA Trial: Baseline survey

The primary purpose of the AAA Trial was to test, using a double blind, placebo controlled, randomised trial, the effect of low dose aspirin in the prevention of cardiovascular events and death in subjects at high risk of cardiovascular events. A secondary objective was to investigate the relationship between late-life inflammatory and rheological biomarker levels with late-life cognitive ability, late-life cognitive change, and estimated lifetime cognitive change.

5.2.1 Study population

The study population of the AAA Trial comprised 3,350 men and women living in central Scotland (Lanarkshire, Edinburgh, and Glasgow), aged 50–75 years. Recruitment to the trial began in 1998 when 165,795 invitations were sent out to members of the general public through their general practices (83% of practices contacted agreed to participate). Screening then took place on 29,147 subjects. At the screening clinic, researchers recorded right and left brachial, posterior tibial, and dorsalis pedis systolic pressures with a standard sphygmomanometer and a Doppler probe. The ankle-brachial pressure index (ABPI) was calculated as the ratio of the lowest pressure in either ankle to the higher of the measurements in the left or right arm. ABPI is both an accurate (Ouriel et al. 1982, Yao et al. 1969) and reliable (Fowkes et al. 1988) marker of generalised atherosclerosis with values ≤ 0.95 shown to be a good predictor of mortality and subsequent cardiovascular events (Heald et al. 2006). A detailed eligibility examination was then carried out on 4,914 subjects with an ABPI ≤ 0.95 to exclude persons for any of the following criteria: current use of aspirin, other antiplatelet, or anticoagulant; severe indigestion; history of myocardial infarction, stroke, angina, or peripheral arterial disease; chronic liver or kidney disease; chemotherapy; contraindications to treatment with aspirin; and an abnormally high or low packed cell volume.

Screening took place at over 20 different clinic sites across Lanarkshire, Glasgow, and Edinburgh with those eligible randomised to either low dose aspirin (100mg enteric coated) or placebo (both preparations provided by Bayer HealthCare). The intention was to follow-up 3,300 subjects for five years for cardiovascular events and death although this figure was surpassed with 3,350 subjects available for randomisation. This population was similar, in terms of age, sex, and level of deprivation, to the general population of Scotland (Price et al. 2008d). A flow chart detailing the recruitment process to the trial is presented in Figure 5.1.

5.2.2 Sample size determination

As the inflammation-cognition investigation was part of a secondary analysis, this had some bearing upon the sample size calculations, which were calculated post-hoc. Nonetheless, the study of baseline inflammatory marker levels as predictors of follow-up cognition was powered at 90% ($\alpha = 0.05$) to detect a standardised effect size of 0.056, assuming a follow-up sample size of 3,350. Allowing for a combined 40% drop-out rate or missing data (n = 2,010), the study was powered at 90% ($\alpha = 0.05$) to detect a standardised effect size of 0.072. This implies that

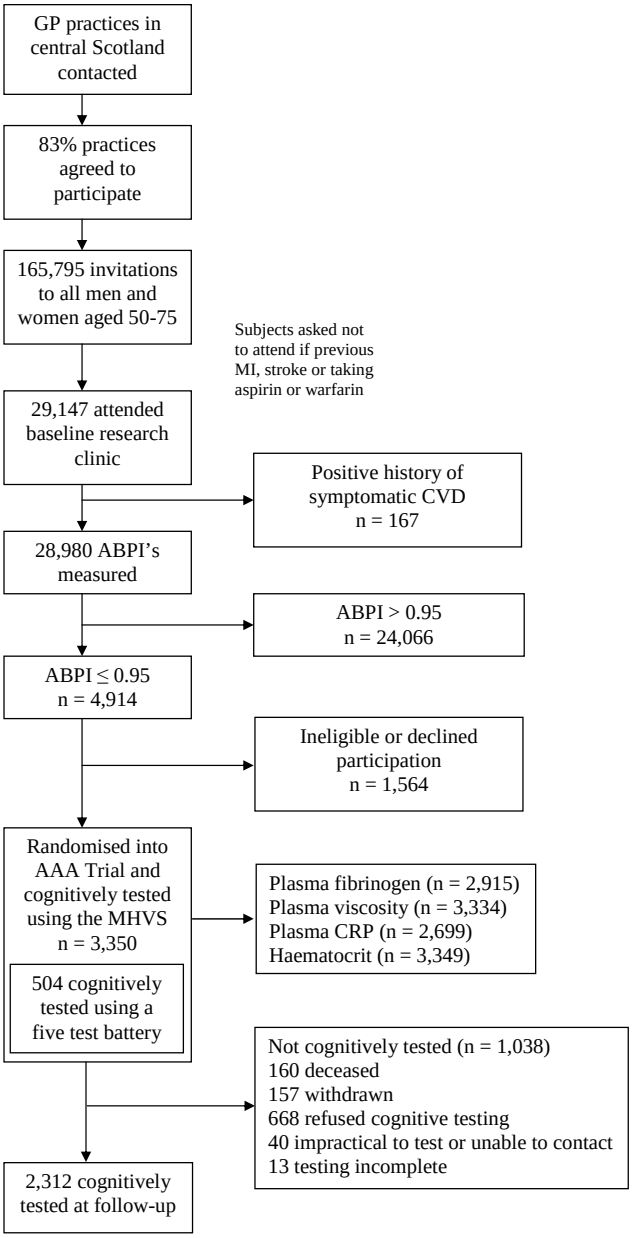

Figure 5.1: AAA Trial recruitment process.

for every standard deviation change in marker level the cognitive score changes by 0.072 standard deviations.

For a sub-group of 504 participants who received extensive cognitive testing at both waves of the study, the Trial was powered at 90% ($\alpha = 0.05$) to detect a standardised effect size of 0.14. After allowing for a sample-size reduction of 20% (n = 403), the study was powered at 90% ($\alpha = 0.05$) to detect an effect of 0.16 between the baseline inflammatory marker levels and five year cognitive change.

5.2.3 Ethical approval

Ethical approval for the AAA Trial was granted by the ethical committees of Lanarkshire, Edinburgh and Glasgow. In addition, all subjects provided written informed consent prior to the start of the trial.

5.2.4 Baseline examination

At the baseline stage of the trial, participants were randomised to aspirin or placebo before being asked to complete a medical history questionnaire. A questionnaire on smoking habits was also used to identify current smokers, previous smokers, or those who had never smoked. Persons who were current or ex-smokers were asked for the average number of cigarettes they smoked per day, and the number of years they had smoked or the number of years since cessation. Other measures taken at this stage included ABPI, systolic and diastolic blood pressure, and a plasma sample for biomarker measurements. All current prescribed or regular medications were also recorded. After a three month period, participants were asked to return for a brief compliance visit and for cognitive testing. At this point, those with missing biomarker or questionnaire data from the baseline visit were re-examined.

5.2.5 Inflammatory and rheological biomarker measurements

Baseline venous blood samples were used to measure plasma viscosity, haematocrit, fibrinogen, and CRP. Assays were performed in the University Department of Medicine, Glasgow Royal Infirmary. Plasma viscosity was assayed in a fresh blood sample anticoagulated with K_2EDTA in a capillary viscometer (Coulter) at 37 °C (Tzoulaki et al. 2006). In the same sample, haematocrit was measured using a microcentrifuge and optical reader (Hawksley) (Tzoulaki et al. 2006). In

stored plasma anticoagulated with trisodium citrate, fibrinogen was assayed by the automated Clauss assay (MDA-180 coagulometer, Organon Teknika), and CRP using a high-sensitivity immunonephelometric assay (Tzoulaki et al. 2006).

5.3 The AAA Trial: Five-year follow-up examination

Approximately five years after the baseline clinical examination all surviving and eligible subjects were invited to attend a follow-up clinical examination. The procedures of this examination are detailed below.

5.3.1 Invitation to study

All subjects had been followed up annually by means of telephone contact since the inception of the trial. Prior to the fifth year phone call, a letter was sent to all participants to inform them about the processes involved for the follow-up cognitive testing. Participants were then contacted to arrange a time and date for a visit to a local research clinic for the cognitive examination. Persons who were unable to make it to the clinic were given the option of a home visit. Those who did not attend the clinic, missed their appointment, or failed to respond to the invitation were re-contacted up to three times or were asked to complete a brief telephone interview to identify a reason for withdrawal/non-participation.

In total, the number of participants who were available for follow-up cognitive testing was 2,312. Of those lost to follow-up (n = 1,038), 160 were deceased, 157 had withdrawn, 668 refused to be cognitively assessed, 40 persons were impractical to test or unable to contact, and 13 had incomplete testing.

5.3.2 Clinical examination

In addition to the cognitive testing, a brief clinical examination was performed on participants at follow-up to measure ABPI and (systolic and diastolic) blood pressure.

5.4 Methods of cognitive function testing

5.4.1 Baseline cognitive testing

Cognitive assessment of the AAA Trial was launched three months after screening and randomisation took place. A vocabulary-based test was administered to all willing participants of the trial with a subset of 504 persons also receiving an extensive five test omnibus of cognitive tests. The same battery was applied to all participants along with another vocabulary-based test at the five year follow-up. Extensive details on the tests used in the cognitive battery are provided in the subsection on the second round of cognitive testing.

5.4.2 Baseline subject recruitment

Of those who attended the three month clinic, 2,733 (81.6%) completed the Mill Hill Vocabulary Scale (MHVS). A subset of this population was then selected for extensive cognitive testing. The selection process for this subset was not randomised. Briefly, research funding became available to perform extensive baseline cognitive testing after approximately one third of subjects had been through the three month clinic. Thereafter, participants were invited sequentially to take the additional cognitive testing until this group totalled a target of 480 in number. In practice, 504 persons ended up taking part in this additional cognitive examination. However, these data clearly introduce the possibility of bias through a non-representative sample.

5.4.3 Baseline medical questionnaire

In an attempt to account for any medical conditions that may have interfered with the subjects performances on the cognitive tests, a brief questionnaire was administered. Subjects were asked to relate information on other relevant diseases such as severe arthritis and vision or hearing difficulties that may have had an affect on cognitive function or the ability to complete the tests.

5.4.4 Baseline cognitive testing

The cognitive function testing, which began in 1999 and was completed in 2001, was carried out by two members of staff (a research nurse and a research fellow) who were trained in the cognitive test procedures by a senior member of academic staff (Psychology consultant on the AAA Trial) from the Department of

Psychology at the University of Edinburgh.

5.4.5 Follow-up cognitive testing

The recruitment of AAA Trial subjects to follow-up cognitive testing began in early 2003. All subjects who were enrolled into the trial at baseline were potentially eligible for participation. Testing took place between May 2003 and April 2006. The mean duration of participation in the trial was five years: 230 participants were tested after four years; 259 after six years; two after seven years; and the remainder at five years. Details of the cognitive test battery and quality control measures for testing are described below.

Before the start of the cognitive function testing, training in the administration of the cognitive tests was provided. Between six and eight trained research nurses were hired at any one point to administer the testing. All nurses were informed about the basic cognitive test procedures by the research fellow who conducted the testing at baseline.

At both waves of cognitive testing an informal assessment of inter-rater agreement was carried out. The lack of formal comparisons between the cognitive testers may have introduced bias into the results although all the testers at both waves were trained by a single supervisor.

5.4.6 The cognitive test battery

The detailed cognitive test battery assessed at both waves of the AAA Trial included five identical neuropsychological tests. A screening test for dementia (the Mini-Mental State Examination) was administered at both baseline and follow-up. However, persons were not removed from the study if they had a low MMSE score. Participants were all community-dwelling and attended the clinic under their own freewill. If they asked to discontinue with the testing at any timepoint, or if their GP contacted the AAA Trial research team asking for their participation to be stopped then this was granted with immediate effect. Similarly, testing was stopped if the research nurse felt that this was causing the participant any distress. A vocabulary-based measure of pre-morbid cognitive ability (the National Adult Reading Test) was also assessed at the 5-year follow-up although this test does differ slightly from the Mill Hill Vocabulary Scale used at baseline. Nonetheless, the correlation between these two similar measures (Crawford et al. 2001) was extremely high ($r = 0.721$). The test administration time was approximately one hour. The individual components of the test battery

are described below in the order they were presented to the participants.

List of Cognitive Tests

Mill Hill Vocabulary Scale

During the visit that occurred three months after commencement of the trial, subjects were asked to complete a combined version of the Junior and Senior Form A synonyms of the Mill Hill Vocabulary Scale (MHVS) (Raven et al. 1998). The MHVS is a self-administered test that contains 44 items. Each item comprises a word for which the subject must identify the closest synonym from a choice of six given alternatives. The words become increasingly esoteric as the test progresses. In common with other vocabulary-based measures of cognition, the MHVS may be thought of as an indicator of pre-morbid or best-ever cognitive ability. As mentioned in Section 1.3.2, vocabulary measures vary little over a lifetime (Crawford et al. 2001, Schaie 2005). Assessment of the MHVS allowed for a baseline comparison of the trial population and the subset, in addition to a crude measure of the difference in cognitive ability of participants who were lost to follow-up. The maximum possible score on the MHVS is 44 (range 0 – 44).

Mini-Mental State Examination

The Mini-Mental State Examination (MMSE) is a commonly used instrument in the screening of cognitive function in both clinical practice and research (Folstein et al. 1975). The test is not sensitive to subtle changes in cognition but it may be used to indicate the presence of cognitive impairment, for example suspected dementia (Crum et al. 1993). Furthermore, it is possible for people with low intelligence or education to score poorly on this test in the absence of cognitive impairment (Tombaugh & McIntyre 1992) whilst well educated people may score well despite having cognitive impairment (Brayne & Calloway 1990). The MMSE is a short test comprised of 20 questions and tasks across the following domains: orientation-time, orientation-place, memory-registration, attention and calculation, memory–recall, praxis–ideational, praxis–copying and drawing, and spontaneous praxis–writing. As an example of a question from the memory–registration domain, the subject is asked to recall immediately a list of three words. The examiner then informs the subject that they will be asked to recall the same three words at a later point in the assessment, while in the praxis–copying domain, the subject is asked to draw a copy of two interlocking pentagons. A single point is awarded for each correct answer with 30 points being the maximum score pos-

sible (range 0 - 30). In healthy cohorts the distribution of MMSE scores tends to exhibit a ceiling effect with the majority of subjects answering all questions correctly. A common cut-off used as a screening measure for dementia is a score of less than 24 points.

Verbal Fluency Test

The Verbal Fluency Test (VFT) assesses executive function via measurement of the number of words, orally presented by a subject within a restricted time interval (Lezak 1995). The VFT consists of three, one-minute word-naming trials. The examiner asks the subject to list as many words as possible beginning with a particular letter of the alphabet within a one minute period (the letters C, F, and L were used in the AAA Trial). An overall score is assigned after summing the total number of acceptable words from all three of the trials. The letters selected for the trials are chosen on the relative frequency of English language words that beginning with these letters. For example, words beginning with the letter C are relatively common, words beginning with F are slightly less common, and words beginning with L are the least common of this trio. Proper nouns are not counted and the same word with a suffix variation (e.g. make and makes) is only counted once. Before the main test begins, the subject is asked to complete a practice example using the letter S, or a similarly common letter for the beginning of a word. After providing several words beginning with this letter, the subject is asked to move onto the timed trials.

Raven's Standard Progressive Matrices

To assess the participants' skills in non-verbal reasoning, Raven's Standard Progressive Matrices (RAVENS) were administered (Raven et al. 1998). This test relies heavily on problem solving skills and requires the subject to decipher puzzles through identification of relationships between complex structures. The RAVENS test comprises five batches of 12 items (60 in total). Each individual item requires the subject to solve a pattern problem that has a piece missing from it. Either six or eight possible options are given for the missing piece with only one correct option per item. The subject must choose one piece per item to complete the pattern. Items increase in difficulty within each batch and from the first to the fifth batch. A single point is assigned to each item, giving a maximum possible score of 60 (range 0 - 60). In the AAA Trial, participants were allowed 20 minutes to complete as many items of the test as possible after having had a brief practice trial involving the first three test items. Participants were also allowed to change

their answers to any question during the 20 minute test period.

Auditory Verbal Learning Test

Immediate and delayed memory was assessed by administration of the Auditory Verbal Learning Test (AVLT) (Lezak 1995). The test involves five presentations with recall of a list of 15 words, one presentation of an alternative list of 15 words, and then a sixth recall trial. The test usually takes in the region of 15 minutes to administer with retention being examined anywhere from 30 minutes, hours, or days later. The order in which the words are recalled is irrelevant. In the AAA Trial, the scores from the first five trials were aggregated to provide a joint measure of immediate and delayed memory. Although not examined in the AAA Trial, the AVLT can provide information about an individual's learning strategies by noting the order of recall response. As there are five trials with 15 items per trial, the maximum possible score on the AVLT is 75 (range 0 - 75).

Trail Making Test, Parts A and B

Parts A and B of the Trail Making Test (TMT) were used to assess mental flexibility via visual conception and visuo-motor tracking (Spreen & Strauss 1991). Part A of the test requires the subject to draw connective lines between consecutive numbers printed randomly on a piece of paper. The subject is not allowed to remove the pen/pencil from the paper and must complete the task as quickly as possible. Part B of the test is similar, except the subject must now follow a sequence of consecutive number-letter combinations e.g. 1-to-A-to-2-to-B-to-3-to-C etc. Again the pen/pencil is not allowed to leave the paper with the aim being to complete the test as quickly as possible. The time taken to completion but not the number of errors was recorded by the examiner. In the case where the subject got stuck, the examiner would prompt them to the next letter/number in the sequence. For all subsequent analyses of the AAA Trial data, only the time to completion of the TMT Part B was used.

National Adult Reading Test

A similar test to the MHVS, the National Adult Reading Test (NART) assesses vocabulary skills – specifically, reading ability (Nelson & Willison 1991). It is commonly used to estimate pre-morbid cognitive function. The NART requires the subject to correctly pronounce a list of 50 words from the English language that are irregular with respect to common rules of pronunciation. The difficulty of the words increases sequentially throughout the test. Some examples of the words

used in the test (in increasing order of complexity) are chord, gaoled, drachm, and demesne. The test administrator notes a correct or incorrect answer for each word, allowing slight variations for regional accents. The marking of the test relies entirely on the ability of the examiner to interpret correctly the participant's responses. As a marking aid, the examiner is provided with the correct pronunciation of all words in both written and audio format. The maximum possible score on the NART is 50 (range 0 – 50).

Digit Symbol-Coding Test

The Digit Symbol-Coding Test (DST) is a subtest from the Wechsler Adult Intelligence Scale (WAIS-III^{UK}) - a clinical instrument designed for evaluating the intellectual ability of adults 16 to 89 years of age (Wechsler 1998a). The purpose of the DST is to measure an individual's processing speed through the recoding of symbols into numbers. The task is laid out with four rows of 25 paired boxes. The top box of each pair contains a symbol and the participant has to use the digit-symbol key at the top of the page to assign the correct number to the blank box below each symbol. Prior to the test, the participant practices on the first seven boxes, which do not count towards the final score. The main test requires the examinee to fill in as many blank boxes with the corresponding symbols as possible in 90 seconds. An overall test score is given upon summation of the correctly completed boxes within the given time limit. The maximum score obtainable is 93 (range 0 – 93). Moreover, participants must complete the test in order from top left to bottom right i.e. they are not allowed to go through the test systematically coding the symbols one at a time.

5.5 Genotypic data

Blood samples collected from AAA Trial participants at the three month visit were used for genotyping. Fifty-two candidate SNPs were selected from thirty different genes for genotyping. SNPs were chosen on the basis of previously reported associations with cognitive ability or because they lay in genes strongly associated with phenotypic variants thought to be important in cognitive processes. A list of all SNPs typed in the study and analysed in this thesis are presented in Table 5.1. Genomic DNA was isolated from whole blood by standard procedure at the Wellcome Trust Clinical Research Facility Genetics Core, Western General Hospital, Edinburgh. Genotyping was carried out by KBioscience (Herts, UK) using their in-house chemistry of Competitive Allele Specific

PCR (KASPar). As discussed in Subsection 4.6.4, in order to generate $APO\epsilon$ alleles, one must create a haplotype using information from the rs429358 and rs7412 SNPs. This was done by exporting the raw SNP information from KBioscience into a Microsoft Excel comma–separated variable file before being read into the computer software package SimHap (Carter et al. 2008b). For individuals with ambiguous phase, SimHap uses biallelic SNP genotype data to impute haplotype frequencies at the individual level. The most likely haplotypes were then selected for each individual with the new data being transferred back into Excel prior to statistical analysis. This haplotype information was then used to generate $APO\epsilon$ alleles for each individual.

Gene	SNP	Gene	SNP
APOϵ	rs429358	*CRP*	rs1800947
	rs7412		rs1130864
TCF-1	rs1169292		rs1205
	rs1169301		rs1417938
	rs2464196	*F13A1*	rs5985
FGA	rs2070022	*IL-1α*	rs2856836
	rs6050		rs3783546
	rs2070016		rs2856838
	rs2070018	*IL-1β*	rs1143643
	rs2070011		rs1143634
FGB	rs4220	*IL-6*	rs2069832
	rs1800788		rs2069840
	rs2227412		rs1800795

Table 5.1: Genotyped SNPs from the AAA Trial

Of the 3,350 men and women recruited into the trial, 2,312 underwent cognitive testing at 5 year follow-up. For the genetic analyses presented in this thesis, the AAA Trial study population consists of 2,091 subjects who completed three or more tests from the five test follow-up cognitive battery in addition to having DNA available for testing. Some persons were also included in the genetic analysis if they had DNA available for testing and a plasma CRP or fibrinogen level in the upper or lower decile of the distribution – irrespective of the availability of cognitive data.

5.6 Data handling and management

5.6.1 Entry of cognitive data

Data from both rounds of cognitive testing were double entered into a computer using Microsoft Access. The cognitive packs were checked prior to data entry for errors, such as scores beyond the range of possible values for the test, and missing values. Under both scenarios, the original paper questionnaires were consulted and checked with regards to whether the participant had left out individual items (and the corresponding test scored as missing), refrained from doing the test, or whether there had been a transcription error in the double data entry. The participants' unique study numbers were used to match the follow-up data with the baseline data for the cognitive and physiological/demographic variables. The data merging was performed by running a query in Microsoft Access. The data were then exported into an Excel file to allow them to be read into the analysis software packages R and SPSS.

5.6.2 Missing data and bias

At the baseline and follow-up visits there were numerous examples of missing values in both the cognitive and demographic/physiological variables. Data imputation was considered for the cognitive tests where persons had three or more scores to impute from. However, no formal analyses were conducted to investigate the assumption of the data being missing at random. A brief analysis was conducted using the Expectation-Maximization (EM) algorithm to impute missing values although this made little difference to the study findings and interpretation. Moreover, not analysing persons with missing data is likely to lead to more conservative estimates of any associations uncovered. The baseline MHVS scores were lower for those who completed fewer cognitive tests (Table 5.2). This was most noticeable when comparing the group with full follow-up cognitive data to those with at least one missing test result ($p < 0.001$). However, as it was not the primary scope of this thesis to investigate missing data, it was deemed more appropriate to list the lack of a formal missing data and imputation analysis as a limitation instead of conducting a crude and ill-considered analysis.

Tests completed	Mean	s.d.	n	t	d.f.	p-value
$\geq$ 1 tests	31.1	4.74	2,191	-2.16	2,731	0.031
0 tests	30.6	4.59	542			
$\geq$ 3 tests	31.1	4.72	2,154	-2.47	2,731	0.014
$\leq$ 2 tests	30.5	4.68	579			
5 tests	31.2	4.71	1,930	-4.11	2,731	<0.001
$\leq$ 4 tests	30.4	4.69	803			

Table 5.2: Comparison of mean MHVS scores by number of cognitive tests completed.

5.6.3 General intelligence factor and Principal Components Analysis

Given the high correlation between each of the five cognitive tests (Table 5.3), a general factor representing the variance common to all tests was created via Principal Components Analysis. The correlations between the TMT and the other tests are negative due to higher scores on the TMT reflecting a poorer cognitive performance i.e. the score reported is the time to completion of the task.

	AVLT	RAVENS	VFT	DST	ln(TMT)
AVLT	1.000				
RAVENS	0.421	1.000			
VFT	0.362	0.400	1.000		
DST	0.425	0.589	0.411	1.000	
ln(TMT)	-0.401	-0.591	-0.389	-0.658	1.000

Table 5.3: Pearson correlations between follow-up cognitive test scores.

It is standard practice in neuropsychological studies to use statistical techniques to reduce the dimension of cognitive test data. For example, if several tests of memory are administered in a cognitive battery then it may be desirable to extract an overall memory component instead of dealing with multiple, highly correlated variables. As mentioned in Subsection 1.3.1, it is recognised that there are strong correlations between most cognitive tests. By using a process called Principal Component Analysis (PCA) it is possible to extract a general ability score.

Mathematically, PCA is defined as an orthogonal linear transformation that transforms the data to a new coordinate system such that the greatest variance by any projection of the data comes to lie on the first coordinate (called the first

principal component), the second greatest variance on the second coordinate, and so on. Conceptually, PCA visualises the data in an n–dimensional space (where n corresponds to the number of variables) before altering the perspective to find the most informative view of the data. This 'most informative' view is reported as the first principal component.

For the AAA Trial, scores from the five cognitive tests at baseline (follow-up) were incorporated into the PCA. Both analyses provided evidence for a single, principal component (Tables 5.4, and 5.5). At baseline (follow-up) the first component explained 52.6% (56.9%) of the total variation of the cognitive data. By examining the eigenvalues it is clear that there is only a single component of interest at both time–points - as a general rule, one would only extract components with $\lambda > 1$. The PCA analysis also prints the component matrix and the component score coefficient matrix. The former matrix indicates the loading of the individual cognitive tests onto the component. This is presented as a correlation between the individual test and the principal component. For the AAA Trial data it can be seen that all of the tests loaded strongly onto the first component (baseline range 0.581 – 0.818), confirming the presence of a general ability factor. This matrix does differ depending on which statistical package the analysis is run in. Whilst all generate the same eigenvalues and g-scores, R and SPSS use different scaling methods to calculate the factor loadings. SPSS tends to generate larger loadings than R although the ratios between loadings are identical. Each of the PCA analyses reported in this thesis were performed using SPSS. The second matrix reports the factor scores used to calculate the g score for each trial participant. For the baseline data of the AAA Trial this gave participant i's g-score as

$$g_i = (0.225 \times \text{bAVLT}_i) + (0.298 \times \text{bRAVENS}_i) + (0.221 \times \text{bVFT}_i) + (0.311 \times \text{bDST}_i) - (0.308 \times \ln(\text{bTMT}_i))$$

These g-scores were then standardised and added to the database. As general intelligence is not something that can be measured directly, g is referred to as a latent variable.

5.6.4 Adjustment variables

The following variables, based on their associations with cardiovascular disease and/or cognition were considered as adjustment variables in the AAA Trial:

Baseline

Principal Component	Eigenvalue (λ)	% Variance explained	Cognitive Test	Factor Loading	Factor Score
1	2.63	52.6	AVLT	0.592	0.225
2	0.81	16.1	RAVENS	0.785	0.298
3	0.73	14.5	VFT	0.581	0.221
4	0.46	9.2	DST	0.818	0.311
5	0.38	7.6	-ln(TMT)	0.810	0.308

Table 5.4: PCA baseline output for the AAA Trial.

Follow-up

Principal Component	Eigenvalue (λ)	% Variance explained	Cognitive Test	Factor Loading	Factor Score
1	2.85	56.9	AVLT	0.659	0.232
2	0.73	14.5	RAVENS	0.809	0.284
3	0.64	12.9	VFT	0.643	0.226
4	0.43	8.6	DST	0.825	0.290
5	0.36	7.1	-ln(TMT)	0.813	0.286

Table 5.5: PCA follow-up output for the AAA Trial.

- Age at the time of cognitive testing in 2003/2006.
- Sex.
- Peak prior cognitive ability, which was estimated using baseline MHVS scores.
- Baseline levels of smoking. Pack-years of smoking were estimated by multiplying the number of 20-cigarette packs smoked per day with the number of years as a smoker. A zero value was entered for life-long non-smokers.
- Diastolic blood pressure.
- Baseline serum levels of total cholesterol.
- ABPI.

5.7 Summary

This chapter has described the recruitment procedures and variable measurements taken in the AAA Trial. After screening participants for an ABPI ≤ 0.95, 3,350

persons were enrolled into either the aspirin or placebo arm of the Trial. All participants had a blood sample taken at baseline of the Trial for biomarker measurements and genotyping. Also at baseline, the population were cognitively assessed using the Mill Hill Vocabulary Scale to provide an estimate of peak cognitive ability. A subset of 504 persons was further assessed using a battery of five cognitive tests, which were then administered to all participants at the Trial's five year follow-up. General intelligence (g) factors were calculated at both time points by means of Principal Components Analyses.

Chapter 6

The Edinburgh Type 2 Diabetes Study (ET2DS)

6.1 Introduction

This chapter describes the baseline methodology and preparation for follow-up for the Edinburgh Type 2 Diabetes Study, a prospective cohort study to analyse the impact of inflammation, microvascular disease and dysregulation of the hypothalamic-pituitary-adrenal axis on cognitive dysfunction. It provides information on the ET2DS study population, the procedures of recruitment at baseline, and the layout of the baseline clinical examination. In addition, the cognitive function testing that was carried out in 2006/2007 is discussed in depth. The chapter draws to a close by describing the methods of data storage, variable manipulation and transformation in preparation for the main analyses. The study details and descriptions presented in this chapter are all referenced from the ET2DS protocol paper (Price et al. 2008b).

6.2 The Edinburgh Type 2 Diabetes Study: Baseline survey

6.2.1 Study design

The relationship between diabetes and cognitive function has not been the primary focus of major longitudinal studies. Moreover, the investigation into diabetes-related variables that are involved with cognitive function has been limited (Allen et al. 2004, Strachan et al. 2003). The primary purpose of the ET2DS was to

test, using a prospective cohort study of persons with Type 2 diabetes, the relationship between inflammation, microvascular disease and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and cognitive dysfunction

6.2.2 Study population

Sampling frame and exclusion criteria

Participants for the ET2DS were selected from a population of 20,000 persons from the Lothian Diabetes Register (LDR). The LDR is a computerised database with details on almost all persons with diagnosed Type 2 diabetes who are living in the Lothian region of Scotland.

Although World Health Organisation criteria need to be met before a patient can be added to the LDR, the ET2DS study team re-validated the existence of diabetes in all potential study subjects. For this confirmatory diagnosis, several diagnoses were accepted as an indication of Type 2 diabetes:

- if the subject was being treated with oral anti-diabetic agents or insulin.
- if the subject was being treated via dietary intervention and they had a DCCT-aligned HbA1c measure of $> 6.5\%$ when tested at the research clinic.

Dietary controlled diabetics who had an HbA1c reading below 6.5% had their clinical records checked by a consultant diabetologist to confirm a correct diagnosis. Additional checks were carried out on persons who satisfied criteria making it more likely for them to be wrongly classified as having Type 2 diabetes. This included persons who had begun insulin treatment within one year of diabetes diagnosis; or who were under 35 years old and being treated with insulin; or who had reported evidence of pancreatic disease/surgery at the clinic. Persons in whom it was not possible to confirm a clinical diagnosis of Type 2 diabetes were excluded from participating in the study. Subject exclusion was also considered under the following circumstances:

- the subject was a non-English speaker (since fluent English is required for some of the cognitive tasks).
- the subject's corrected visual acuity was worse than 6/36 for distance vision or if they were unable to read large print text, as at least moderate visual function is required to complete some of the cognitive tasks.

- the subject was unwilling to give consent (or judged by clinical research staff to be unable to give fully-informed consent).
- the subject was not physically able to complete the clinical and cognitive examination.

Study population

Using the LDR as the sampling frame, elderly adults aged between 60 and 75 years (as of 1^{st} August 2006) were used to generate the study population. Eligible subjects were chosen from sex and five year age banded groups. Initially, 5,454 persons were invited to take part in the study; invitations were sent by the custodians of the LDR over a one year period from 20^{th} June 2006 to 1^{st} June 2007. The response rate for these invites was 60% with 2,104 failing to reply with an additional 64 invites being returned as a result of non-residence at the address provided in the LDR records. Of those who did reply, 1,252 (38%) were happy to volunteer as study participants. For those who failed to reply to the invitation or who did not wish to participate in the study a fresh list of randomly selected subjects was chosen from the stratified LDR records. Patient confidentiality was maintained with names and addresses of only those willing to participate being supplied to the ET2DS team.

Those interested in taking part in the study were sent a questionnaire for completion via post and also an appointment time for a visit to the research clinic. To ensure maximal turnout for the clinical examination, an offer to organise all travel arrangements was made. All travel expenses were fully reimbursed and a flexible appointment scheme was provided for each subject to arrange a date when it would be convenient for them to come to the clinic.

A reminder phone call was made by a member of the team the day before the scheduled appointment to finalise confirmation or to re-schedule the visit. In total, 1,077 of the 1,252 volunteers attended the baseline clinic. For the 175 who did not attend: the ET2DS team were unable to re-contact 56 individuals, 111 were unable or unwilling to attend for clinical examination when invited, five repeatedly failed to attend their research clinic appointment, and three had died

Further withdrawals were made from the study due to: an inability to complete the cognitive or physical examinations (n = 4), a failure to meet the criteria for being diagnosed as suffering from Type 2 diabetes (n = 7). Of this group two dietary-controlled patients with an HbA1c $< 6.5\%$ could not be confirmed as having diabetes, four subjects were believed to have Type 1 and not Type 2 diabetes, and one subject had a previous pancreatic neuroendocrine tumour. These

exclusions resulted in a final population of 1,066 subjects who were both willing and eligible to take part in the ET2DS. A flow chart detailing the recruitment process to the ET2DS is presented in Figure 6.1.

6.2.3 Sample size determination

A priori, the targeted sample size of 1,000 was large enough to be powered at 90% ($\alpha = 0.05$) to detect a Pearson correlation of 0.10 and above between the cognitive test scores and the independent variables. At the four year follow-up of the study, even if the sample size is reduced to 800, the ET2DS will still be powered to detect correlations of 0.12 or greater.

In multiple linear regression analyses with four covariates and an R^2 of 0.25, the study is powered at 90% ($\alpha = 0.05$) to detect a 0.01 increase in R^2 upon the addition of another covariate. The study will retain a similar power to detect a 0.01 increase in R^2 at follow-up assuming sample attrition of 20% i.e. n = 800. This implies that the study will be able to detect a variable that explains 1% or more of the variance in the cognitive test scores.

6.2.4 Ethical approval

Ethical permission was obtained from the Lothian Medical Research Ethics Committee.

6.2.5 Baseline examination

Prior to the baseline clinic, participants had been asked to observe an overnight fast. Upon arrival at the research facility, written informed consent was provided for study participation. The assessment began with the collection of a urine sample and a blood sample. Following a short break for breakfast, the participants underwent a physical examination and cognitive assessment.

The physical examination was carried out by one of six trained nurses and measurement technicians, using standard operating procedures. A specially designed form for data collection was used with great care being taken to ensure consistency both between and within the nurses assessments. Moreover, subjects were asked to submit the questionnaire that had been mailed to them along with their invitation to the clinic. This contained information on demographic characteristics, educational attainment, diabetes history and treatment, cardiovascular disease and other co-morbidities, medications, alcohol intake, smoking habits, em-

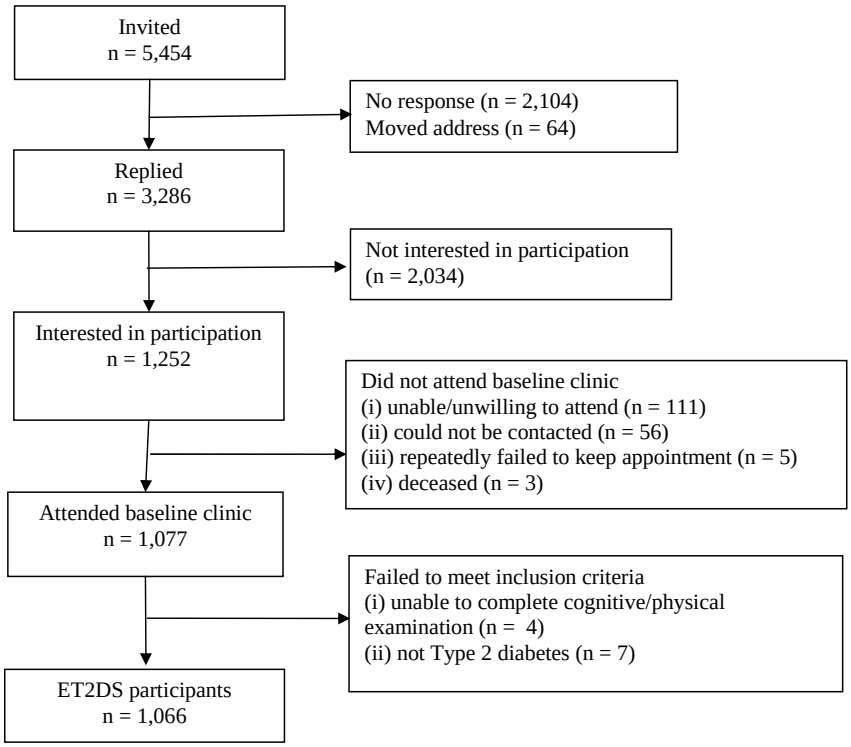

Figure 6.1: ET2DS recruitment process.

ployment/occupation, stress, satisfaction with life, subjective social status and personality.

6.2.6 Inflammatory and rheological biomarker measurements

At baseline, a venous blood sample was taken after an overnight fast for DNA extraction, and measurement of biomarkers. Assays to measure plasma CRP, fibrinogen, IL-6, and TNF-α were performed in the University Department of Medicine, Glasgow Royal Infirmary. In stored plasma anticoagulated with trisodium citrate, fibrinogen was assayed by the automated Clauss assay (MDA-180 coagulometer, Organon Teknika), and CRP using a high-sensitivity immunonephelometric assay (Tzoulaki et al. 2006). TNF-α and IL-6 antigen levels were determined by high-sensitivity ELISA kits from R&D Systems, Oxon UK.

6.2.7 Assessment of cardiovascular risk factors

Questionnaire

The baseline questionnaire completed by all participants asked for details about current and previous medical diagnoses and treatments for angina, myocardial infarction, stroke, and several other conditions. Both the year of diagnosis or event and the hospital or general practice attended were requested in the form. This enabled the study team to validate the data through comparison with Information Services Division (ISD) Scotland and LDR data.

Several criteria were used in combination to define a myocardial infarction. These included

- subject recall of a doctor's diagnosis of the event.
- a positive WHO chest pain questionnaire for MI.
- ECG evidence of ischaemia (Minnesota codes 1.1 to 1.3, 4.1 to 4.2, 5.1 to 5.3 or 7.1)
- a prior hospital discharge code for MI (ICD10 codes I21-I23, I252).

A positive recording for MI was made if either two of the first three criteria were met, or if the first and last criteria were met. The corresponding criteria for angina included

- subject recall of a doctor's diagnosis of the condition or of being on regular medication for angina
- a positive WHO chest pain questionnaire for angina
- ECG evidence of ischaemia
- a prior hospital discharge code for ischaemic heart disease (ICD10 codes I20-I25).

A positive recording for angina was made again, it two of the first three criteria were met, or if both the first and last criteria were met. A composite coronary heart disease (CHD) measure was defined for persons who were coded as either an MI or angina sufferer.

A history of stroke was assessed using data from the self-report questionnaire. The participants had to answer 'yes', 'no', or 'not sure' as to whether they had had a stroke. For the analysis, those in the 'not sure' category were treated as missing data. Where a positive answer was given, the date of the stroke and the hospital where the subject was treated were also asked for. Hospital records were then checked to confirm the stroke diagnosis by use of ICD codes. Of the 90 self-reported strokes, 36 were found to have no cerebrovascular code, the remainder had a code that met criteria for ICD-stroke or ICD-other cerebrovascular disease. These 36 persons were included in the analyses, which may have been a source of bias. However, excluding the stroke variable as a covariate made very little difference to the results reported.

A questionnaire of smoking history and the WHO Chest Pain (Rose et al. 1977) and Edinburgh Claudication Questionnaires (Leng & Fowkes 1992) were also completed at baseline. For the analysis in this thesis, smoking was treated as a categorical variable: never-smoked, current smoker, or ex-smoker.

Physical examination

The physical examination measured systolic and diastolic brachial blood pressures in the right arm (± 2 mmHg). Subjects were assessed in the supine position with their arm resting at the level of the mid-sternum, using a standard stethoscope and an aneroid, 6 inch dial, desk standing sphygmomanometer (Acceson^{TM}, AC Cossor & Son (Surgical) Ltd, Harlow, UK).

The individual components used to derive body mass index were assessed as follows: standing height was measured to the nearest mm, without shoes, using a wall-mounted vertical rule; Weight was assessed to the nearest 0.1 kg

without outdoor clothing or shoes using SECA 761 electronic weighing scales. BMI was calculated as the fraction of weight in kg to height^2 in m^2. The ankle brachial pressure index was calculated in an identical manner to the AAA Trial. Right and left brachial, posterior tibial and dorsalis pedis systolic pressures were recorded with the subject in the supine position and after at least 5 minutes rest, using the aneroid sphygmomanometer and a doppler probe (Dopplex® advanced pocket Doppler, Huntleigh Healthcare Ltd., Cardiff, UK). An ABPI measure was derived for each subject by dividing the lowest of the ankle pressures by the higher of the two arm pressures. To assess inter-observer variation in ABPI, a total of 20 subjects had repeat measurements performed independently in a single day by all six of the clinic staff.

6.3 Methods of cognitive function testing

6.3.1 Baseline cognitive testing

At baseline, a battery of psychometric tests providing a comprehensive and validated assessment of cognitive function and mood state was administered. Individual testers were trained and then observed for validation by one of the ET2DS investigators. A detailed discussion on the cognitive test battery that was used is provided below.

6.3.2 The cognitive test battery

In the ET2DS an omnibus of seven cognitive tests was completed by all willing participants: Logical Memory test, Faces and Families Recognition Subtest, Letter-Number Sequencing, Matrix Reasoning, Verbal Fluency Test, Digit Symbol Test, and Trail Making Test, Part B. A full description of the latter three tests is provided in Subsection 5.4.6 with details on the first four presented below. In addition to the battery of tests, measures of vocabulary (MHVS - see Subsection 5.4.6 for details), anxiety and depression, and general function (MMSE - see Subsection 5.4.6 for details) were also obtained.

Logical Memory

The Logical Memory test (LM) is a measure of immediate and delayed verbal declarative memory from the Wechsler Memory Scale (WMS-III^{UK}) (Wechsler 1998b). The test administrator reads two short stories, stopping after each one to allow for recall from the test participant. Both stories contain 25 elements with

the second story being administered twice. Participants are informed that they will be asked to recall details from the stories again later (a delay of around 25–35 minutes). In the ET2DS, only the first story of the Logical Memory Test was completed i.e. immediate and delayed recall was measured for Story A, which contained 25 elements. The two individual scores were summed to create an overall memory component, which had a scoring range 0 – 50.

Faces and Families Recognition Subtest

To test the participants' abilities in immediate and delayed non-verbal memory, the Faces and Families Recognition Subtest from the WMS-III^{UK} was administered (Wechsler 1998b). Participants are initially shown a sequence of 24 photographs of faces. Each photograph is presented for two seconds with the participant being told to try and remember all 24. Immediately after, a second batch of 48 photographs is shown to the participant. All 24 of the faces to be remembered were contained in the batch and the examinee has to tell the examiner whether or not they had seen each of the 48 faces previously. The maximum score for the immediate recall is 48 (range 0 – 48). Around 25 minutes later, after completing some other cognitive tests, the examiner shows the participant a new set of 48 faces. The aim is again to identify the 24 initial faces although this time some of the false faces from the immediate recall test are re-used in addition to some new faces. The scoring range is identical to the immediate memory recall and no time restriction is placed on the response to either of the tasks. In the ET2DS, scores from both tasks were combined to create an overall non-verbal memory component (FACES). The scoring range for this variable was 0 – 96.

Matrix Reasoning

To assess the participants' non-verbal reasoning skills, the Matrix Reasoning (MR) subtest from the WAIS-III^{UK} was administered (Wechsler 1998a). This test is similar to the Raven's Progressive Matrices used in the AAA Trial in that the participant must examine a pattern arrayed in a matrix with one missing piece. The elements of the matrix follow a particular pattern or rule. The task is to work out the rules and to apply them to find out what the missing piece should be. For each item (there are 26 in total) the subject must correctly select the missing piece from the six possible answer options (five possible pieces or 'don't know'). The range of scores possible is from 0 to 26. Three practice items are administered prior to the start of the test although these do not count towards the total score. Moreover, if a subject incorrectly answers either question 4 or

5 then the examiner takes them back to questions 1–3 (in reverse order) and re-tests them until they obtain two correct answers consecutively before returning to the question that had been incorrectly answered. The test terminates under three possible scenarios: four consecutive scores of zero, four scores of zero on five consecutive items, or completion of all 26 items.

Letter-Number Sequencing

Another subtest from the WAIS-III^{UK}, Letter-Number Sequencing (LNS) was used to assess working memory (Wechsler 1998a). Participants listen while the tester reads mixed strings of numbers and letters. The aim is for the participant to repeat the list to the tester, starting with the numbers in numerical order, and then the letters in alphabetical order. The test is split into seven sections of three items. Section one contains three sets of single letter, single number combinations. The sections become increasingly more complex with section seven containing eight pieces of information - four numbers and four letters. Participants score a point for each item within a section for which they give the correct response i.e. the maximum possible score is 21 (range 0 – 21). The test is stopped at the end of the seventh trial or when the participant fails to correctly answer any of the items within one of the seven sections.

Hospital Anxiety Depression Scale

The Hospital Anxiety Depression Scale (HADS) assesses recent mood states (Zigmond & Snaith 1983). It contains seven items for anxiety and seven for depression. The items are presented in alternate order beginning with an anxiety question. There are four possible responses for each ranging from very negative to very positive options. For example, the first anxiety item asks the participant to respond to the statement "I feel tense or wound up" by selecting one of:

- Most of the time,
- A lot of the time,
- From time to time, occasionally,
- Not at all.

Three points are allocated if the most negative option is selected, two for the second most negative, one for the third most, and zero for a positive response. The maximum possible score on each scale is 21, with probable anxiety or depression at scores of 11 or more. In the ET2DS analysis, only the depression scores were used (HADS-D).

6.4 Genotypic data

Baseline blood samples collected from ET2DS participants were used for genotyping. Sixteen candidate SNPs were selected from twelve different genes for genotyping. SNPs were again chosen on the basis of previously reported associations with cognitive ability or because they lay in genes strongly associated with phenotypic variants thought to be important in cognitive processes. A list of the SNPs analyzed in this thesis are presented in Table 6.1. Genomic DNA was isolated from whole blood by standard procedure at the Wellcome Trust Clinical Research Facility Genetics Core, Western General Hospital, Edinburgh. Genotyping was carried out by KBioscience (Herts, UK) using their in-house chemistry of Competitive Allele Specific PCR (KASPar).

Gene	SNP	Gene	SNP
CRP	rs1130864	*IL-1*	rs554344
	rs1205		rs580253
	rs1800947	*IL-6*	rs8192284
FGA	rs2070016	*TNF-α*	rs1800630
FGB	rs4220	*TCF-1*	rs2464196
	rs2227412		

Table 6.1: Genotyped SNPs from the ET2DS.

6.5 Data handling and management

6.5.1 Entry of cognitive data

Data from the baseline questionnaire and data collection forms were coded and entered into a Microsoft Access database. The results of plasma assays were entered onto the same master database, either from paper records (biochemistry and haematology) or from electronic files provided by the participating laboratories. The majority of data from paper records were double entered with discrepancies resolved by reference to the original paper documentation; for the remaining data, a random sample of records was double-checked. A small amount of baseline data that was found to be missing was collected at the one year clinic (primarily clarification of incomplete or inconsistent personal and clinical details) and entered onto the master database. Questionnaire data and plasma assays collected at the one year clinic visit were entered initially onto a separate Microsoft Access database and checked for inconsistencies prior to transfer onto the master database. All

data, including the master database, were stored securely on dedicated university computers and backed up on a dedicated university server.

6.5.2 General intelligence factor and Principal Components Analysis

As with the AAA Trial, given the high correlation between each of the seven cognitive tests in the battery, a general cognitive factor representing the variance common to all the cognitive tests was created via Principal Components Analysis.

The results of the PCA showed the first component to explain 44% of the total variation of the dataset. By examining the component eigenvalues it was clear that there was only a single component of interest. It is also apparent from Table 6.2 that all of the tests loaded strongly onto this component (range 0.454 – 0.794). The g score attributed to each individual was derived from the factor scores, and were calculated using the formula below. These scores were then standardised and added to the database.

$$g_i = (0.216 \times MR_i) + (0.230 \times LNS_i) + (0.217 \times VFT_i) + (0.245 \times DST_i) - (0.258 \times \ln(TMT_i)) + (0.147 \times FACES_i) + (0.172 \times LM_i)$$

Principal Component	Eigenvalue (λ)	% Variance explained	Cognitive Test	Factor Loading	Factor Score
1	3.08	44.0	MR	0.666	0.216
2	0.91	13.0	LNS	0.710	0.230
3	0.78	11.2	VFT	0.670	0.217
4	0.69	9.8	DST	0.755	0.245
5	0.65	9.3	-ln(TMT)	0.794	0.258
6	0.54	7.8	FACES	0.454	0.147
7	0.34	4.9	LM	0.529	0.172

Table 6.2: PCA output for the ET2DS.

6.5.3 Adjustment variables

The following variables, based on their associations with cardiovascular disease and/or cognitive outcomes, were used as covariates in the ET2DS:

- Age in years at the time of cognitive testing in 2006.

- Sex.
- Peak prior cognitive ability, which was estimated using the MHVS.
- Smoking status – measured as current smoker, ex-smoker, or never smoked. All of cigars, pipes, and cigarettes were considered as smoking. In addition, persons who were listed as non-smokers but who had given up in the past 6 months were recoded as current smokers.
- Diastolic blood pressure.
- Serum levels of total cholesterol.
- Glycated haemoglobin – HbA1c.
- ABPI.
- Coronary Heart Disease – defined as history of angina or MI.
- Stroke – defined using the self-report questionnaire data.
- Depression, which was measured using the HADS.
- Duration of diabetes, which was measured in years from the self-reported date of onset to the date of visit to the clinic.
- Daily alcohol consumption per normal drinking day in the past 12 months, which was categorised as: 0 drinks; 1 – 2 drinks; 3 – 4 drinks; 5 – 6 drinks; 7 – 9 drinks; or $\geq$ 10 drinks
- Self-reported highest educational attainment, which was categorised as: University/college degree course; Other professional/technical qualification after leaving school; Secondary school; or Primary school.
- BMI.

6.6 Summary

Chapter 6 has introduced the Edinburgh Type 2 Diabetes Study, its study design and recruitment policy. In total, 1066 persons were enrolled in the study. These persons were assessed at the study baseline for cognitive ability (using a seven test battery), pre-morbid cognitive ability (using the Mill Hill Vocabulary Scale), and four inflammatory biomarker measurements (CRP, fibrinogen, TNF-α, and

IL-6). Extensive demographic, physiological, and genetic information was also gathered at the baseline assessment.

Chapter 7

Replication cohorts: The Edinburgh Artery Study (EAS) and The 1936 Lothian Birth Cohort (LBC1936)

7.1 Introduction

In addition to the AAA Trial and the ET2DS, two other elderly Scottish cohorts were considered for the genetic analysis of *CRP* and fibrinogen SNPs with late-life cognitive ability. These studies were the Edinburgh Artery Study and the 1936 Lothian Birth Cohort. A brief description of both studies, including subject selection and cognitive test details are provided below. Many of the details described in this chapter can be attributed to the PhD thesis of Dr. Snorri Björn Rafnsson[1], and the 1936 Lothian Birth Cohort protocol paper (Deary et al. 2007), both of which provide an extensive guide to the respective studies.

7.2 The Edinburgh Artery Study

The principal objective of the Edinburgh Artery Study (EAS) was to examine the relationship between clinical cardiovascular disease, vascular risk factors, and cognitive function in older people.

[1]Cardiovascular diseases, risk factors and cognitive decline in the general population, PhD thesis, Dr. S.B. Rafnsson, Centre for Population Health Sciences, University of Edinburgh, 2006

7.2.1 Study population

The EAS population constituted residents of the City of Edinburgh who were between 55–74 years in age. Participants were randomly selected from age–sex registers of eleven Edinburgh general practices, which represented the whole spectrum of socioeconomic strata in all parts of the city. A priori sample size calculations indicated a target of 272 subjects to be randomly selected from each general practice, using four age-sex specific bands to stratify equal numbers into each group (34 males and females in each five year age band). General practitioners were then asked to review a list of 2,720 patient names, which had been obtained from the practices, to exclude persons they considered unable to participate in the study. For example, persons with advanced mental or physical illness, those who had changed general practice, or those who had died. In total, 353 (13%) subjects were excluded and replaced by other randomly selected participants.

All subjects eligible for the study were invited to attend a University of Edinburgh clinic for medical examination (n = 2,709). A media campaign was undertaken, after which, potential participants were sent a letter inviting them to take part in the study. Those who responded positively were sent by post an appointment date, a map of the University area, and detailed description of the clinical examination. Where letters of invitation were returned by the post office, those persons were replaced by alternative randomly selected subjects (n = 163). Non-repliers to the first invitation were sent a second invitation letter, and those who had confirmed participation but were unable to attend the examination were offered a new appointment at a subsequent date. Persons who were unable to attend the clinic at the University were given the option of a home visit. Full refunds were provided to all participants for any travel expenses incurred.

The enrolment stage of the EAS began in 1987 and ended in 1988, after a total of 809 men and 783 women were successfully invited to participate (total n = 1,592). Several follow-up visits were subsequently organised with the first wave of cognitive testing occurring in 1998/1999, by which point the available sample size was 1,209. The second wave of cognitive testing was administered between 2002/2003 when the available sample size had shrunk to 601 participants. Given the reduced numbers at follow-up, in addition to the lack of complete cognitive data, information was only used from the first wave of cognitive testing. Moreover, after accounting for non-participation, this left a sample of 534 persons with both cognitive scores and genotyping data for the analysis.

7.2.2 Sample size determination

Given that the SNP-cognition associations did not form part of the primary analysis for the EAS, post hoc calculations were conducted to identify the statistical power of the study. Using the sample size of 534 subjects, the EAS had 80% power to detect a standardised effect size of 0.12 ($\alpha = 0.05$) for the relationship between baseline biomarker levels and follow-up cognition.

7.2.3 Ethical approval

Ethical approval for the EAS was granted by the Lothian Health Board Ethics of Medical Research Sub-Committee for Medicine and Clinical Oncology. As with the AAA Trial and ET2DS, written informed consent was provided by each participant prior to the clinical examination.

7.2.4 Baseline examination

All eligible subjects were invited to a baseline examination which involved a detailed collection of sociodemographic information, medical history, and clinical data. Plasma CRP was measured at baseline in 384 of the 534 (71.9%) participants using a high-sensitivity assay in a BN ProSpec nephelometer (Dade Behring, Milton Keynes, UK). Of the other variables measured in the EAS, age and sex were included in the analysis as covariates, whilst the descriptive statistics for cholesterol, smoking status, and blood pressure were recorded to allow for population comparisons between the various cohorts.

7.2.5 The cognitive test battery

Eleven years after the baseline visit in 1987/1988 to the clinic, participants were asked to take part in a cognitive test examination. The battery of tests used included:

- Raven's Standard Progressive Matrices – RAVENS.
- Verbal Fluency Test – VFT.
- Digit Symbol Test – DST.
- Logical Memory – LM.

Whilst these tests have been described in detail previously, there was a slight variation with the assessment of Logical Memory. For the EAS two stories were read (instead of one, as in the ET2DS) with delayed but not immediate memory assessed. Information from the four test battery was aggregated for the derivation of a general intelligence g-factor. The methodology used for this was PCA, which has been described in detail previously. Briefly, the results yielded a single principal component of relevance (Table 7.1), the scores of which were standardised and saved as a g-factor. Each of the tests loaded strongly onto g and the individual scores were calculated using the formula below.

$$g_i = (0.364 \times \text{RAVENS}_i) + (0.318 \times \text{VFT}_i) + (0.377 \times \text{DST}_i) + (0.288 \times \text{LM}_i)$$

Principal Component	Eigenvalue (λ)	% Variance explained	Cognitive Test	Factor Loading	Factor Score
1	2.18	54.5	RAVENS	0.793	0.364
2	0.80	20.1	VFT	0.693	0.318
3	0.59	14.6	DST	0.822	0.377
4	0.43	10.8	LM	0.628	0.288

Table 7.1: PCA output for the EAS.

An estimate of pre-morbid cognitive ability was also assessed at the first wave of cognitive testing in the EAS using the National Adult Reading Test (NART).

7.2.6 Genotypic data

Blood samples from the baseline examination were used for the genetic analysis. Genotyping was carried out using a 5'–nuclease assay (TaqMan) by Helen Ireland in the laboratories of Professor Steve Humphries and Dr. Aroon Hingorani at University College London.

7.3 The 1936 Lothian Birth Cohort

A unique epidemiological study, the 1936 Lothian Birth Cohort (LBC1936) comprises survivors of the 1947 Scottish Mental Survey now living in Lothian, Scotland. The survey tested nearly all (70,085 of a possible 75,211 (93.2%)) Scottish school children born in 1936 when they were mostly age 11 (on June 4th 1947) using the Moray House Test, Number 12 – a validated test of general cognitive

function. Survivors from the initial survey who were living in the Lothian area were recruited for cognitive testing when they were aged around 70 years. Having measures of cognition at age 11 and again nearly 60 years later provides a rare and extremely exciting opportunity to assess change in cognitive ability over what is nearly a lifetime's duration.

7.3.1 Study Population

Subject Identification

To identify potential participants from the 1947 survey, the Lothian Health Board investigated Community Health Indices (CHI) on behalf of the LBC team. CHI numbers provide a unique identifier for persons living in a specific area who are registered with a general practice. Using this resource, the Lothian Health Board were requested to identify all persons on the Lothian CHI who were born in 1936. Unfortunately, CHI details may not be fully correct due to a time lag between updating the system after deaths and migration to or from a region. The number of subjects identified by the Health Board who may have taken part in the 1947 survey was 3,810.

Given that researchers are not allowed to make direct contact with people identified from their CHI data, initial contact was made via the Lothian Health Board on behalf of the LBC team. This constituted a letter from the Health Board, an invitation letter – written and signed by the LBC Study Director, Professor Ian Deary, a letter explaining the motivation behind the LBC study, and a reply slip with a pre-paid reply envelope addressed to the LBC study team at the University of Edinburgh. Persons were asked for their contact details in addition to their date of birth, country of schooling, and schools attended. Moreover, persons were given an option as to whether they were interested in receiving more details about the LBC study. Invitations were posted in batches of between 200–300 at a time to aid a swift response from the LBC team.

Subject Recruitment

Of the initial 3,810 persons identified from the CHI, 3,686 were contacted about potential study participation. This began in June 2004 and ran until November 2006. The loss of numbers can be explained by the flaws in using the CHI that were identified previously. Nonetheless, of this group, a response was obtained from 1,703 (46.2%) persons, 286 of whom requested no further contact regarding the study. An analysis was performed to try and identify reasons for

non-compliance: 46 were ineligible, 9 refused on medical grounds, 22 failed to provide sufficient information to determine their eligibility, and 66 failed to state whether they were willing to take part or not. All 66 persons in this final category ended up being excluded from the study for other reasons including: medical grounds (n = 5), schooled in another country (n = 59), and not born in 1936 (n = 2). This left 209 persons who were seemingly eligible but unwilling to take part in the study. After these exclusions a total of 1,132 persons were eligible to partake in the LBC1936 study.

The non-responders from the initial invitation were re-contacted in an attempt to increase participation and the response rate. However, given the confidential nature of the CHI, this meant that everyone in the sample had to be sent a second letter. After updates to the CHI, this resulted in letters being sent to 1,741 persons. A lower response rate (n = 615, 35.3%) was observed for this repeat procedure but a further 94 eligible and willing persons were identified.

In a final recruitment drive, a media campaign was launched to identify additional participants who had been missed from the CHI-based approach. In February 2007 an advert for volunteers was placed in a freely-distributed Edinburgh newspaper with a further advert being placed in an Edinburgh-based evening newspaper in March 2007. Response to the adverts was low with less than 100 replies received. The eligibility details of these replies have been incorporated into the figures presented above.

Study Population

Taking the positive respondents from the two mailing campaigns and the media promotion left 1,226 persons eligible and interested to take part in the study. Prior to being cognitively tested at the research clinic, 85 persons withdrew from the study. An inability to contact or arrange a clinical appointment before the end of testing in May 2007 resulted in a loss of a further 50 individuals. This left a 1936 Lothian Birth Cohort study population of 1,091 who had complete cognitive data and questionnaire data.

7.3.2 Sample size determination

The LBC1936 sample size of 1,091 provided 90% power ($\alpha = 0.05$) to detect a standardised effect size of 0.098 for the cross-sectional association between biomarker levels and cognition at age-70. Power calculations for the secondary, genetic analysis are discussed in the results section.

7.3.3 Ethical approval

Ethical permission for the LBC1936 was granted by the Multi-Centre Research Ethics Committee for Scotland and the Lothian Research Ethics Committee. All subjects were required to give written, informed consent prior to study participation.

7.3.4 Baseline examination

Cognitive and clinical testing along with a general health interview were conducted on all subjects at the Wellcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh. Testing was carried out by a trained psychologist and a research nurse. Upon arrival, the participants were introduced to the psychologist and reminded about the study and the measures that were going to be assessed. An opportunity to ask any questions was given before the completion of written, informed consent for study participation. The measurements assessed at the visit are summarised below in the chronological order in which they were administered. Depending on the availability of staff, the position of the physical examination varied in the sequence of events. All individuals were allowed a break of no less than 15 minutes for refreshments during their visit to the clinic.

Social and medical history and medications

The first interview given required the subjects to provide current contact details, educational history (school leaving age, further and higher education, and their highest qualification obtained), main occupation (and that of their spouse for women), and age at retirement. Additional questions ascertained each participant's smoking history and current alcohol consumption. A structured interview was then given to assess history of disease and a list of current medications.

Moray House Test No. 12

Initially administered when most of the cohort were aged 11 in 1947 (Scottish Council for Research in Education 1949), the Moray House Test Number 12 (MHT) was re-administered nearly 60 years later when the cohort were aged about 70. The same test was used on both occasions with the same instructions and time limits. Two small changes were made for items where the content was now archaic (Deary et al. 2000). Despite often being referenced as a measure of 'verbal reasoning' ability, the MHT also includes items of a variety of types: following

directions (14 items), same-opposites (11), word classification (10), analogies (8), practical items (6), reasoning (5), proverbs (4), arithmetic (4), spatial items (4), mixed sentences (3), cypher decoding (2), and other items (4). The maximum possible score on the MHT is 76 (range 0 – 76). The scores from the original 1947 MHT were obtained through the Scottish Council for Research in Education's office in Glasgow, to enable an accurate and comprehensive electronic index of all persons who completed the MHT.

Physical examination and interview

The LBC cohort was heavily phenotyped at the physical examination and interview stage. However, as only age and sex were adjusted for in the analyses, details of the characteristics measured were not discussed extensively. Nonetheless, to provide a general comparison between the LBC1936 and the other cohorts, some measures were presented. Briefly, sitting and standing systolic and diastolic blood pressure were assessed using an Omron 705IT monitor. Smoking status was determined from the interview with each subject being asked to state whether they were a current or ex-smoker, or if they had never smoked.

7.3.5 Inflammatory and rheological biomarker measurements

Blood samples were taken to allow for measurement of the acute-phase inflammatory proteins C-reactive protein and fibrinogen. CRP was assessed using a dry slide immuno-rate method on the OrthoFusion 5.1 F.S analysers. Fibrinogen was measured using an automated Clauss assay (TOPS coagulometer, Instrumentation Laboratory) (Hart et al. 2005).

7.3.6 The cognitive test battery

Whilst an extensive battery of cognitive tests were administered to the LBC1936 population, only tests in common with those from the AAA Trial and ET2DS were considered for the analysis in this thesis. Briefly, these included

- Matrix Reasoning – MR.
- Letter Number Sequencing – LNS.
- Verbal Fluency Test – VFT.
- Digit Symbol Test – DST.

- Logical Memory – LM

Measurement of LM differed from the ET2DS and the EAS with both stories being read and recalled immediately and also after a delay. A general intelligence, g-factor, was derived using the information from the five cognitive tests. As with the other studies, PCA indicated the presence of a single principal component (Table 7.2). Each of the individual tests loaded strongly onto the general factor (range 0.640 – 0.775), which accounted for 48.4% of the variance of the entire data. The g-scores were calculated using the formula below before being standardized and saved onto the database.

$$g_i = (0.290 \times MR_i) + (0.320 \times LNS_i) + (0.292 \times VFT_i) + (0.264 \times DST_i) + (0.267 \times LM_i)$$

Principal Component	Eigenvalue (λ)	% Variance explained	Cognitive Test	Factor Loading	Factor Score
1	2.42	48.4	MR	0.702	0.290
2	0.78	15.5	LNS	0.775	0.320
3	0.66	13.3	VFT	0.707	0.292
4	0.62	12.3	DST	0.640	0.264
5	0.52	10.5	LM	0.645	0.267

Table 7.2: PCA output for the LBC1936.

7.3.7 Genotypic data

Blood samples were taken for DNA extraction from white blood cells. Genomic DNA was isolated from whole blood by standard procedure at the Wellcome Trust Clinical Research Facility Genetics Core, Western General Hospital, Edinburgh. Genotyping was carried out by KBioscience (Herts, UK) using their in-house chemistry of Competitive Allele Specific PCR (KASPar).

7.4 Summary

This chapter provided a brief summary of the Edinburgh Artery Study and the 1936 Lothian Birth Cohort, which were used as replication cohorts for the inflammation-cognition genetic analyses. Both studies were set in central Scotland. The EAS contained 534 persons who were cognitively tested, using a four

test battery, at the study's eleven year follow-up in addition to having genotyping data available. Biomarker measures of plasma CRP were made at the baseline stage of the study. The 1936 Lothian Birth Cohort contained 1,091 persons from the Lothian region of Scotland who undertook cognitive testing at age-11 and again at age-70. Biomarker measurements, genotyping details, and other physiological variables were also assessed age-70.

Chapter 8

Regression analysis of biomarker levels and cognition.

8.1 AAA Trial Results

8.1.1 Introduction

This section begins by describing the general characteristics of the AAA Trial population. This includes a comparison between those in the subset who were cognitively tested at baseline and the remainder of the population. Bivariate correlations are then presented showing the raw, unadjusted associations between the biomarker levels and cognitive test scores. Multivariate linear regression is then used to assess in more detail the relationships between the late-life biomarker levels and cognition. The first set of models adjust for age and sex, before the addition of covariates to control for cardiovascular risk factors, estimated premorbid cognitive ability, and in a subset model, baseline cognitive ability. Finally, a section is devoted to the $APO\epsilon$ gene and its association with cognition and CRP.

8.1.2 Descriptive statistics and variable summaries

A summary of the predictor and adjustment variables are presented in Table 8.1 with the follow-up cognitive test scores summarised in Table 8.2. Categorical variables were described with regard to the frequency at each level of the variable. The distribution of continuous variables was explored using basic descriptive statistics, Q-Q Plots, and formal tests for normality. Where visually obvious outliers were present they were removed. Where dependent or independent variables were found not to follow the normal distribution (CRP and TMT - both of which were right-skewed), natural logarithm transformations were conducted. Conse-

quently, the median and inter-quartile range are reported for the raw values of these variables. Histograms and Q-Q Plots of the raw and transformed CRP values can be seen in Figure 8.1.

	AAA Trial population (maximum n = 3,350)	Cognitively tested population (maximum n = 2,312)	Cognitive change subset (maximum n = 504)
Age (years)	61.9 (6.70)	61.7 (6.55)	63.0 (6.85)
Male - n (%)	954 (28.5)	623 (26.9)	135 (26.8)
ABPI	0.86 (0.09)	0.86 (0.09)	0.87 (0.09)
Systolic BP (mmHg)	147.9 (21.59)	147.4 (21.44)	142.7 (19.41)
Diastolic BP (mmHg)	83.7 (10.74)	83.7 (10.71)	80.5 (9.76)
Cholesterol (mmol/l)	6.18 (1.09)	6.27 (1.08)	6.20 (1.16)
Smoking Status - n (%)			
Never Smoked	1087 (32.4)	733 (31.7)	153 (30.4)
Current Smoker	1101 (32.9)	776 (33.6)	183 (36.3)
Ex-smoker	1162 (34.7)	803 (34.7)	168 (33.3)
Fibrinogen (g/l)	3.29 (0.71)	3.27 (0.71)	3.36 (0.72)
CRP (mg/l)	2.00 (0.92–4.39)	1.85 (0.89–4.08)	1.98 (0.89–4.18)
Plasma Viscosity (mPa.s)	1.27 (0.07)	1.27 (0.07)	1.27 (0.07)
Haematocrit - % (s.d.)	42.3 (3.25)	42.2 (3.16)	42.6 (3.15)

Table 8.1: AAA Trial baseline descriptive statistics (mean (s.d.) or median (IQR) unless otherwise stated).

Test	n	Subset (n = 504)	n	Population (n = 2,312)
AVLT	389	68.5 (15.53)	2,182	63.0 (16.81)
RAVENS	387	38.0 (8.84)	2,101	34.7 (9.32)
VFT	403	41.3 (11.54)	2,228	37.4 (12.78)
DST	405	42.4 (11.34)	2,285	40.0 (11.67)
TMT	401	85 (70–115)	2,252	97 (76–129)
g	378	0.36 (0.92)	1,998	0.00 (1.00)
MHVS	501	32.7 (4.58)	2,191	31.1 (4.74)

Table 8.2: AAA Trial follow-up cognitive test scores (baseline MHVS scores).

Compared to the entire AAA Trial population, there was little difference in the demographics of the group of subjects who went on to be cognitively tested at follow-up. The mean age of the trial participants was just under 70 years with women accounting for almost three quarters of the population. The ABPI was low with a mean value of 0.86 (s.d. 0.09). The mean blood pressure (systolic/diastolic) was 148/84, and the mean cholesterol reading was ∼6.2mmol/l. The final adjustment variable listed in the table was smoking, which had an approximate equal distribution of persons across all states (current smoker, ex-smoker, and never smoked).

The descriptive statistics for the biomarkers all fall within a normal, healthy range. The median CRP was 2.00 mg/l which is within healthy bounds in terms of risk of developing cardiovascular disease. A similar interpretation can be made from the fibrinogen output where the mean value was elevated, but not substantially so, from a healthy reading. Haematocrit, which is the proportion of blood volume occupied by red blood cells, was raised in females with a mean value of 41.4% compared to a healthy reference estimate of 38% (Purves et al. 2004). By contrast, the mean haematocrit for men was 44.8%, which is well within the normal range for a healthy adult (Purves et al. 2004). Finally, the plasma viscosity readings had identical mean values of 1.27mPa.s across the three groups presented.

Comparing the cognitively tested subset and the cognitively tested population, there are few demographic differences between the two groups. The subset was a little over a year older on average and in slightly better health with lower blood pressure and cholesterol measurements. Their biomarker measurements were elevated for CRP and fibrinogen. Although not significantly different, the latter association may be due to a greater proportion of the subset being current smokers. Nonetheless, these descriptives reflect a seemingly healthy, ageing

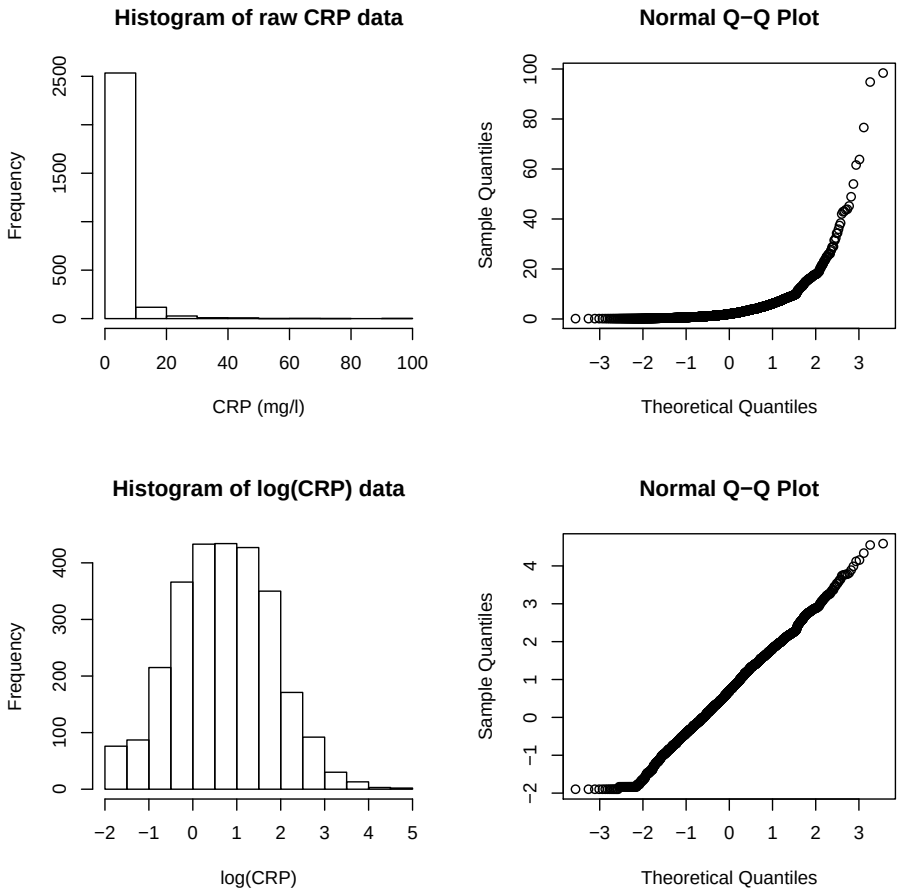

Figure 8.1: Histogram and Q-Q Plot of the raw and natural log-transformed CRP values.

cohort.

Contrasting the mean cognitive scores of the subset with the cognitively tested population showed substantial differences in both baseline MHVS and follow-up cognitive scores. The subset consistently and significantly outperformed the main population on all of the tests, scoring a mean of 0.36 standard deviations above the whole sample for general intelligence.

8.1.3 Correlations between biomarker levels and cognitive test scores

To assess the relationship between the cognitive test scores and biomarker levels, a series of bivariate Pearson correlations were calculated. In addition to the four markers, plasma viscosity was re-analyzed after adjusting for fibrinogen in order to explore the properties of its residual component. The only assumption that needs to be met when calculating Pearson correlations is that of a normal distribution for each of the variables. However, in addition to providing the statistical power to detect small correlations, the large sample size of the study dominates any problems regarding lack of normality of the variables.

The raw correlations between the cognitive test scores and biomarker levels (Table 8.3) were all quite small (maximum $r = 0.113$ for the plasma viscosity-TMT correlation, $p < 0.001$) and in the direction of increased marker levels associating with decreased cognitive test scores. Significant correlations were found between all of the markers and the majority of the cognitive tests. One exception was the VFT, which only correlated significantly with CRP ($p < 0.05$). Furthermore, CRP was the only biomarker to correlate significantly with MHVS scores.

After adjustment for age and sex, the correlations decreased in magnitude although most remained statistically significant (Table 8.4). Small but strongly significant correlations were found for both fibrinogen and CRP with RAVENS, DST, TMT, and g ($p < 0.01$). Plasma viscosity associated significantly with scores on the DST, TMT, and with g ($p < 0.01$). These associations became non-significant after adjustment for fibrinogen levels with the exception of the TMT correlation. CRP again correlated significantly with the baseline MHVS scores ($p < 0.001$). Inverse correlations for all tests except the TMT, in which high scores reflect lower cognitive ability, indicated that higher levels of the biomarkers were associated with poorer cognitive performance. No significant correlations were found between haematocrit levels and any of the cognitive tests.

The age and sex-adjusted correlations are equivalent to standardised beta weights from a linear regression and provide an indication of how much variation

in the cognitive test scores can be explained by the biomarkers. Explicitly, a correlation of 0.10 suggests that 1% of the variation in cognitive ability is being explained by the biomarkers.

Finally, there were no large correlations ($r > 0.30$) between the covariates. The solitary exception was the correlation between systolic and diastolic blood pressure ($r = 0.675$, $p < 0.001$). Subsequent modelling therefore adjusted for all of the covariates except systolic blood pressure, which was excluded in an attempt to control for problems of collinearity.

Cognitive Test	ln(CRP)	Fibrinogen	Plasma Viscosity	Haematocrit	Plasma Viscosity (adjusted)
AVLT	-0.026	-0.046*	-0.039‡	-0.079‡	-0.021
RAVENS	-0.098‡	-0.098‡	-0.064*	0.046*	-0.023
VFT	-0.056*	-0.031	-0.041	-0.010	-0.030
DST	-0.107‡	-0.103‡	-0.087‡	-0.073	-0.046*
ln(TMT)	0.099‡	0.111‡	0.113‡	0.068†	0.071†
g	-0.106‡	-0.099‡	-0.088‡	-0.053*	-0.049*
MHVS	-0.092‡	-0.030	-0.037	-0.033	-0.026
n-range	1,669 – 1,902	1,797 – 2,046	1,987 – 2,274	1,997 – 2,284	1,794 – 2,013

Table 8.3: Correlations between AAA Trial follow-up cognitive test scores (baseline MHVS) and baseline plasma biomarker levels. (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$)

Cognitive Test	ln(CRP)	Fibrinogen	Plasma Viscosity	Haematocrit	Plasma Viscosity (adjusted)
AVLT	-0.021	-0.039	-0.032	0.024	-0.008
RAVENS	-0.088‡	-0.060†	-0.035	0.019	-0.006
VFT	-0.054*	-0.025	-0.036	-0.006	-0.025
DST	-0.105‡	-0.082‡	-0.069†	-0.003	-0.036
ln(TMT)	0.097‡	0.091‡	0.098‡	0.029	0.068†
g	-0.100‡	-0.077†	-0.069†	0.006	-0.034
MHVS	-0.093‡	-0.034	-0.050	0.017	-0.019

Table 8.4: Age and sex-adjusted correlations between AAA Trial follow-up cognitive test scores (baseline MHVS) and baseline plasma biomarker levels. (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$)

8.1.4 Linear regression analysis of baseline marker levels and follow-up cognitive test scores

The associations identified by the correlations were analyzed further via a series of linear regressions that modelled the biomarker levels against follow-up cognition, actual five year cognitive change across individual tests, and estimated cognitive change. In each of the models, age and sex were entered as covariates with further adjustments being made when modelling five year change (baseline cognitive scores) and estimated change (MHVS). The use of prior scores as covariates is better than modelling with the actual change in cognitive ability, as this reduces the spurious correlation between the baseline and follow-up cognitive scores (Campbell & Kenny 1999). For the estimated cognitive decline models, the use of a robust and validated approximation of peak cognitive ability (MHVS) enabled the assessment of decline from best ever cognitive function (Deary et al. 2004). Analyses were repeated to account for the role of cardiovascular risk factors by adjusting all models for ABPI, diastolic blood pressure, cholesterol level, and smoking (measured in pack years). Plasma viscosity calculations were also repeated after adjusting for its main constituent, fibrinogen, to enable an investigation of its residual component. Finally, all biomarkers were trimmed for outliers, points beyond ± 3.5 standard deviations from the mean, prior to the analysis. Full results for all of the models described are shown in Tables 8.5, and 8.6 with the significant output discussed in the text.

CRP regression results

After age and sex-adjustment, raised plasma CRP levels were significantly associated with poorer functioning on:

- RAVENS (standardised β = -0.085, p $<$ 0.001),
- VFT (standardised β = -0.054, p $<$ 0.05),
- DST (standardised β = -0.095, p $<$ 0.001),
- TMT (standardised β = 0.093, p $<$ 0.001),
- g (standardised β = -0.095, p $<$ 0.001).

Additional adjustment for the MHVS weakened the associations slightly, with the VFT result becoming non-significant:

- RAVENS (standardised β = -0.045, p $<$ 0.05),

- DST (standardised β = -0.069, $p < 0.001$),
- TMT (standardised β = 0.063, $p < 0.01$),
- g (standardised β = -0.048, $p < 0.05$).

In the fully saturated models only two of the associations remained significant at the $p = 0.05$ threshold:

- DST (standardised β = -0.047, $p < 0.05$),
- TMT (standardised β = 0.045, $p < 0.05$).

Fibrinogen regression results

After age and sex-adjustment, raised plasma fibrinogen levels were significantly associated with poorer functioning on:

- AVLT (standardised β = -0.045, $p < 0.05$),
- RAVENS (standardised β = -0.066, $p < 0.01$),
- DST (standardised β = -0.083, $p < 0.001$),
- TMT (standardised β = 0.090, $p < 0.001$),
- g (standardised β = -0.081, $p < 0.01$).

Additional adjustment for the MHVS to attain an estimate of lifetime cognitive decline weakened the associations slightly, although all remained statistically significant apart from the AVLT:

- RAVENS (standardised β = -0.052, $p < 0.01$),
- DST (standardised β = -0.080, $p < 0.001$),
- TMT (standardised β = 0.082, $p < 0.001$),
- g (standardised β = -0.069, $p < 0.001$).

In the fully saturated models including the four cardiovascular risk factors, the associations were again weakened but still statistically significant. The only exception was the β weight for the RAVENS test, which showed a trend association:

- RAVENS (standardised β = -0.045, $p < 0.10$),

- DST (standardised β = -0.060, p < 0.01),
- TMT (standardised β = 0.067, p < 0.01),
- g (standardised β = -0.057, p < 0.01).

Plasma viscosity regression results (unadjusted)

Not surprisingly, the plasma viscosity regression results showed a similar pattern to the fibrinogen output. Upon age and sex-adjustment, elevated plasma viscosity associated significantly with decreased cognitive function on:

- DST (standardised β = -0.068, p < 0.001),
- TMT (standardised β = 0.089, p < 0.001),
- g (standardised β = -0.065, p < 0.01).

However, this marker did seem to be less sensitive to adjustment for MHVS scores with neither magnitude nor significance of the associations being overly affected:

- DST (standardised β = -0.061, p < 0.001),
- TMT (standardised β = 0.082, p < 0.001),
- g (standardised β = -0.063, p < 0.001).

Even upon full adjustment for the cardiovascular covariates, the weight of the standardised βs diminished only slightly:

- DST (standardised β = -0.049, p < 0.01),
- TMT (standardised β = 0.069, p < 0.001),
- g (standardised β = -0.048, p < 0.05).

Haematocrit regression results

The regression models for haematocrit yielded a solitary significant association with the cognitive scores. Consistent with the findings for the other biomarkers, this was with the TMT but only in the estimated lifetime change model after additional adjustment for MHVS.

- TMT (standardised β = 0.044, p < 0.05).

Plasma viscosity regression results (adjusted for fibrinogen)
The model estimating the residual, or non-fibrinogen component, of plasma viscosity yielded significant associations with only the TMT. Again, increased marker levels were associated with cognitive dysfunction.

- TMT (standardised $\beta = 0.066$, $p < 0.01$),

Interestingly, adjustment for MHVS scores resulted in an increase to the absolute weight for the general intelligence factor, which jumped from a non-significant -0.036 ($p = 0.15$) in the age and sex-adjusted model to -0.048. As with the unadjusted plasma viscosity model, the association with the TMT was significant.

- TMT (standardised $\beta = 0.069$, $p < 0.01$).
- g (standardised $\beta = -0.048$, $p < 0.05$).

In the fully saturated models, the TMT results still held whilst the associations with g weakened slightly showing a statistical trend:

- TMT (standardised $\beta = 0.062$, $p < 0.01$),
- g (standardised $\beta = -0.039$, $p < 0.10$).

Throughout the modelling process, strongly significant associations persisted between all of the markers and the TMT scores (the mental flexibility domain). Consistent associations also persisted for the DST and the general intelligence factor, g.

Baseline biomarker levels and actual 5-year cognitive change.

When cognitive decline was measured in the subset of study participants who undertook detailed cognitive testing at both baseline and follow-up (Table 8.6), several associations were found to be statistically significant. Raised plasma CRP showed weak associations with an *improvement* in performance on the AVLT (standardised $\beta = 0.078$, $p < 0.05$), and decreased performance on RAVENS (standardised $\beta = -0.066$, $p < 0.05$) and the VFT (standardised $\beta = -0.052$, $p < 0.10$). These associations were retained upon covariate adjustment in the saturated models. Plasma fibrinogen showed an inverse trend association with change in VFT scores (standardised $\beta = -0.053$, $p < 0.10$), whilst plasma viscosity and haematocrit showed some sign of increased levels being related to decline on the TMT (standardised $\beta = 0.079$, $p < 0.05$, and 0.071, $p < 0.10$, respectively). These associations remained as statistical trends in the fully adjusted models.

Removal of persons with acute inflammation

To discount the possibility of acute inflammation biasing the findings between CRP and cognition, the analyses were repeated after the exclusion of subjects with a CRP measurement greater than 10 mg/l. CRP values above this threshold are regarded as being indicative of acute inflammation (Ockene et al. 2001). However, the number of subjects with CRP levels > 10 mg/l was only 112 (4.8% of the original population of 2,312) and after exclusion of these subjects from the dataset, the results remained similar (Table 8.7). Nonetheless, in addition to a small decrease in the already small effect sizes, the previously significant association with VFT (at $p < 0.05$) did not persist.

	AVLT	RAVENS	VFT	DST	ln(TMT)	g
Inflammatory Marker	Standardised β (Standard Error)					
ln(CRP)						
+ Age and Sex	-0.020 (0.023)	-0.085 (0.023)‡	-0.054 (0.023)*	-0.095 (0.021)‡	0.093 (0.022)‡	-0.095 (0.023)‡
+ MHVS	0.016 (0.22)	-0.045 (0.020)*	-0.016 (0.021)	-0.069 (0.020)‡	0.063 (0.021)†	-0.048 (0.020)*
+ MHVS + covariates	0.021 (0.022)	-0.034 (0.020)	-0.012 (0.021)	-0.047 (0.020)*	0.045 (0.021)*	-0.032 (0.020)
Fibrinogen						
+ Age and Sex	-0.045 (0.022)*	-0.066 (0.023)†	-0.027 (0.023)	-0.083 (0.020)‡	0.090 (0.021)‡	-0.081 (0.023)†
+ MHVS	-0.035 (0.021)	-0.052 (0.020)†	-0.020 (0.020)	-0.080 (0.019)‡	0.082 (0.020)‡	-0.069 (0.019)‡
+ MHVS + covariates	-0.034 (0.021)	-0.045 (0.020)	-0.023 (0.021)	-0.060 (0.019)†	0.067 (0.020)†	-0.057 (0.019)†
Plasma Viscosity						
+ Age and Sex	-0.038 (0.021)	-0.028 (0.022)	-0.034 (0.022)	-0.068 (0.020)‡	0.089 (0.021)‡	-0.065 (0.022)†
+ MHVS	-0.032 (0.020)	-0.023 (0.019)	-0.031 (0.020)	-0.061 (0.018)‡	0.082 (0.019)‡	-0.063 (0.018)‡
+ MHVS + covariates	-0.024 (0.021)	-0.012 (0.019)	-0.026 (0.020)	-0.049 (0.019)†	0.069 (0.020)‡	-0.048 (0.019)*
Haematocrit						
+ Age and Sex	0.027 (0.023)	0.020 (0.024)	-0.007 (0.025)	0.003 (0.022)	0.031 (0.023)	0.006 (0.024)
+ MHVS	0.026 (0.022)	0.011 (0.021)	-0.022 (0.022)	-0.010 (0.021)	0.044 (0.022)*	-0.009 (0.020)
+ MHVS + covariates	0.044 (0.023)	0.031 (0.021)	-0.017 (0.023)	0.016 (0.021)	0.022 (0.022)	0.015 (0.021)
Plasma Viscosity (Fibrinogen adjusted)						
+ Age and Sex	-0.015 (0.024)	-0.001 (0.025)	-0.026 (0.026)	-0.042 (0.023)	0.066 (0.024)†	-0.036 (0.025)
+ MHVS	-0.019 (0.023)	-0.008 (0.022)	-0.036 (0.023)	-0.041 (0.021)	0.069 (0.022)†	-0.048 (0.021)*
+ MHVS + covariates	-0.013 (0.024)	-0.002 (0.022)	-0.029 (0.024)	-0.036 (0.022)	0.062 (0.023)†	-0.039 (0.022)

Table 8.5: AAA Trial regression of plasma biomarker levels against follow-up cognitive test scores (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$). Additional covariates: smoking (in pack years), diastolic BP, ABPI, and cholesterol.

	AVLT	RAVENS	VFT	DST	ln(TMT)	g
Inflammatory Marker	Standardised β (Standard Error)					
ln(CRP)						
+ Age and Sex	0.078 (0.036)*	-0.066 (0.031)*	-0.052 (0.031)	-0.046 (0.027)	0.033 (0.037)	-0.017 (0.025)
+ covariates	0.091 (0.036)*	-0.074 (0.032)*	-0.055 (0.032)	-0.045 (0.028)	0.029 (0.038)	-0.017 (0.025)
Fibrinogen						
+ Age and Sex	0.006 (0.035)	-0.059 (0.030)	-0.053 (0.030)	-0.028 (0.027)	0.034 (0.035)	-0.040 (0.024)
+ covariates	0.012 (0.036)	-0.062 (0.031)	-0.058 (0.030)	-0.026 (0.027)	0.026 (0.035)	-0.038 (0.024)
Plasma Viscosity						
+ Age and Sex	-0.049 (0.033)	0.003 (0.030)	-0.004 (0.030)	-0.045 (0.026)	0.079 (0.034)*	-0.034 (0.023)
+ covariates	-0.042 (0.035)	0.001 (0.031)	-0.012 (0.031)	-0.035 (0.027)	0.063 (0.036)	-0.026 (0.024)
Haematocrit						
+ Age and Sex	0.009 (0.039)	0.030 (0.034)	0.018 (0.034)	0.015 (0.030)	0.071 (0.030)	-0.004 (0.026)
+ covariates	0.033 (0.041)	0.022 (0.036)	0.015 (0.036)	0.033 (0.032)	0.048 (0.042)	0.008 (0.028)
Plasma Viscosity (Fibrinogen adjusted)						
+ Age and Sex	-0.054 (0.037)	0.017 (0.033)	0.023 (0.033)	-0.039 (0.028)	0.063 (0.038)	-0.018 (0.026)
+ covariates	-0.051 (0.038)	0.019 (0.034)	0.014 (0.034)	-0.031 (0.029)	0.053 (0.039)	-0.012 (0.026)

Table 8.6: AAA Trial regression of plasma biomarker levels against five year cognitive change (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$). Additional covariates: smoking (in pack years), diastolic BP, ABPI, and cholesterol.

	AVLT	RAVENS	VFT	DST	ln(TMT)	g
Inflammatory Marker	Standardised β (Standard Error)					
ln(CRP)						
+ Age and Sex	-0.029 (0.023)	-0.066 (0.023)†	-0.041 (0.024)	-0.083 (0.022)‡	0.064 (0.023)†	-0.083 (0.024)‡
+ MHVS	0.002 (0.22)	-0.030 (0.020)	-0.014 (0.021)	-0.062 (0.020)†	0.038 (0.021)	-0.044 (0.020)*
+ MHVS + covariates	0.010 (0.022)	-0.017 (0.020)	-0.009 (0.022)	-0.039 (0.020)	0.020 (0.022)	-0.027 (0.020)

Table 8.7: AAA Trial regression of plasma CRP levels (< 10mg/l) against follow-up cognitive test scores (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$). Additional covariates: smoking (in pack years), diastolic BP, ABPI, and cholesterol.

8.1.5 Regression assumptions

There are several assumptions that need to be satisfied in order to carry out a linear regression analysis. These include:

- a linear relationship between dependent and independent variables,
- independence of the errors (no serial correlation),
- homoscedasticity (constant variance) of the errors,
- normality of the error distribution.

If any of the above assumptions are violated then the predictions and confidence intervals generated from the regression model may be inefficient or biased. These four assumptions were checked and satisfied for all models presented. A detailed description of the output generated is given for the age and sex-adjusted model of CRP against DST.

8.1.6 Regression diagnostics.

Test for linearity
In linear regression with a single independent variable, a scatter plot of the dependent versus independent variable provides an indication of the linearity of the relationship. When regression is extended to models with more than one independent variable, partial residual plots, which account for the impact of the competing independent variables, may be plotted for each variable. These partial residual plots take the form

$$(\text{Residuals} + \beta_i \text{x}_i) \text{ versus } \text{x}_i$$

where

- Residuals = residuals from the full model,
- β_i = regression coefficient from the i^{th} independent variable in the full model,
- x_i = the i^{th} independent variable.

Figure 8.2 shows the plots from the CRP-DST model. Given the extremely large number of points in the model, it was very difficult to observe any trends, linear

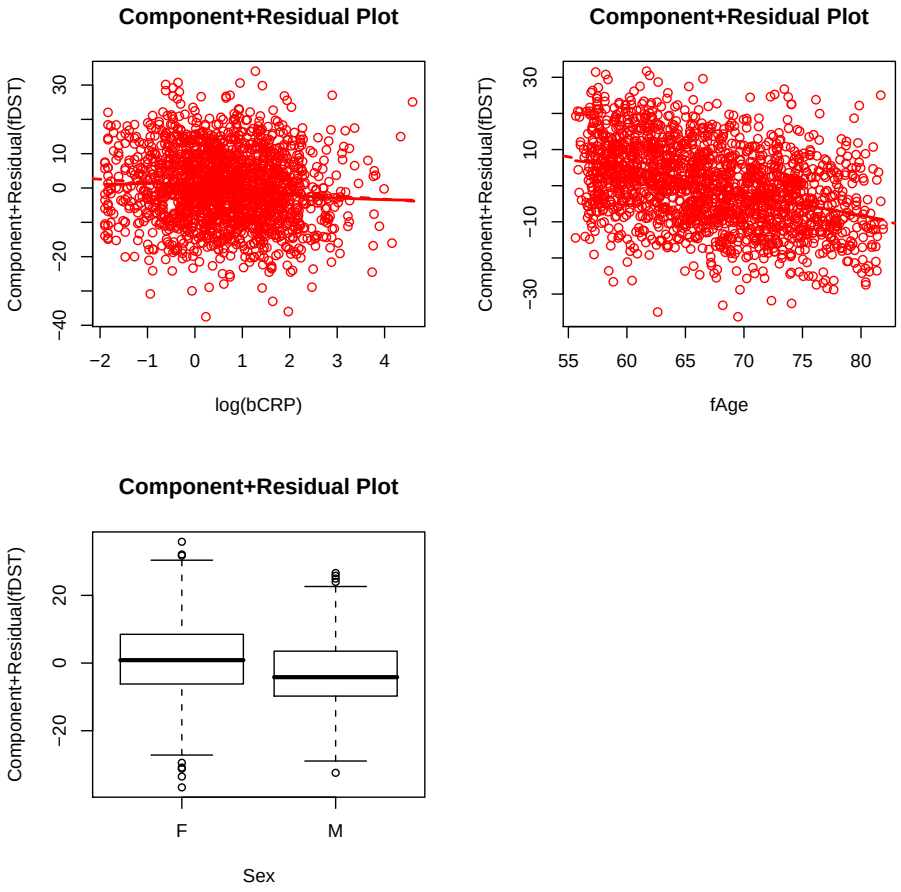

Figure 8.2: Partial residual plots for the CRP-DST model.

or otherwise. Nonetheless, it does not seem unreasonable that the assumption was satisfied for the model.

Test for independence of the errors
To test for autocorrelation amongst the residuals a Durbin-Watson statistic can be calculated. As this statistic is meaningful when the data form a time-series, there was no need to calculate it for any of the models presented in this thesis.

Test for homoscedasticity
Linear regression makes an assumption of constant variance for the error terms in the model. A plot of the studentized residuals against the fitted values of the model indicates any change in variance by predicted values. For example, a funnel shaped plot shows that the model provides less accurate estimates as the values of the dependent variable increase. Other errors possible from this plot include a bow-shaped distribution that would suggest the addition of a quadratic term into the model. However, as can be seen from Figure 8.3, there was no evidence to suggest non-constant variance in the CRP-DST model.

Test for normality
The final of the four assumptions is that the model residuals follow a normal distribution. Violations of normality compromise the estimation of coefficients and the calculation of confidence intervals. To test this assumption it is standard practice to graph a Q-Q Plot of the residuals. Formal tests of normality may also be conducted using, for example, the Shapiro-Wilk test. In addition to identifying outlying data points from the Q-Q Plot, Cook's distance may also be plotted to show points with large influence on the regression analysis. It can often be informative to drop such points before repeating the analysis to see how this impacts upon the coefficients and confidence intervals generated. Figure 8.4 shows the Q-Q Plot of the residuals for the CRP-DST model. As the residuals followed the 45° line quite closely it was reasonable to assume that they were approximately normally distributed.

8.2 *APOϵ* analyses

As described in Subsection 4.6.4, *APOϵ* is one of the few genetic markers to be consistently associated with cognitive ability (Bertram & Tanzi 2008). Specifically, carriers of the $\epsilon 4$ allele (as opposed to $\epsilon 2$ or $\epsilon 3$ carriers) are found to be at greater risk of developing cognitive decline and dementia. The $\epsilon 4$ allele is also

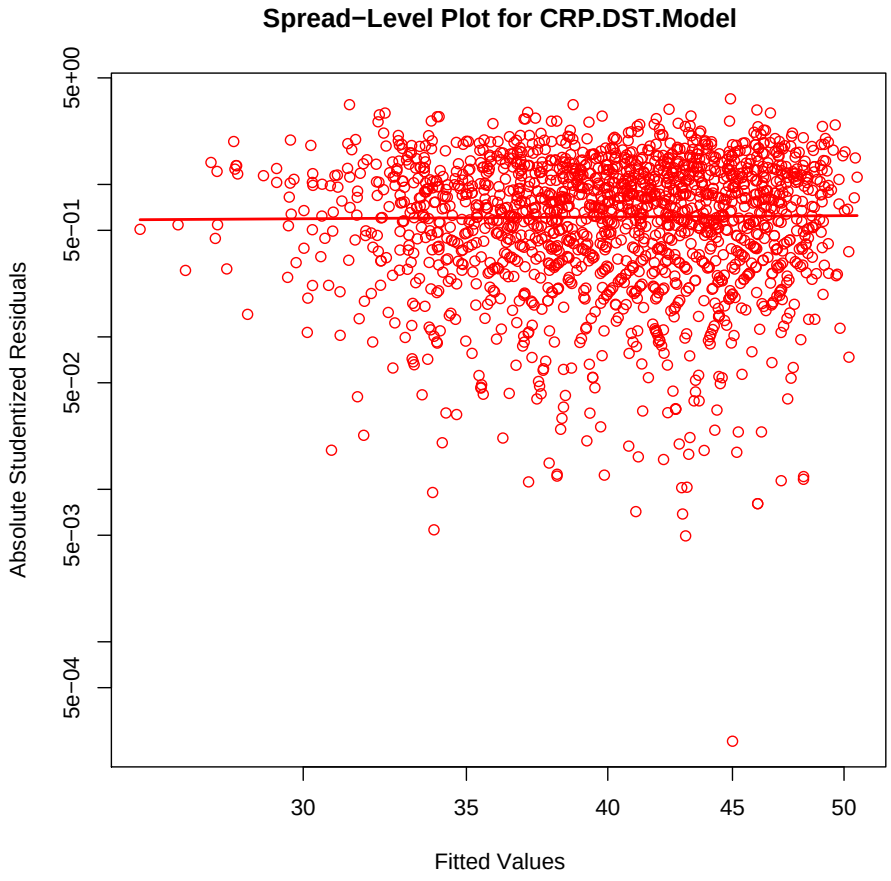

Figure 8.3: Plot for non-constant error variance for the CRP-DST model.

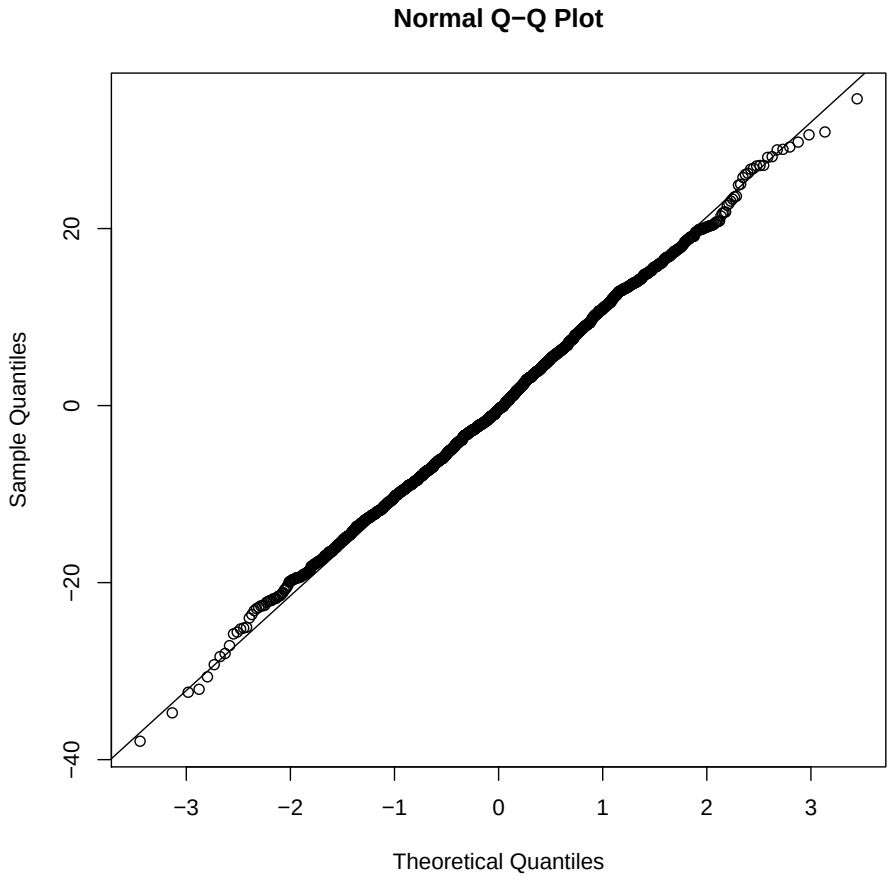

Figure 8.4: Normal Q-Q Plot for the residuals from the CRP-DST model.

associated with lower values of circulating CRP (März et al. 2004). Using the data from the AAA Trial, a series of analyses were conducted to answer three questions:

- Is *APOϵ* allele status associated with cognitive ability?
- Do plasma CRP levels vary by *APOϵ* status?
- Are *APOϵ4* stratified CRP levels associated with cognitive ability?

***APOϵ* and cognitive ability**

From the study population of 2,091 the distribution of *APOϵ* alleles is presented in Table 8.8. The overall proportions of $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ carriers were 0.097, 0.766, and 0.137, which is in accordance with population-level figures reported in the literature (Rebeck et al. 1993). Whilst the majority of persons had at least one copy of the $\epsilon 3$ allele, 30 were found to have two copies of the high risk $\epsilon 4$ allele with 47 having two copies of the low risk $\epsilon 2$ allele. The number of persons with data missing for *APOϵ* status was 396.

	$\epsilon 2$	$\epsilon 3$	$\epsilon 4$
$\epsilon 2$	47	196	39
$\epsilon 3$		1,019	364
$\epsilon 4$			30

Table 8.8: Distribution of *APOϵ* alleles in the AAA Trial population.

To test for differences between *APOϵ* and cognition, age and sex-adjusted ANCOVA was used to compare the mean cognitive scores of the $\epsilon 4$ carriers versus non-$\epsilon 4$ carriers. The ANCOVA models were built using the 'lm' function in R. The results from these analyses showed non-significant associations between $\epsilon 4$ status and each of the individual cognitive tests (p-range 0.09 – 0.34), g ($p = 0.08$), and the MHVS ($p = 0.73$). Nonetheless, the age and sex-adjusted means were all in the direction of poorer cognitive performance for *APOϵ4* carriers.

Despite the lack of an *APOϵ*-cognition association, age and sex-adjusted ANCOVA was used to try and identify the high risk and low risk genotypes for decreased cognitive ability. These analyses again showed non-significant results for *APOϵ* status (Appendix A). Examining the *APOϵ* genotypes, it was found that the $\epsilon 4\epsilon 4$ carriers had poorer cognitive scores relative to all other genotypes, and in particular with the protective $\epsilon 2\epsilon 2$ genotype. There were also consistent trends for impaired performance with carriers of the $\epsilon 2\epsilon 4$ and $\epsilon 3\epsilon 4$ genotypes. Nonetheless, the relationship between the VFT scores and *APOϵ* actually pointed towards

better cognitive performance for those in the $\epsilon 4\epsilon 4$ group. The overall ability of the models to explain variation in cognition tended to range between 8.9% and 16.2% although the model for VFT only explained 0.09% of the variance. This may help explain the unusual findings between this test and the $\epsilon 4$ allele. Overall, the main explanatory power lay with the age and sex covariates; the $APO\epsilon$ gene explained a maximum of 0.6% of the variance (for the TMT model).

The assumptions for ANCOVA surround the normal distribution of the response variable within each group and equal variances between the groups. Boxplots of g by $APO\epsilon$ genotype were used to check for equality of variance between groups. This was formally assessed using Bartlett's test, which yielded a K^2 value of 5.79 on 5 degrees of freedom, p = 0.33. The assumption of normality was assessed for all groups by visually inspecting Q-Q Plots and histograms for deviation from the normal distribution.

Plasma CRP levels by *APOϵ*

To test for differences between mean CRP levels by $APO\epsilon$ status, an age and sex-adjusted ANCOVA model was used. The fit of the model was tested using the methods described previously. Using the $\epsilon 2\epsilon 2$ group as the reference category, significantly lower CRP levels were found for those in the $\epsilon 3\epsilon 4$ and $\epsilon 4\epsilon 4$ groups.

```
lm(formula = log(bCRP) ~ Age + Sex + APOE, data = AAA)


Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.702440   0.338317   2.076   0.0380 *
Age          0.002198   0.004466   0.492   0.6227
SexM        -0.017980   0.064327  -0.280   0.7799
APOEe2e3    -0.030113   0.191653  -0.157   0.8752
APOEe2e4    -0.324447   0.248730  -1.304   0.1923
APOEe3e3    -0.169827   0.176586  -0.962   0.3364
APOEe3e4    -0.432215   0.183494  -2.355   0.0186 *
APOEe4e4    -0.703580   0.281490  -2.499   0.0125 *
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1
```

These analyses were repeated to investigate the relationship between $APO\epsilon$ and fibrinogen levels, plasma viscosity, and haematocrit but with null findings (overall ANCOVA p = 0.14, 0.81, 0.97, respectively).

***APOϵ* stratified CRP by cognition**

To mirror the analysis performed by Schram et al., which showed no evidence for an $APO\epsilon$:CRP interaction with cognition (Schram et al. 2007), a series of linear regression analyses were undertaken. An $APO\epsilon$ term ($\epsilon 4$ versus non-$\epsilon 4$) and an $APO\epsilon$–CRP interaction term were added to the age and sex-adjusted models described previously. For each model, both the interaction term and the $APO\epsilon$ term were found to be non-significant (p-value range for the interactions 0.15–0.80).

8.3 ET2DS Results

8.3.1 Introduction

This section provides a summary of the ET2DS data and the methods that were used to analyse the associations between the inflammatory biomarker levels and cognitive ability. Descriptive statistics are used to summarise each of the independent biomarker variables, dependent cognitive test variables, and adjustment variables. Correlations are then presented between the biomarkers and the cognitive test scores. This is followed by the main analysis, which used linear regression methods to investigate the contemporaneous associations between inflammation and cognition, and estimated cognitive decline.

8.3.2 Descriptive statistics and variable summaries

Tables 8.9 and 8.10 give the baseline characteristics of the ET2DS population. The mean age of the population was 67.9 years (s.d. 4.20), and approximately half of the sample (n = 547, 51.3%) was male. The mean blood pressure and cholesterol measurements were low, which is most likely a consequence of the medication being taken to control for cardiovascular risk factors. The mean BMI of the sample was 31.4, which places the majority of participants as being overweight or obese. Finally, whilst the mean duration of Type 2 diabetes was 8 years (s.d. 6.46), the range of reported values was large, varying between 6 months and 43 years.

All cognitive and inflammatory biomarker data were approximately normally distributed with the exception of the TMT scores, CRP, IL-6, and TNF-α levels, which were positively skewed and therefore, transformed using natural logarithms. A general inflammation factor (Inflam) was derived from the four inflammatory biomarkers by means of an unrotated principal components analysis. All four markers loaded strongly onto the first principal component (0.439-0.805), which

Test	n	mean (s.d.)
MR	1,052	12.8 (5.28)
LNS	1,048	9.7 (2.75)
VFT	1,060	36.9 (12.83)
DST	1,057	49.2 (14.77)
TMT	1,052	104 (81–139)
FACES	1,059	65.8 (7.88)
LM	1,050	25.2 (8.17)
g	1,021	0.00 (1.00)
MHVS	1,049	30.9 (5.23)

Table 8.9: ET2DS cognitive test scores.

accounted for 49.7% of the total variance (Table 8.11). The four markers and the inflammatory factor were all trimmed for outliers, points exceeding 3.5 standard deviations from the mean, prior to the correlation and regression analyses.

8.3.3 Correlations between biomarker levels and cognitive test scores

To investigate the relationship between the cognitive test scores and the biomarker levels a series of bivariate Pearson correlations were calculated. As with the AAA Trial, the correlations between the covariates were minimal with none exceeding $\pm$ 0.30 except between systolic and diastolic blood pressure ($r = 0.39$, $p < 0.001$). Further analyses of the covariate associations with the inflammatory marker levels and the general inflammatory factor found age, sex, ABPI, CHD, duration of diabetes, HADS-D, stroke, and BMI to be consistently significant predictors. In combination, the covariates explained 4.4% (adjusted R^2) of the variation in ln(TNF-α) levels, between 10.2% and 13.7% of the other inflammatory markers, and 17.6% of the general inflammatory factor. The predictive power of the covariates increased when assessing the cognitive test scores, explaining between 6.6% and 16.1% of the variation in the individual tests and 22.5% of the variation in g.

The raw associations between the biomarkers and cognition are presented in Table 8.12. The magnitude of the CRP and fibrinogen correlations were fairly small, never exceeding $\pm$ 0.1. Significant associations were noted between both markers and MR ($p < 0.01$), and between fibrinogen and TMT ($p < 0.01$), and g ($p < 0.05$)

Compared to the downstream markers, the correlations for the upstream

ET2DS Population (n = 1066)			
Variable	Mean (s.d.)	Variable	Mean (s.d.)
Age (years)	67.9 (4.20)	Daily alcohol - n (%)	
Male - n (%)	547 (51.3)	0 drinks	309 (29.0)
ABPI	0.98 (0.21)	1-2 drinks	471 (44.1)
Systolic BP (mmHg)	133.3 (16.44)	3-4 drinks	178 (16.7)
Diastolic BP (mmHg)	69.1 (9.01)	5-6 drinks	56 (5.3)
Cholesterol (mmol/L)	4.31 (0.90)	7-9 drinks	21 (2.0.)
HbA1c (%)	7.40 (1.12)	10 + drinks	7 (0.7)
BMI (kg/m^2)	31.4 (5.69)	Education - n (%)	
Smoker - n (%)		Primary School	171 (16.0)
Current	153 (14.4)	Secondary School	307 (28.8)
Ex-smoker	499 (46.8)	Professional Qualification	581 (54.5)
Never smoked	414 (38.8)	University/College	7 (0.7)
Duration of diabetes	8.1 (6.46)	Fibrinogen (g/l)	3.65 (0.74)
CHD (Yes) - n (%)	330 (31.0)	CRP (mg/l)	1.86 (0.87–4.37)
Stroke (Yes) – n (%)	90 (8.4)	IL-6 (pg/ml)	2.87 (1.97–4.46)
HADS-D score	3.9 (2.89)	TNF-α (pg/ml)	1.07 (0.69–1.62)

Table 8.10: ET2DS descriptive statistics (mean (s.d.) or median (IQR) unless otherwise stated).

Principal Component	Eigenvalue (λ)	% Variance explained	Biomarker	Factor Loading	Factor Score
1	1.97	49.3	ln(CRP)	0.803	0.407
2	0.99	24.6	Fibrinogen	0.755	0.383
3	0.60	15.0	ln(IL-6)	0.753	0.382
4	0.45	11.1	ln(TNF-α)	0.434	0.220

Table 8.11: PCA output for the ET2DS general inflammation factor.

markers and the general inflammation factor were around twice the size. Significant results were found for all three markers on all of the cognitive tests excluding LM, DST (TNF-α), and VFT (Inflam). All of the markers apart from fibrinogen correlated significantly with the MHVS estimate of pre-morbid cognitive ability ($p < 0.05$ or $p < 0.01$). The inverse nature of the associations again support the hypothesis that raised biomarker levels relate to decreased cognitive ability. After adjustment for age and sex, the correlations became smaller but retained statistical significance in almost all cases (Table 8.13).

Cognitive Test	ln(CRP)	Fibrinogen	ln(IL-6)	ln(TNF-α)	Inflam.
MR	-0.076†	-0.087†	-0.133‡	-0.134‡	-0.146‡
LNS	-0.012	-0.029	-0.092†	-0.074*	-0.068*
VFT	-0.020	-0.011	-0.086†	-0.067*	-0.060
DST	-0.009	-0.024	-0.164‡	-0.042	-0.079*
ln(TMT)	0.035	0.093†	0.187‡	0.107†	0.142‡
FACES	0.021	-0.017	-0.091†	-0.083†	-0.048
LM	0.049	-0.009	-0.032	-0.043	-0.006
g	-0.035	-0.075*	-0.182‡	-0.125‡	-0.137‡
MHVS	-0.081*	-0.044	-0.077*	-0.088†	-0.095†
n-range	998 – 1,037	1,018 – 1,057	1,019 – 1,058	1,019 – 1,058	994 – 1,033

Table 8.12: Correlations between ET2DS cognitive test scores and plasma biomarker levels. (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$)

Cognitive Test	ln(CRP)	Fibrinogen	ln(IL-6)	ln(TNF-α)	Inflam.
MR	-0.053	-0.052	-0.118‡	-0.114‡	-0.115‡
LNS	-0.008	-0.011	-0.078*	-0.057	-0.051
VFT	-0.018	-0.002	-0.079*	-0.060	-0.052
DST	-0.049	-0.042	-0.155‡	-0.032	-0.097†
ln(TMT)	0.050	0.089	0.174‡	0.092†	0.139‡
FACES	0.019	-0.040	-0.084†	-0.080*	-0.069*
LM	0.028	-0.021	-0.025	-0.039	-0.015
g	-0.051	-0.071*	-0.168‡	-0.109‡	-0.134‡
MHVS	-0.064*	-0.029	-0.077*	-0.086†	-0.082†

Table 8.13: Age and sex-adjusted correlations between ET2DS cognitive test scores and plasma biomarker levels. (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$)

8.3.4 Linear regression analysis of cross-sectional biomarker–cognition associations

Analogous to the AAA Trial analysis, linear regression was used to assess the association between the markers and age and sex-adjusted cognitive test scores. Further adjustments were made for MHVS scores to enable an estimate of lifetime cognitive change, and for additional covariates to control for depressed mood; total plasma cholesterol; BMI; ABPI; diastolic brachial blood pressure; smoking; self-reported duration of diabetes; coronary heart disease – myocardial infarction or angina; typical daily alcohol consumption per drinking day in the last year; highest level of self-reported education; stroke; and glycated haemoglobin (HbA1c).

Age and sex-adjusted regression results of the biomarker levels against the cognitive test scores are shown in Table 8.14. The results for the downstream markers fibrinogen and CRP tended to be weakly significant or non-significant with the exception of the fibrinogen-TMT association (standardised $\beta = 0.088$, $p < 0.01$). By contrast, the standardised βs for the upstream markers, IL-6 and TNF-α, were of a greater magnitude (significantly greater for IL-6 with DST and g, and for both IL-6 and TNF-α with TMT ($p < 0.05$)). The standardised βs for the upstream markers were also significantly different from zero for the majority of the tests.

IL-6

- MR (standardised β -0.113, $p < 0.001$)
- LNS (standardised β -0.075, $p < 0.05$)
- VFT (standardised β -0.077, $p < 0.05$)
- DST (standardised β -0.152, $p < 0.001$)
- TMT (standardised β 0.173, $p < 0.001$)
- FACES (standardised β -0.084, $p < 0.01$)
- g (standardised β -0.161, $p < 0.001$)

TNF-α

- MR (standardised β -0.120, $p < 0.001$)
- TMT (standardised β 0.091, $p < 0.01$)

- FACES (standardised β -0.074, $p < 0.05$)
- g (standardised β -0.106, $p < 0.001$)

A similar pattern to the upstream markers was found for the overall marker of inflammation with five of the eight βs being significant at the $p = 0.05$ threshold.

General Inflammation Factor

- MR (standardised β -0.122, $p < 0.001$)
- DST (standardised β -0.095, $p < 0.01$)
- TMT (standardised β 0.140, $p < 0.001$)
- FACES (standardised β -0.075, $p < 0.05$)
- g (standardised β -0.133, $p < 0.001$)

All inflammatory measures, with the exception of fibrinogen, associated significantly with the MHVS, which was again used as an indicator of peak prior cognitive ability (standardised β range -0.065 to -0.085, $p < 0.01$).

Results of the regression models investigating marker levels and estimated lifetime change in cognition are also shown in Table 8.14. After age, sex, and MHVS-adjustment, only the fibrinogen-TMT association remained for the downstream markers. However, this association persisted after further covariate adjustment in the saturated model (standardised $\beta = 0.067$, $p < 0.05$).

For the upstream markers, adjustment for MHVS scores weakened both the magnitude and statistical significance of the associations. Nonetheless, the strongest associations from the age and sex-adjusted models were retained after accounting for both MHVS and the other covariates, particularly when using IL-6 as the marker of inflammation.

IL-6

- MR (standardised β -0.077, $p < 0.05$),
- DST (standardised β -0.083, $p < 0.01$),
- TMT (standardised β 0.117, $p < 0.001$),
- FACES (standardised β -0.056, $p < 0.10$),
- g (standardised β -0.087, $p < 0.001$).

TNF-α

- MR (standardised β -0.088, $p < 0.01$),
- TMT (standardised β 0.049, $p < 0.10$),
- g (standardised β -0.052, $p < 0.05$ – MHVS-adjusted model only).

General Inflammation Factor

The general inflammatory factor retained significant associations after adjustment for MHVS (DST and g: $p < 0.05$) and also in the fully adjusted models with:

- MR (standardised β -0.066, $p < 0.05$),
- TMT (standardised β 0.093, $p < 0.01$),
- g (standardised β -0.049, $p < 0.10$).

	MR	LNS	VFT	DST	ln(TMT)	FACES	LM	g
Inflammatory Marker	Standardised β (Standard Error)							
ln(CRP)								
+ Age and Sex	-0.063 (0.031)*	-0.008 (0.032)	-0.020 (0.032)	-0.047 (0.031)	0.052 (0.031)	-0.020 (0.031)	0.027 (0.032)	-0.053 (0.031)
+ MHVS	-0.036 (0.028)	0.017 (0.029)	0.008 (0.029)	-0.023 (0.028)	0.035 (0.029)	-0.002 (0.030)	0.050 (0.029)	-0.014 (0.025)
+ MHVS + covariates	<0.001 (0.031)	0.036 (0.032)	0.018 (0.031)	0.015 (0.031)	0.022 (0.031)	-0.008 (0.033)	0.050 (0.033)	0.011 (0.027)
Fibrinogen								
+ Age and Sex	-0.053 (0.031)	-0.008 (0.031)	-0.004 (0.032)	-0.033 (0.031)	0.088 (0.031)†	-0.031 (0.031)	-0.005 (0.032)	-0.064 (0.031)*
+ MHVS	-0.051 (0.028)	-0.003 (0.029)	<0.001 (0.028)	-0.028 (0.028)	0.084 (0.028)†	-0.027 (0.029)	0.001 (0.029)	-0.050 (0.025)*
+ MHVS + covariates	-0.038 (0.030)	0.006 (0.031)	0.006 (0.030)	0.025 (0.030)	0.067 (0.030)*	-0.020 (0.032)	0.003 (0.032)	-0.023 (0.027)
ln(IL-6)								
+ Age and Sex	-0.113 (0.030)‡	-0.075 (0.031)*	-0.077 (0.031)*	-0.152 (0.030)‡	0.173 (0.030)‡	-0.084 (0.030)‡	-0.018 (0.031)	-0.161 (0.030)‡
+ MHVS	-0.084 (0.027)†	-0.043 (0.028)	-0.045 (0.028)	-0.130 (0.027)‡	0.147 (0.028)‡	-0.063 (0.029)*	0.009 (0.029)	-0.115 (0.024)‡
+ MHVS + covariates	-0.077 (0.030)*	-0.022 (0.032)	-0.029 (0.031)	-0.083 (0.031)†	0.117 (0.030)‡	-0.056 (0.033)	0.003 (0.032)	-0.087 (0.027)†
ln(TNF-α)								
+ Age and Sex	-0.120 (0.030)‡	-0.055 (0.031)	-0.057 (0.031)	-0.029 (0.030)	0.091 (0.030)†	-0.074 (0.030)*	-0.041 (0.031)	-0.106 (0.031)‡
+ MHVS	-0.087 (0.027)†	-0.017 (0.028)	-0.013 (0.028)	0.006 (0.028)	0.064 (0.028)*	-0.051 (0.029)	-0.010 (0.029)	-0.052 (0.025)*
+ MHVS + covariates	-0.088 (0.029)†	-0.009 (0.030)	-0.017 (0.029)	0.034 (0.029)	0.049 (0.029)	-0.032 (0.031)	-0.021 (0.031)	-0.038 (0.026)
General Inflammation Factor								
+ Age and Sex	-0.122 (0.031)‡	-0.051 (0.031)	-0.048 (0.032)	-0.095 (0.031)‡	0.140 (0.031)‡	-0.075 (0.031)*	-0.015 (0.032)	-0.133 (0.031)‡
+ MHVS	-0.089 (0.028)†	-0.016 (0.029)	-0.011 (0.029)	-0.065 (0.028)*	0.114 (0.029)‡	-0.052 (0.030)	0.015 (0.029)	-0.079 (0.025)†
+ MHVS + covariates	-0.066 (0.031)*	0.004 (0.033)	0.001 (0.032)	-0.008 (0.032)	0.093 (0.032)†	-0.055 (0.034)	0.008 (0.034)	-0.049 (0.028)

Table 8.14: Regression of ET2DS plasma biomarker levels against cognitive test scores (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$). Additional covariates: smoking (current, ex, or non smoker), diastolic BP, cholesterol, HbA1c, ABPI, CHD, stroke, HADS-D (depression), duration of diabetes, daily alcohol consumption per normal drinking day in the last year, self-reported highest educational attainment, and BMI.

8.4 Summary

This chapter presented a summary of the AAA Trial and ET2DS variables. Correlations were used to provide raw estimates of the associations between the biomarkers and cognition. The main analyses for both studies modelled the biomarker levels against late-life cognitive ability, late-life cognitive change, and estimated lifetime cognitive change. Small but significant associations were found in both studies: between CRP, fibrinogen, plasma viscosity and measures of processing speed, mental flexibility, and g in the AAA Trial; and between IL-6, TNF-α, a general inflammation factor and processing speed, and g in the ET2DS. These prospective and cross-sectional associations tended to persist after adjustments were made for potential confounding variables and also for the vocabulary-based estimate of peak cognitive ability.

Chapter 9

Genetic association analysis and causal inference.

9.1 Introduction

One of the ultimate aims of epidemiological research is to uncover causal pathways of disease and to identify interventions that will delay or even ameliorate the progression of disease. In the situation where disease mechanisms are understood, randomised controlled trials are among the most powerful ways to test treatment effects. However, the working mechanisms of many diseases are unclear and until such knowledge is available, treatments and cures are likely to remain elusive. When analysing data from prospective cohort studies, one of the most difficult things to control for is whether an identified association between phenotype/exposure and outcome is spurious and actually the result of confounding.

In recent times, the advancement of genome wide association studies has led to large sections of the genome being typed at relatively low cost. With a price reduction also occurring for candidate gene analyses, a genetic epidemiology approach is becoming increasingly common in the quest to identify causal associations.

To apply this approach to the inflammation, rheology and cognition data requires the identification of genetic variants (SNPs) that affect synthesis of the inflammatory/rheological markers. The relationship between these SNPs and cognitive outcomes can then be tested. Given that genotypic information is determined at conception, it cannot be subjected to the influence of confounding variables. Therefore, if significant associations can be shown between genotype, phenotype, and outcome then this would provide evidence for a causal role of inflammation/rheology in cognition. Unfortunately, these types of analyses are unable to identify the mechanisms through which the associations are involved.

However, determining the evidence for causal or non-causal relationships will better inform the design of future studies and help unravel the complex processes that drive cognitive decline.

One approach that relates a phenotype variable to an outcome variable via a genetic predictor of the phenotype is Mendelian randomisation (Davey Smith & Ebrahim 2003). This method may be thought of as a 'natural' randomised controlled trial that assigns subjects to groups based on their genetic profile. Explicitly, Mendelian randomisation models the phenotype variable against genetic predictors before considering its association with the outcome variable. As potential confounder variables are not likely to be associated with the genetic variants, they should be distributed equally across the genotypes.

The primary aim of this chapter is to use genetic information to assess the evidence for a causal association between elevated levels of CRP and fibrinogen and decreased cognitive ability.

9.2 Classical Two-Stage Least-Squares (2SLS) Regression

The classical statistical methodology that runs in parallel with Mendelian randomisation is two-stage least-squares regression (2SLS). In order to run a successful 2SLS regression the genetic variants must be strong predictors of the intermediate variable. The strength of the genetic 'instrument' is measured using the F statistic from the regression of SNP on marker level (Lawlor et al. 2008). If the F statistic exceeds 10 then the SNP is said to be a strong instrument and good predictor of the plasma biomarker level. The second stage of the regression then models the SNP-stratified biomarker levels against the cognitive outcome variable. Using the SNP and biomarker data for CRP and fibrinogen, a classical Mendelian randomisation analysis was conducted on the AAA Trial data.

9.2.1 Fibrinogen SNPs

In addition to measuring plasma levels of fibrinogen, eight fibrinogen SNPs were genotyped in the AAA Trial. Five of the SNPs were located in the fibrinogen alpha (*FGA*) gene with the other three in the fibrinogen beta (*FGB*) gene. A summary of each of the SNPs is printed in Table 9.1. To compare the representativeness of the AAA Trial sample with the general population, the minor allele frequency (MAF) for the CEU (European) HapMap population are also printed in this table.

A quick comparison suggests little difference between the genotype distributions of the two groups.

Gene	SNP	n	Major allele	Minor allele	MAF	MAF CEU	HWE p-value
FGA	rs2070022	2,038	C	T	0.20	0.14	0.17
	rs6050	2,014	A	G	0.25	0.24	0.59
	rs2070016	2,044	T	C	0.15	0.16	0.93
	rs2070018	2,042	T	C	0.13	0.13	0.43
	rs2070011	2,030	G	A	0.38	0.37	0.89
FGB	rs4220	2,029	G	A	0.17	0.19	0.94
	rs1800788	2,024	C	T	0.19	0.23	0.57
	rs2227412	2,030	A	G	0.19	0.12	0.56

Table 9.1: AAA Trial fibrinogen SNP descriptives

Prior to conducting the 2SLS regression, the SNPs were checked for Hardy-Weinberg Equilibrium and for pairwise LD. Hardy-Weinberg Equilibrium was tested for using the HWE.exact command in the genetics library of R. This test is equivalent to a Chi-square test of independence with the null hypothesis implying random mating within the population. Deviation from the null may be caused by violations such as inbreeding, assortative mating (mating between persons with similar phenotypes), a small population sample (genetic drift), or by population structure and genotyping error. The HWE p-value for each of the fibrinogen SNPs is presented in Table 9.1 with none being below the 0.05 significance threshold.

To test for LD between the fibrinogen SNPs, the LD function in the genetics library of R was used. This tests for pairwise LD between SNPs using three distinct measures:

D: The raw difference in frequency between the observed number of 'AB' pairs and the expected number.

$$\mathrm{D} = \mathrm{p(AB)} - (\mathrm{p(A)} \times \mathrm{p(B)})$$

D': A scaled version of D that spans the range [-1, 1].

$$\mathrm{D'} = \frac{\mathrm{D}}{\mathrm{D_{max}}}$$

where, if D > 0:

$$D_{max} = \min\ (p(A)p(b),\ p(a)p(B))$$

or if D < 0:

$$D_{max} = \max(-p(A)p(B),\ -p(a)p(b))$$

r: The correlation coefficient between the markers

$$r = \frac{-D}{\sqrt{(p(A) \times p(a) \times p(B) \times p(b))}}$$

where

- p(A) is defined as the observed probability of allele 'A' for marker 1,
- p(a) = 1 – p(A) is defined as the observed probability of allele 'a' for marker 1,
- p(B) is defined as the observed probability of allele 'B' for marker 2,
- p(b) = 1 – p(B) is defined as the observed probability of allele 'b' for marker 2,
- and p(AB) is defined as the probability of the marker allele pair 'AB'.

Two measures (D' and r) were monitored to assess both the independence of the SNPs and also their predictive power of one another. It is useful to consider both measures when determining LD status, but particularly the r value. For example, two SNPs where one has a very small MAF may have a high D' but a very low r. In general, two SNPs are said to be in high LD if the squared correlation (r^2) exceeds 0.80. For the eight fibrinogen SNPs, rs2070022 and rs2227412 were in high LD (D' = 0.94, r = 0.89, r^2 = 0.80). Of the other SNPs, most showed a strong dependence relationships (D' typically greater than 0.90) but fairly low correlations.

9.2.2 *CRP* SNPs

In the AAA Trial, four SNPs were typed from the *CRP* gene: rs1205, rs1800947, rs1130864, and rs1417938. The SNP descriptives again showed little deviation from the CEU population in terms of MAFs (Table 9.2). Moreover, there was no departure from Hardy-Weinberg Equilibrium with a minimum p-value of 0.11. The LD mapping found strong dependence relations between all four of the SNPs with rs1417938 and rs1130864 also sharing a high r^2 of 0.992.

Gene	SNP	n	Major allele	Minor allele	MAF	MAF CEU	HWE p-value
CRP	rs1205	2,025	C	T	0.33	0.34	0.11
	rs1800947	2,026	G	C	0.06	0.07	0.20
	rs1130864	2,011	C	T	0.30	0.30	0.59
	rs1417938	2,022	T	A	0.30	0.33	0.83

Table 9.2: AAA Trial *CRP* SNP descriptives

9.2.3 Strong 'instruments'

To identify strong instruments for the 2SLS analysis, age and sex-adjusted ANCOVA was used to model the SNPs against the biomarker levels. The F statistics from the analyses are presented in Table 9.3 and show that none of the fibrinogen SNPs satisfy the assumption for the 2SLS model. One of the *CRP* SNPs, rs1205, had a large F statistic of 12.46, which enabled its usage for further calculations.

Gene	SNP	F-statistic	p-value
FGA	rs2070022	3.80	0.022
	rs6050	4.61	0.010
	rs2070016	9.87	$4.2e^{-05}$
	rs2070018	0.004	0.99
	rs2070011	4.02	0.018
FGB	rs4220	9.42	$8.4e^{-05}$
	rs1800788	1.90	0.15
	rs2227412	1.91	0.15
CRP	rs1205	12.46	$4.2e^{-06}$
	rs1800947	9.19	$1.1e^{-04}$
	rs1130864	7.83	$4.1e^{-04}$
	rs1417938	7.29	$7.0e^{-04}$

Table 9.3: Strong 'instruments' in the AAA Trial

9.2.4 Ordinary 2SLS analysis in the AAA Trial

To conduct the main 2SLS regression analysis, the tsls command from the sem library in R was used. The code and results for the 2SLS model with rs1205 as the instrument and g as the dependent variable is presented verbatim in the output below. The outcome of importance is the regression coefficient and p-value for log(bCRP). In this instance the data yielded a non-significant p-value of 0.62. The p-values for CRP from the models examining the individual cognitive test scores were also found to be non-significant: AVLT (p = 0.32); RAVENS (p = 0.66); VFT (p = 0.63); DST (p = 0.26); and ln(TMT) (p = 0.51). Adjustment for age and sex made no difference to the lack of significant findings. A positive β weight for the log(bCRP) term also implies that after stratification for rs1205, age, and sex, that increased CRP is associated with increased cognitive performance.

```
> library(sem)
> summary(tsls(follow.g~log(bCRP), ~rs1205, data=data))

 2SLS Estimates

Model Formula: follow.g ~ log(bCRP)

Instruments: ~rs1205

Residuals:
     Min.   1st Qu.    Median      Mean   3rd Qu.      Max.
-3.15e+00 -6.70e-01  6.57e-02  1.61e-17  7.33e-01  3.24e+00

            Estimate Std. Error t value Pr(>|t|)
(Intercept) -0.06841     0.1282 -0.5337   0.5936
log(bCRP)    0.09936     0.1981  0.5015   0.6161

Residual standard error: 1.0174 on 1499 degrees of freedom
```

9.2.5 Ordinary 2SLS analysis in the ET2DS

Taking an identical approach to the AAA Trial analysis, a classical 2SLS was run on the ET2DS data for both fibrinogen and CRP. In this study, three fibrinogen and three *CRP* SNPs were typed. Their descriptives are presented in Table 9.4. Comparison with the HapMap output shows similar minor allele frequencies. The

HWE and LD structure of the SNPs were also analysed. Once more, the SNPs were all found to be in HWE and despite highly dependent associations being found, there was no evidence to suggest any pairwise LD.

Gene	SNP	n	Major allele	Minor allele	MAF	MAF CEU	HWE p-value
FGA	rs2070016	1,008	T	C	0.15	0.16	0.90
FGB	rs4220	1,007	G	A	0.17	0.19	0.91
	rs2227412	1,028	A	G	0.17	0.12	0.58
CRP	rs1205	1,012	C	T	0.33	0.34	0.26
	rs1800947	1,017	G	C	0.05	0.07	0.18
	rs1130864	1,016	C	T	0.30	0.30	0.16

Table 9.4: ET2DS fibrinogen and *CRP* SNP descriptives

Modelling the SNPs against their respective biomarker levels showed no evidence for strong instruments. The largest F-statistics for fibrinogen and *CRP* were 3.47 (rs4220) and 8.48 (rs1800947), respectively. Given these findings it was deemed inappropriate to continue to the main 2SLS analysis for the ET2DS data.

9.2.6 Discussion on classical 2SLS

The Mendelian randomisation approach to test for causal associations between the biomarkers CRP and fibrinogen and cognitive ability gave null findings. These are consistent with findings for CRP with several other outcomes such as insulin resistance, Type 2 diabetes (Brunner et al. 2008), metabolic syndrome (Timpson et al. 2005), carotid intima-media thickness (Kivimäki et al. 2008), and coronary heart disease (Davey Smith et al. 2004).

In addition, classical modelling of Mendelian randomisation problems is limited by several factors. These include its inability to account for measurement errors and intra-individual variation in the phenotype. Moreover, the nature of classical testing requires caution and great care when interpreting output. Whereas a Bayesian approach will give the strength of evidence for or against a hypothesis, the classical approach is designed towards a dichotomous outcome i.e. rejecting or failing to reject the null hypothesis. In the situation where there is insufficient power to detect a significant association – which is likely to be the case for the AAA Trial and ET2DS analyses – a Bayesian approach will output a Bayes Factor close to 1 implying a lack of evidence to make a decision either way. By contrast, the classical approach will always allow a conclusion to be drawn with the usual cut-off p-value for rejecting H_0 being set to 0.05.

9.3 Introduction to Bayesian Statistics

In classical statistical methodology, parameters are treated as fixed variables. By contrast, a Bayesian approach models these parameters as random variables with probability distributions. To illustrate this point, Crawford and Garthwaite present a simple example (Crawford & Garthwaite 2007):

"suppose m is the (unknown) mean IQ in a specified population. Then a Bayesian might say 'the probability that m is less than 90 is .95' while classical statistics does not permit a probabilistic statement about m. With the classical approach, m is either less than 90 or it is not, and there is no random uncertainty, so the probability that m is less than 90 is either 1 or 0 and nothing in between."

To explain Bayesian methods mathematically, from probability theory if we let

- θ = unobservable population parameters of interest
- y = the observed sample data drawn from the population
- p(.|.) = conditional probability distribution
- p(.) = marginal distribution

then

$$p(y) = \int p(y \mid \theta)p(\theta)d\theta$$

gives the marginal density of the data. By extension using Bayes Theorem we can write

$$\begin{aligned} p(\theta \mid y) &= \frac{p(y|\theta)p(\theta)}{p(y)} \\ &= \frac{p(y|\theta)p(\theta)}{\int p(y|\theta)p(\theta)d\theta} \\ &\propto p(y \mid \theta)p(\theta) \end{aligned}$$

which provides the posterior density of θ given the observed data y.

A Bayesian approach requires the modeller to specify a set of priors, p(θ), before conducting an analysis. A prior is a probability statement surrounding prior

knowledge of a parameter before any data have been collected. Prior distributions may be selected from results of past experiments, or from expert knowledge. This prior information is then combined with the likelihood to generate a posterior distribution for the random variable.

9.4 Bayesian hypothesis testing

Assume that the hypothesis to be tested is

$$\mathrm{H}_0 : \theta \in \Theta_0 \text{ versus } \mathrm{H}_A : \theta \in \Theta_A$$

and that the prior probabilities on each of the hypotheses are

$$\pi_0 = \Pr(\mathrm{H}_0) = \Pr(\theta \in \Theta_0)$$

$$\pi_A = \Pr(\mathrm{H}_A) = \Pr(\theta \in \Theta_A)$$

Taking π_A/π_0 gives the prior odds on H_A against H_0. After collecting experimental data, a Bayesian will update the prior odds to the posterior odds,

$$\frac{\pi_0 \int_{\Theta_A} p(y \mid \theta_A) p(\theta_A) d\theta_A}{\pi_A \int_{\Theta_0} p(y \mid \theta_0) p(\theta_0) d\theta_0}$$

before comparing the two models using a Bayes Factor (BF),

$$\mathrm{BF} = \frac{\Pr(\mathrm{H}_A \mid y)}{\Pr(\mathrm{H}_0 \mid y)} = \frac{\int_{\Theta_A} p(y \mid \theta_A) p(\theta_A) d\theta_A}{\int_{\Theta_0} p(y \mid \theta_0) p(\theta_0) d\theta_0}.$$

To interpret Bayes Factors, a value > 1 provides support for H_A as opposed to H_0 and vice-versa. Computationally, it is often easier to work with $\log_{10}$ BF. The weight of evidence presented by Bayes Factors is nicely summarised by Jeffreys (Jeffreys 1961)(Table 9.5). Unfortunately, the choice of prior distribution in combination with the likelihood function often results in non-trivial joint posterior distributions that require numerical techniques such as Markov-Chain Monte Carlo to solve.

9.5 Introduction to Markov-Chain Monte Carlo

Markov chain Monte Carlo (MCMC) is a computational tool that is used to generate samples from a probability distribution. After a large number of steps,

Bayes Factor	Evidence against H_0
1 to 3.2	Not worth more than a bare mention
3.2 to 10	Substantial
10 to 100	Strong
> 100	Decisive

Table 9.5: Strength of evidence for Bayes Factors.

and when the chain approaches convergence, it can be used as a sample for the sought after distribution. Most chains require a burn-in phase with the quality of the sample improving as the number of steps increases.

9.5.1 Markov Chain

A (First-order) Markov chain is a stochastic process, which possesses the Markov property that its current value, X_{n+1} is conditional on value X_n but not the values of X_0, , X_{n-1}. Formally, this can be presented as

$$\Pr(X_{n+1} = x \mid X_n = x_n, \ldots, X_1 = x_1) = \Pr(X_{n+1} = x \mid X_n = x_n).$$

9.5.2 Monte Carlo

The Monte Carlo part of MCMC refers to the computational algorithm that is used to aggregate the results of the individual Markov chain output. By taking random draws from the Markov chain-derived distribution they can generate a distribution for the random variable of interest. Classic Monte Carlo techniques used in Bayesian statistics include the Metropolis Hastings algorithm and Gibbs sampling.

9.6 Bayesian 2SLS analysis in the AAA Trial

The Bayesian model used for the analysis was based on a model created by Professor Paul McKeigue. In the initial application McKeigue et al. tested the association of Uric acid with metabolic syndrome through the Uric acid transporter SNP rs1312967[1]. The differences between that model and the inflammation-cognition model defined here include a continuous instead of dichotomous outcome, and the use of multiple SNPs as instrument predictors.

[1]http://homepages.ed.ac.uk/pmckeigu/mendelrand/index.html

9.6.1 Model parameterization

Firstly, consider the model of CRP levels (x_i) against *CRP* SNPs (g_i):

$$x_i = \alpha_0 + \alpha_g g_i + \epsilon_i$$

Where i runs from 1 to 2,091, and $\epsilon_i \sim \mathrm{N}(0,\ \sigma_i^2)$. Now let the model of the outcome variable, follow-up g scores (y_i) against the SNP stratified CRP levels be written as:

$$y_i = \beta_0 + \beta_x x_i + \beta_\epsilon \epsilon_i$$

To speed up the mixing of the MCMC sampler and to provide more interpretable output, the second model was re-parameterized as:

$$y_i = \beta_0 + \beta_x(\alpha_0 + \alpha_g g_i + \epsilon_i) + \beta_\epsilon \epsilon_i = \beta_0 + \gamma_x(\theta\langle x_i \mid g_i\rangle + \epsilon_i)$$

Where

- $\gamma_x = \beta_x + \beta_\epsilon$
- $\theta = \frac{\beta_x}{\gamma_x}$
- and $\langle x_i \mid g_i\rangle = \alpha_0 + \alpha_g g_i$

In this model, θ is the ratio of causal to crude effect of plasma CRP on g. By setting θ equal to 0 and 1 we obtain the models for a causal and a confounded relationship respectively. Testing the null hypothesis that $\theta = 1$ vs. $\theta = 0$, we obtain a Bayes Factor for the evidence in favour of a causal or non-causal association between CRP and g. The model was extended to include age and sex as covariates and also to incorporate multiple SNPs in unison. All 2,091 subjects were included in the model with missing values for CRP, genotype, and g being sampled from their joint posterior distribution.

The model was run using the JAGS[2] (Just Another Gibbs Sampler) software interfaced with R version 2.8.1. Prior to running the model, the variables were defined and prepared in R. The file describing this preparation and the running of the model is presented in Appendix B.

[2]http://www-ice.iarc.fr/∼martyn/software/jags/

9.6.2 Bayesian 2SLS results for CRP

The most important output of the model is the log-likelihood function for θ (Figure 9.1). The critical point on the graph is when the ratio of causal to crude effect is equal to 1. At this point the log-likelihood is approximately -0.5. The negative sign implies that the evidence is in favour of a non-causal association with the magnitude of 0.5 corresponding to a likelihood ratio of around 3 to 1 ($e^{0.5}$ to 1). This corresponds to weak to non-existent evidence. However, the direction of the association when taken in tandem with the results from the classical 2SLS approach does suggest that the association is more likely to be built on confounding than causal evidence.

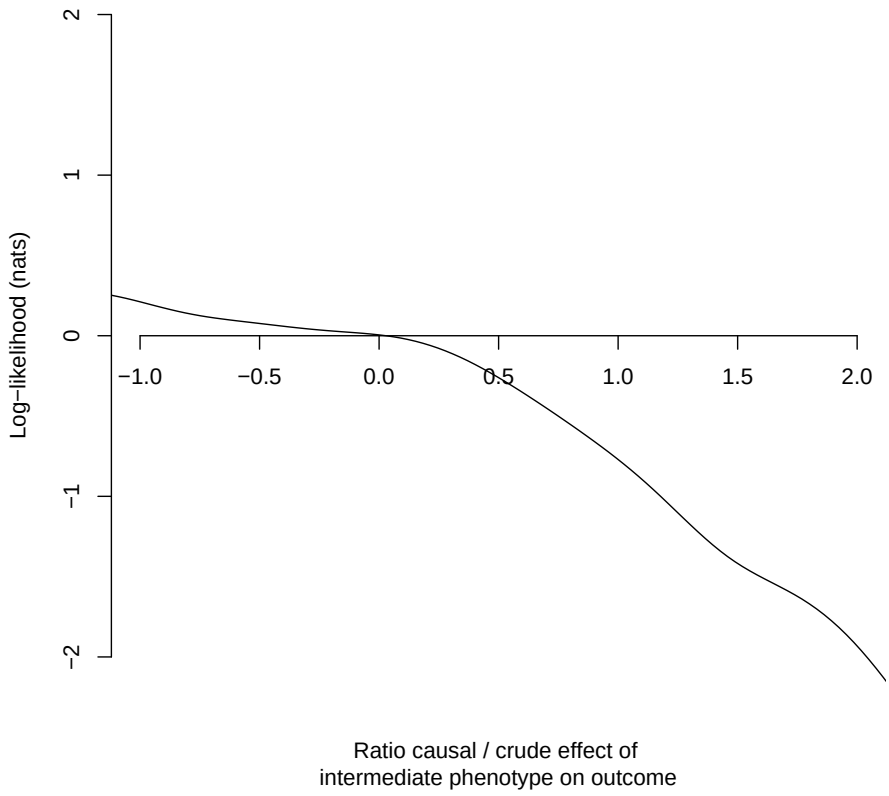

Figure 9.1: Log-likelihood plot of the causal effect of CRP on cognition.

9.6.3 Model diagnostics

To check the reliability and fit of the model, several diagnostic criteria were monitored. Traceplots display the parameter values against the iteration number (Figures 9.2 and 9.3). Model convergence should result in the plot values deviating around the mode of the posterior distribution. In the CRP model, there do not appear to be any visible trends in the traceplots suggesting non-convergence. Nonetheless, it is possible that the chain may have converged at a local region instead of covering the whole posterior distribution space.

Autocorrelation was examined for both of the chains generated (Figures 9.4 and 9.5). If sequential draws from a chain are highly correlated, this can result in the sampler taking a long time to explore the complete posterior distribution. When the autocorrelations are high for the parameter of interest, the traceplot becomes a poor diagnostic for convergence. The autocorrelation structure for θ in the CRP model shows dampening as the number of lags in both chains increases. This suggests that the model has been parameterized suitably.

Finally, Gelman-Rubin plots were produced to calculate the potential scale reduction factor. This factor is computed by sampling from each of the two chains that were initialised. After discarding the first 50 draws, the within and between chain variances are calculated. The estimated variance of the parameter can then be derived as a weighted sum of the within and between chain variance. When this factor has a large value (> 1.2) then we should consider running the chains out longer to improve convergence to the stationary distribution. Whilst the 97.5 percentile for θ is raised slightly, the median value is close to 1, suggesting convergence of the chain.

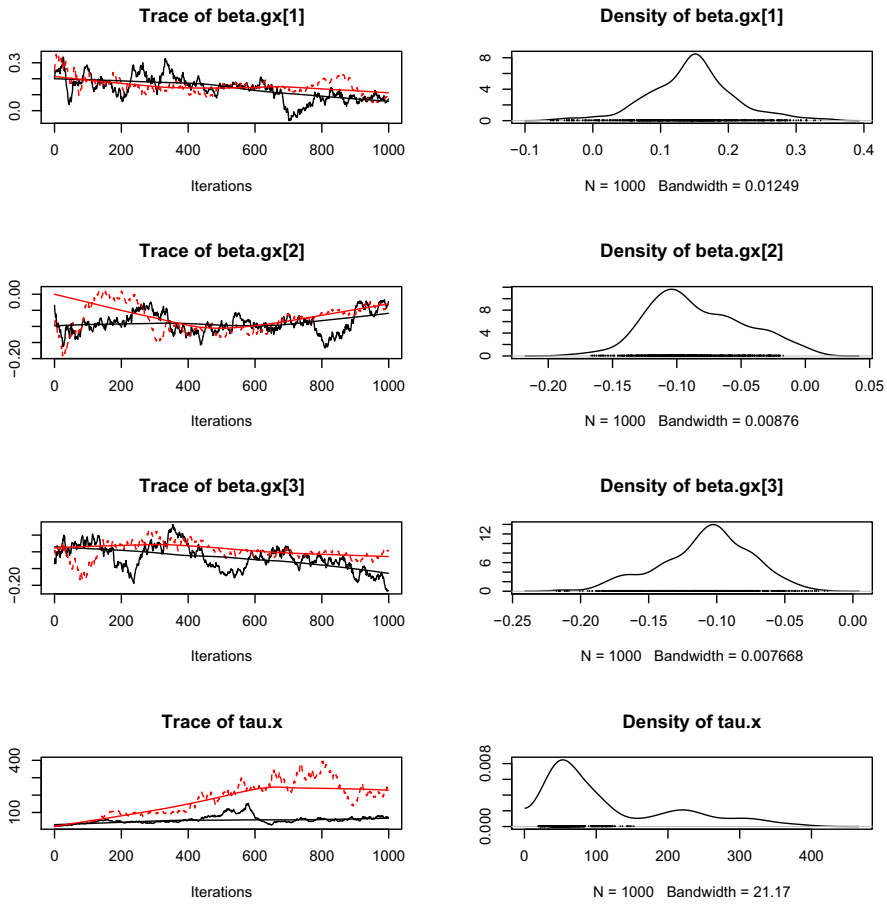

Figure 9.2: Traceplots for the causal CRP-cognition model (1).

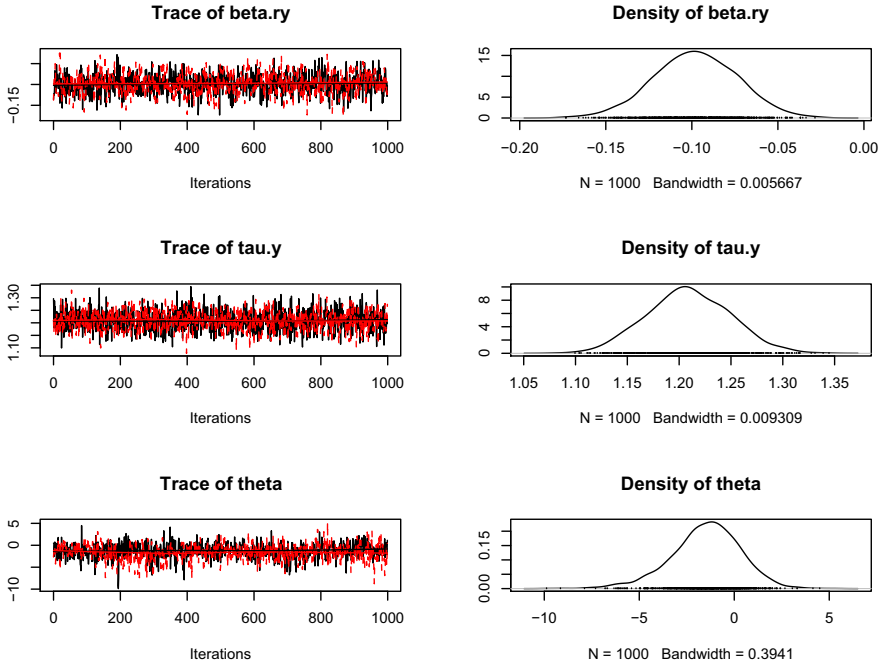

Figure 9.3: Traceplots for the causal CRP-cognition model (2).

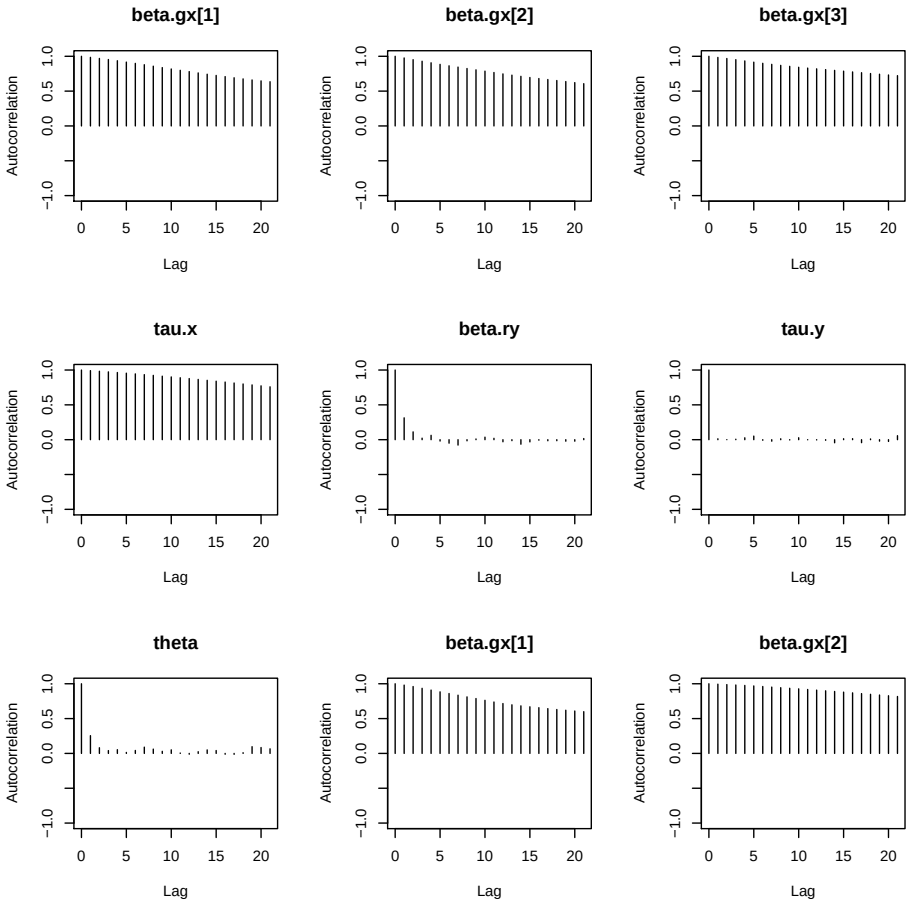

Figure 9.4: Autocorrelation plots for the causal CRP-cognition model (1).

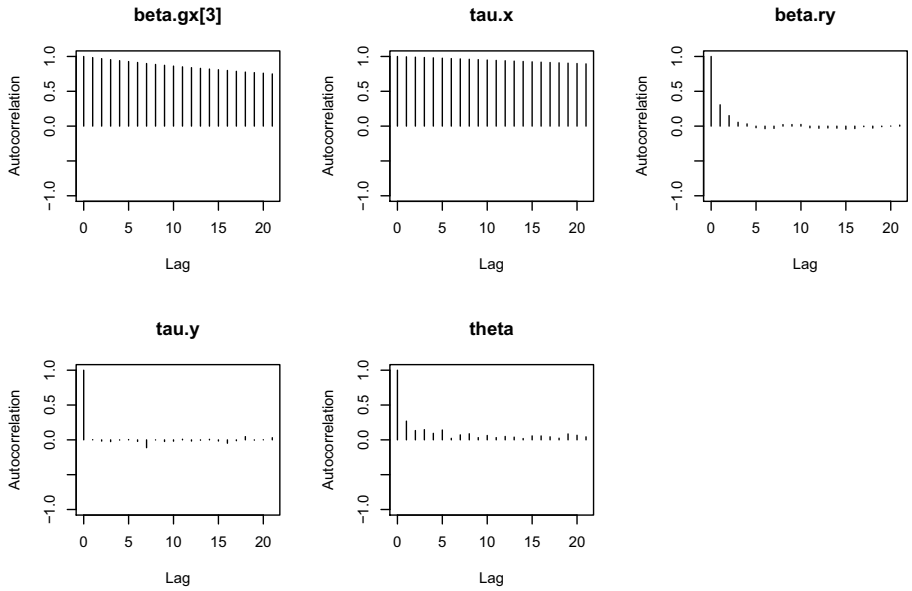

Figure 9.5: Autocorrelation plots for the causal CRP-cognition model (2).

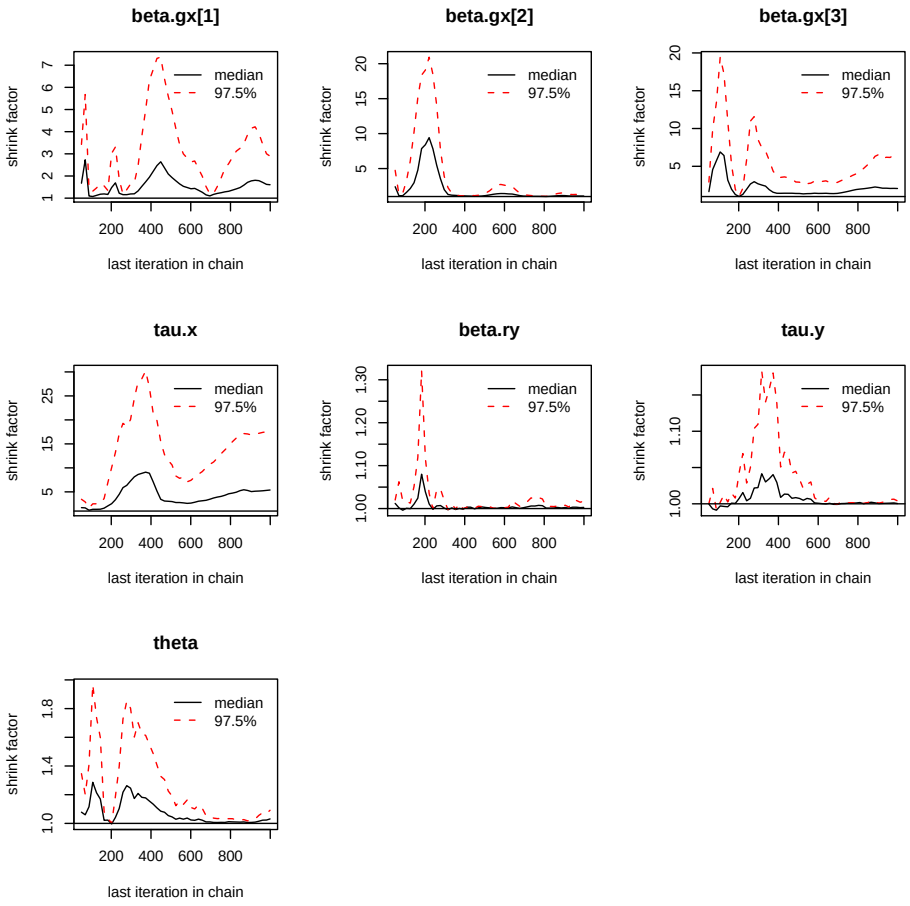

Figure 9.6: Gelman diagnostics for the causal CRP-cognition model.

9.6.4 Bayesian 2SLS results for fibrinogen

An identical approach was taken to analyse the fibrinogen data and g from the AAA Trial. The likelihood function for θ is presented in Figure 9.7 and shows an extremely flat profile. The point estimate is fractionally above zero, which suggests evidence in favour of a causal association. However, the magnitude of the estimate is so small that it translates into no strength whatsoever to favour the causal over the crude model. All of the diagnostic plots for the model were satisfactory.

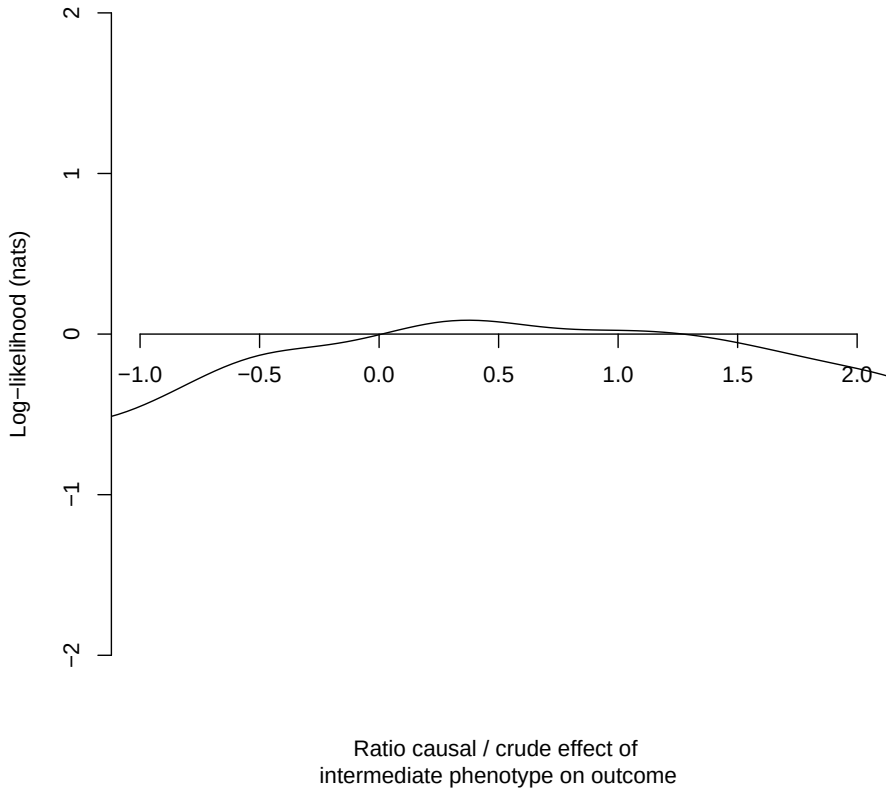

Figure 9.7: Log-likelihood plot of the causal effect of fibrinogen on cognition.

9.6.5 Limitations of the 2SLS approach

There are several commonly reported limitations of a Mendelian randomisation approach for genetic analyses (Davey Smith & Ebrahim 2003). These include:

- pleiotropy and the multi-function of genes. It is possible that the SNP(s) under investigation affect the outcome through pathways not involving the intermediate phenotype.

- canalization. This describes the situation where the possible negative impact of gene variation on normal development is softened by developmental compensation.

- the genetic variant being tested could be in high LD with the causal SNP. If the true causal SNP lies in the same gene then the casual region will still have been identified. However, if the causal variant is located in a different gene then this could have a major impact upon potential therapies and hypothesised mechanisms.

One further potential problem of Mendelian randomisation for both Bayesian and classical methods is the inability to directly assess SNP-outcome associations. This may be an issue when investigating late-life biomarker levels, especially CRP, due to the dynamic nature of their levels. For example, CRP is involved in many different physiological processes and is affected by multiple factors, especially in later life. Furthermore, the mediating factors which cause elevated CRP may result in CRP-cognition associations that are not causally related to CRP. It is certainly plausible that late-life CRP measurements are a confounded measure of lifetime exposure to CRP. Whilst the Mendelian randomisation process should distribute this confounded variation equally across groups, the use of genetic variants that are involved in CRP synthesis may be a better measure of CRP level than the marker itself. Such an approach will be particularly beneficial when the number of persons homozygous for the minor allele is small, due to small amounts of confounding on CRP levels having a potentially great influence on any analyses performed.

In the absence of serial biomarker measurements across an individual's lifetime, it is debatable whether a single, late-life measure gives a true reflection of lifetime exposure to circulating marker levels. It was subsequently chosen to examine the direct associations between SNPs and cognitive outcomes, believing them to be a better indicator of the biomarker levels than the actual biomarker measures.

9.7 Direct SNP–cognition associations

9.7.1 Aims

To test the hypothesis of there being a direct association between inflammatory marker SNPs and cognitive ability, data from the two main studies (AAA Trial and ET2DS) and the replication cohorts (EAS and LBC1936) were analysed. The combined sample size from these populations was 4,776 (AAA Trial: n = 2,091, ET2DS: n = 1,060, EAS: n = 534, LBC1936: n = 1,091). Furthermore, the additional inflammatory SNPs typed in the AAA Trial and ET2DS were also analysed in relation to late-life cognitive ability and estimated lifetime decline.

9.7.2 Statistical methods and results

As discussed previously, several SNPs from the *CRP*, *FGA*, and *FGB* genes were genotyped in the four cohorts. A recap of these are presented in Table 9.6. The study descriptives are presented in Table 9.7.

Comparing the cognitive scores across cohorts (Table 9.8), the ET2DS group scores significantly lower than the other three cohorts ($p < 0.05$). The only exception was the VFT scores in the AAA Trial and ET2DS, which were similar. A substantial difference is seen between DST scores in the AAA Trial and EAS compared to the other two cohorts due to a 90 second time allowance in the former studies with a 120 second time limit in the ET2DS and LBC1936. It was not possible to compare LM scores across the ET2DS, EAS, and LBC1936 due to only delayed recall being assessed in the EAS, and with the ET2DS subjects only being assessed for immediate and delayed recall on one of the two stories.

The AAA Trial had substantially fewer men and a greater proportion of current smokers than the ET2DS and LBC1936 (Table 9.7). The EAS also contained a high proportion ($\sim$40%) of current smokers. The only other noticeable difference across cohorts was the low blood pressure and cholesterol measurements of the ET2DS participants. This is likely to be a consequence of the increased medication this group were taking to control their cardiovascular risk factors. Prior to the analysis, CRP and fibrinogen levels were trimmed for outliers by excluding values exceeding 3.5 standard deviations in either direction from the mean.

The first stage of the modelling used age and sex-adjusted linear regression to assess the association between CRP and fibrinogen levels and cognitive ability. Additional adjustment was also made for estimated or actual pre-morbid ability (MHVS scores in the AAA Trial and ET2DS, NART scores in the EAS, and

age-11 MHT scores in the LBC1936) to examine the association between late-life marker levels and estimated (AAA Trial, ET2DS, EAS) or actual (LBC1936) lifetime cognitive change. The AAA Trial, and ET2DS results have already been presented and described in detail. However, slightly different figures are presented here as a result of the prior AAA Trial and ET2DS calculations being based on the larger, cognitively assessed samples of 2,312 and 1,066, irrespective of the availability of genotypic data. The AAA Trial, ET2DS, and LBC1936 analyses were powered at over 90% for a two-sided significance test ($\alpha = 0.05$) to detect a standardised β coefficient of 0.10. The EAS was powered at 80% for a two-sided significance test ($\alpha = 0.05$) to detect a standardised β coefficient of 0.15.

Age and sex-adjusted ANCOVA was used to test for differences in CRP (fibrinogen) level by *CRP* (fibrinogen) SNP genotype. Prior to the analysis, SNPs in the EAS and LBC1936 were assessed for deviation from Hardy-Weinberg equilibrium and also for patterns of LD. Age and sex-adjusted ANCOVA was used to test for associations between genotype and cognitive ability. Assuming a minor allele frequency (MAF) of 0.26 (0.2), which is the mean MAF of the *CRP* (fibrinogen) SNPs in the HapMap CEU population, the AAA Trial, ET2DS, and LBC1936 cohorts were able to detect a genetic contribution of 1% of the variance in the cognitive phenotype with over 90% power ($\alpha = 0.05$) with the EAS powered at over 80% to detect 1.5% of the variance in the cognitive phenotype ($\alpha = 0.05$).

9.8 CRP results

9.8.1 Plasma CRP–cognition regression

The regression results showed strong associations between CRP levels and cognition for all of the AAA Trial tests ($p < 0.05$) except the measure of memory (Table 9.8). For the other studies, sporadic associations were detected: ET2DS - MR ($p < 0.05$); EAS - VFT and DST ($p < 0.01$); LBC1936 - DST ($p < 0.05$). The only consistent result across all studies was between CRP level and the vocabulary-based estimate of prior cognitive ability, and the age-11 MHT score in the LBC1936 ($p < 0.05$). After adjusting the models for estimated or actual prior intelligence, the significant associations disappeared in the ET2DS, EAS and LBC1936.

9.8.2 Plasma CRP–*CRP* SNP associations

The second stage of the analysis looked at the relationship between the *CRP* SNPs and plasma CRP levels. All SNPs passed Hardy-Weinberg equilibrium ($p > 0.05$) although rs1130864 and rs1417938 in the AAA Trial (and rs3093068 and rs3093077 in the LBC1936) were found to be in high LD (D' = 0.99 (0.98), r^2 = 0.99 (0.94)). The -$\log_{10}$ p-values of the SNPs modelled against age and sex-adjusted CRP levels are shown in Figure 9.8.

All four SNP-biomarker associations from the AAA Trial reached Bonferroni-adjusted significance (p range $4.2e^{-06}$ - $7.0e^{-04}$). Similarly, rs1205 and rs1800947 were significant in the ET2DS ($p = 4.8e^{-05}$ and $2.2e^{-04}$). However, just one of the SNPs in the LBC1936 was associated with CRP level (p = 0.0068 for rs1130864) and two of the three EAS SNPs showed borderline significant relationships (p = 0.041 for rs1205 and p = 0.056 for rs3093077).

9.8.3 *CRP* SNP–cognition associations

The main analysis used ANCOVA to model age and sex-adjusted cognitive scores by *CRP* SNP genotypes. Applying a Bonferroni correction alters the nominal p-value from 0.05 to 0.002, 0.002, 0.003, and 0.001 in the AAA Trial, ET2DS, EAS, and LBC1936 groups, respectively. Using these criteria, no significant difference in cognitive score by genotype was found for any of the tests in any of the four studies (Table 9.9). Considering a nominal significance threshold of p = 0.05, three significant results were found in the AAA Trial: rs1417938 with RAVENS and VFT (p = 0.034 and 0.017); and its LD partner rs1130864 with VFT (p = 0.016). Three significant results were also found in the ET2DS: rs1130864 with DST, TMT, and FACES (p = 0.012, 0.030, and 0.017).

Further adjustment for the estimates/measure of pre-morbid IQ resulted in a general decreased significance in the findings (Table 9.10). However, the VFT associations with rs1130864 and rs1417938 in the AAA Trial remained significant (at a nominal p = 0.05 threshold) along with the ET2DS rs1130864–FACES association, which was significant even after considering a Bonferroni correction to the p-value (p = 0.0010).

9.9 Fibrinogen results

9.9.1 Plasma fibrinogen–cognition regression

The standardised βs and standard errors from the regression of plasma fibrinogen levels against cognitive ability are presented in Table 9.11. The AAA Trial results showed strongly significant associations between fibrinogen levels and scores on the RAVENS, DST, TMT, and g ($p < 0.001$) These relationships became stronger, with the exception of the RAVENS test, after further adjustments for MHVS. There was no evidence of an association between fibrinogen levels and MHVS ($p = 0.20$).

For the ET2DS, fibrinogen associated significantly with the MR ($p < 0.10$), TMT ($p < 0.01$) and g ($p < 0.05$). As with the AAA Trial results, adjustment for MHVS made little difference to the strength of these associations ($p < 0.10$, 0.01, and 0.10, respectively).

In the LBC1936 cohort, significant associations were found between fibrinogen levels and the MHT-11 scores ($p < 0.01$). Weakly significant results were observed for LNS, DST, LM, and g (p range 0.037 - 0.087). These associations were attenuated after adjustment for MHT-11 scores.

9.9.2 Plasma fibrinogen–fibrinogen SNP associations

The second stage of the analysis looked at the relationship between the fibrinogen SNPs and plasma fibrinogen levels. All SNPs typed passed Hardy-Weinberg equilibrium ($p > 0.05$) although rs2070022 and rs2227412 in the AAA Trial were found to be in high LD (D' = 0.94, $r^2 = 0.80$). The $-\log_{10}$ p-values of the SNPs modelled against age and sex-adjusted fibrinogen levels are shown in Figure 9.9. Three SNPs from the AAA Trial were significant after a Bonferroni correction for multiple testing: rs6050, rs2070016, and rs4220 ($p = 0.0039$, $2.5e^{-06}$, and $1.2e^{-05}$, respectively). In the ET2DS, rs4220 associated significantly with plasma fibrinogen levels whilst rs2070016 and rs2227412 were borderline significant (Bonferroni p-value and nominal p-value, respectively). The associations for the LBC1936 SNPs were all non-significant.

9.9.3 Fibrinogen SNP–cognition associations

In the main stage of the analysis, fibrinogen SNPs were modelled against the cognitive outcomes. The ANCOVA p-values for the models testing age and sex-adjusted cognitive scores by genotype groups are reported in Table 9.12. Applying

a Bonferroni correction alters the nominal p-value from 0.05 to 0.0009, 0.0019, and 0.0024 in the AAA Trial, ET2DS, and LBC1936 groups, respectively. Using these criteria, a significant difference in cognitive score by genotype was found for the VFT, DST, and g scores by rs2227412 in the ET2DS ($p = 1.6e^{-03}$, $8.0e^{-05}$, and $3.3e^{-04}$, respectively) but not for any of the results in the AAA Trial or LBC1936.

Using the nominal significance threshold of $p = 0.05$, significant results were found between rs2227412 and six of the eight ET2DS cognitive tests (maximum $p = 0.024$); between rs2070022, which is in high LD with rs2227412, and VFT in the AAA Trial; and between rs2070016 and LNS in the LBC1936. Trends ($p < 0.10$) were also observed between rs2070022 and AVLT and between rs4220 and VFT in the AAA Trial. None of the SNPs associated significantly with the MHVS or MHT measures.

Further adjustment for the estimates/measure of pre-morbid IQ resulted in a general decreased significance in the findings (Table 9.13). Again the results indicated an association between cognition and rs2227412 in the ET2DS and between rs2070016 and LNS scores in the LBC1936. Furthermore, a new association appeared between rs2227412 and DST scores ($p = 0.039$) and between rs4220 and VFT scores ($p = 0.023$) in the AAA Trial cohort.

In light of these findings, standardised residuals from regression models of age and sex on cognitive test scores were compared across SNPs showing significant or borderline significant ANCOVA results (Appendices C.1, C.2, C.3). This was done to try and identify a cognitive risk genotype for each SNP. The standardised differences between high and low risk genotypes are presented in Figure 9.10. A similar procedure was undertaken to obtain standardised age and sex-adjusted fibrinogen levels by fibrinogen genotype (Table 9.14).

Contrasting results were found for the rs2070016 genotypes. The CC genotype associated with decreased performance in the AAA Trial and ET2DS cohorts but with increased performance in the LBC1936. However, the CC group in the AAA Trial was much larger than the corresponding ET2DS or LBC1936 group (40 versus 21 versus 17 persons, respectively). The association between rs2070016 and plasma fibrinogen showed significantly higher levels in individuals with a CC genotype in the AAA Trial cohort ($p = 3.1e^{-05}$), higher levels in the LBC1936, but lower levels in the ET2DS.

For rs4220 the AA genotype was consistently implicated with poorer cognitive scores in the AAA Trial and ET2DS cohorts. Again, a slight increase in performance was noted in the LBC1936. The AA genotype was associated with higher

fibrinogen levels compared to G allele carriers in the AAA Trial ($p = 0.008$). There was no evidence for a significant difference in fibrinogen level by AA versus other genotype in the ET2DS or LBC1936.

The heterozygous GA genotype of the rs2227412 SNP associated with poorer cognition in all of the ET2DS tests. A similar pattern was also observed for the AAA Trial and LBC1936 cohorts, albeit with much smaller and non-significant differences. The VFT differences in both of these cohorts were in the opposite direction, suggesting a possible protective effect. The GA genotype was linked to slightly higher plasma fibrinogen levels than the combined homozygote level in the AAA Trial and ET2DS and with higher levels than the GG genotype in the LBC1936. Finally, the rs2070022 SNP in the *FGA* gene, which is in high LD with rs2227412 showed decreased memory performance but increased executive function performance for those with a TT genotype.

9.10 SNP-cognition associations for other genes.

In addition to the *CRP*, *FGA*, and *FGB* genes, SNP-cognition associations were investigated in the AAA Trial and the ET2DS using variants from the *TCF-1*, *IL-6*, *F13A1*, *IL-1α*, *IL-1β*, and *TNF-α* genes.

A list of the SNPs, complete with MAFs and Hardy-Weinberg Equilibrium descriptives, are presented in Table 9.15. All SNPs were found to be in HWE ($p > 0.05$). The three *TCF-1* SNPs and the *IL-6* pair rs2069832 and rs1800795 in the AAA Trial, and the two *IL-1* SNPs in the ET2DS were found to be in LD (r^2's all > 0.80).

Age and sex-adjusted ANCOVA was used to assess the association between each of the SNPs and the cognitive test scores in the respective studies (Table 9.16). This resulted in only a few significant findings:

- rs2856838 (*IL-1α*) and TMT scores in the AAA Trial ($p < 0.05$),
- rs8192284 (*IL-6*) and DST scores in the ET2DS ($p < 0.05$),
- rs1800630 (*TNF-α*) and TMT scores in the ET2DS ($p < 0.10$),
- rs1800630 (*TNF-α*) and LM scores in the ET2DS ($p < 0.01$).

All of these associations persisted upon adjustment for MHVS scores with the exception of the trend association between rs1800630 and the TMT.

- rs2856838 (*IL-1α*) and TMT scores in the AAA Trial ($p < 0.10$).

- rs8192284 (*IL-6*) and DST scores in the ET2DS ($p < 0.05$).
- rs1800630 (*TNF-α*) and LM scores in the ET2DS ($p < 0.05$).

9.11 Summary

The aim of this chapter was to present the genetic evidence for an association between inflammation and age-related cognitive decline. Three methodologies were used for the analysis including both a classical and Bayesian approach to Mendelian randomisation. These methods were then rejected in favour of a direct analysis of biomarker SNPs against cognitive ability.

The results of the direct SNP-cognition associations showed strongly significant results for the fibrinogen beta rs2227412 SNP with the majority of the cognitive tests in the ET2DS. The findings for this SNP were not replicated across the other cohorts although other fibrinogen SNP-cognition associations were found. Despite one or two weakly significant associations between cognitive ability and SNPs in the *CRP* gene, the overall suggestion is for a non-causal relationship. Similar null findings were made for the other inflammatory genes investigated. However, the coverage of variants from these genes was not nearly as comprehensive as for *CRP* and fibrinogen.

		Study			
Gene	SNP	AAA Trial	ET2DS	EAS	LBC1936
CRP	rs1205	•	•	•	•
	rs1800947	•	•		•
	rs1130864	•	•	•	•
	rs1417938	•			
	rs3093077			•	•
	rs3093068				•
FGA	rs2070022	•			
	rs6050	•			
	rs2070016	•	•		•
	rs2070018	•			
	rs2070011	•			
FGB	rs4220	•	•		•
	rs1800788	•			
	rs2227412	•	•		•

Table 9.6: Summary of genotyped SNPs from the four cohorts.

	AAA Trial (n = 2,091)	ET2DS (n = 1,060)	EAS (n = 534)	LBC1936 (n = 1,091)
Age (years)	67.2 (6.5)	67.9 (4.2)	74.0 (5.3)	69.6 (0.80)
Male - n (%)	571 (27.3)	547 (51.6)	268 (50.6)	548 (50.2)
Systolic BP (mmHg)	147.1 (21.3)	133.3 (16.4)	141.0 (22.6)	149.8 (19.2)
Diastolic BP (mmHg)	83.7 (10.7)	69.1 (9.0)	76.8 (12.2)	81.4 (10.3)
Cholesterol (mmol/l)	6.17 (1.06)	4.31 (0.90)	7.06 (1.29)	5.46 (1.16)
Smoking Status - n (%)				
Current Smoker	601 (28.7)	151 (14.2)	212 (39.8)	144 (13.2)
Ex-Smoker	700 (33.5)	497 (46.9)	113 (21.2)	473 (43.4)
Never Smoked	790 (37.8)	412 (38.9)	208 (39.0)	474 (43.4)
C-reactive protein (mg/l)	1.82 (0.89–4.06)	1.86 (0.87–4.37)	1.60 (0.79–3.39)	3.00 (1.50–6.00)

Table 9.7: Descriptive statistics for the four cohorts (mean (s.d.) or median (IQR) unless otherwise stated). Mean ages at follow-up are presented for the AAA Trial and EAS cohorts.

AAA Trial						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
AVLT	63.6 (16.44)	1675	-0.13	0.35	0.72	0.32
RAVENS	34.9 (9.27)	1635	-0.77	0.2	$\mathbf{1.0e^{-04}}$	**0.0097**
VFT	37.5 (12.69)	1715	-0.57	0.28	**0.043**	0.46
DST	40.4 (11.52)	1743	-1.02	0.23	$\mathbf{7.80e^{-06}}$	$\mathbf{1.60e^{-04}}$
ln(TMT)	4.6 (0.41)	1723	0.035	0.01	$\mathbf{3.50e^{-05}}$	**0.001**
g	0.007 (1.01)	1551	-0.09	0.02	$\mathbf{3.00e^{-05}}$	**0.0067**
MHVS	31.2 (4.71)	1680	-0.39	0.1	$\mathbf{1.70e^{-04}}$	-
ET2DS						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
MR	12.8 (5.25)	1028	-0.28	0.14	**0.045**	0.19
LNS	9.7 (2.75)	1024	-0.018	0.074	0.81	0.55
VFT	37.0 (12.82)	1036	-0.22	0.35	0.53	0.79
DST	49.3 (14.83)	1033	-0.59	0.39	0.13	0.41
ln(TMT)	4.7 (0.42)	1028	0.019	0.011	0.094	0.23
FACES	65.8 (7.88)	1035	-0.13	0.21	0.52	0.94
LM	25.3 (8.17)	1026	0.19	0.22	0.39	0.091
g	0.006 (1.00)	997	-0.046	0.027	0.088	0.57
MHVS	31.0 (5.24)	1025	-0.3	0.14	**0.038**	-
EAS						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
RAVENS	33.3 (9.73)	361	-0.35	0.47	0.46	0.43
VFT	38.7 (12.92)	370	-1.81	0.66	**0.0065**	0.23
DST	40.1 (11.51)	323	-1.7	0.6	**0.0047**	0.18
LM	35.2 (13.65)	364	0.35	0.69	0.62	**0.046**
g	0.014 (1.01)	318	-0.091	0.054	0.097	0.99
NART	31.6 (9.73)	369	-1.74	0.5	$\mathbf{5.60e^{-04}}$	-
LBC1936						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
MR	13.5 (5.10)	1047	-0.35	0.18	0.058	0.55
LNS	10.9 (3.17)	1040	-0.12	0.11	0.30	0.94
VFT	42.5 (12.50)	1047	-0.14	0.45	0.76	0.38
DST	56.7 (12.94)	1046	-1.16	0.46	**0.012**	0.25
LM	71.5 (17.97)	1049	-0.69	0.64	0.28	0.77
g	0.006 (1.00)	1032	-0.068	0.035	0.054	0.84
MHT-11	49.0 (11.79)	990	-1.44	0.44	**0.0011**	-

Table 9.8: Age and sex (* and vocabulary)-adjusted regression of plasma CRP levels against cognitive test scores in the four study cohorts (Unstandardised βs and standard errors – p-values less than 0.05 highlighted in bold).

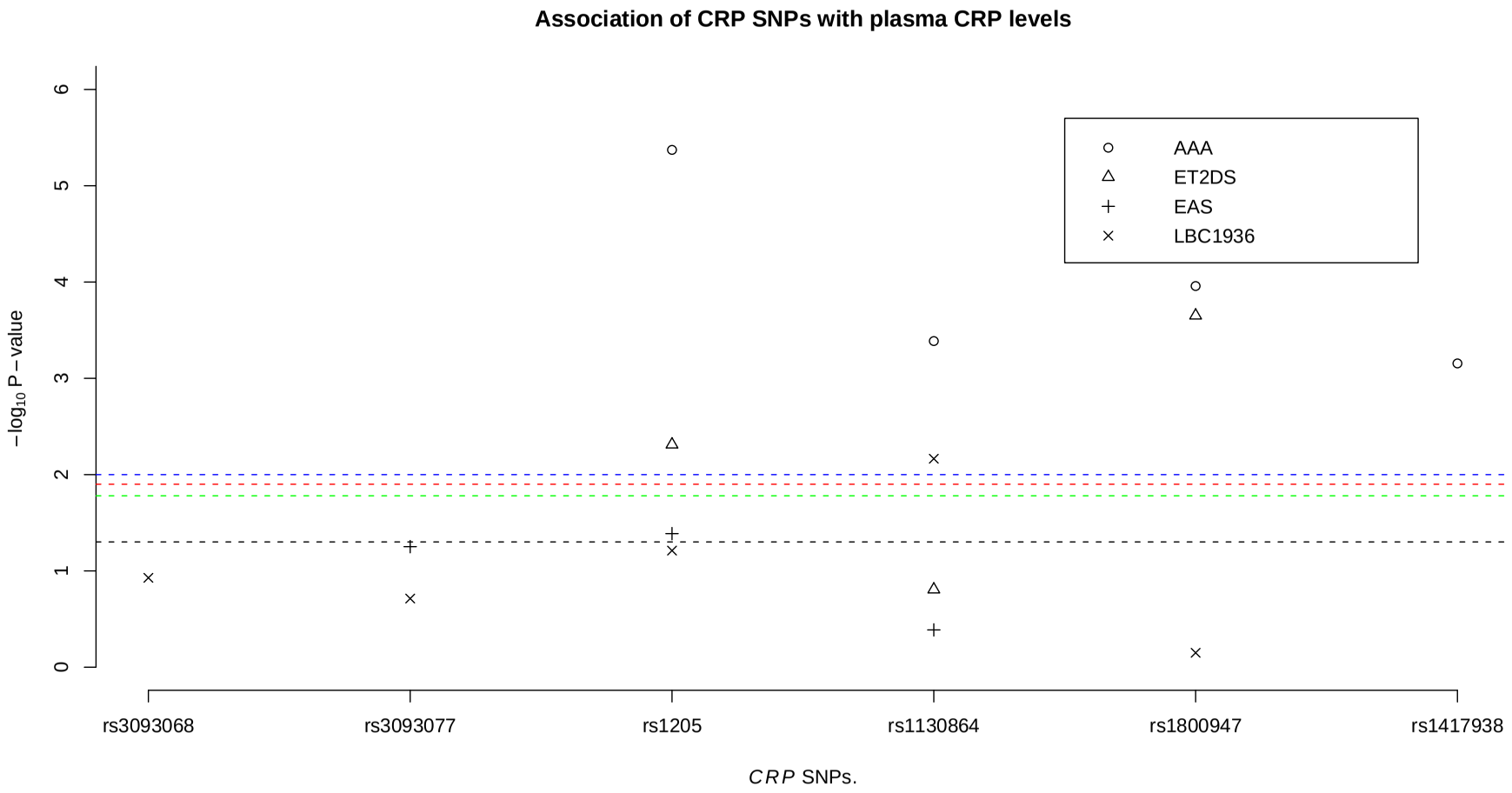

Figure 9.8: -$\log_{10}$ p-values for the age and sex-adjusted ANCOVA associations between *CRP* SNPs and plasma CRP levels. The nominal significance threshold (p = 0.05) is indicated by a black line. Bonferroni adjusted thresholds are indicated by the blue (LBC1936), red (AAA Trial), and green (ET2DS and EAS) lines.

AAA Trial				
Cognitive Test	rs1205	rs1800947	rs1130864	rs1417938
AVLT	0.67	0.27	0.57	0.64
RAVENS	0.73	0.14	0.082	**0.034**
VFT	0.56	0.22	**0.016**	**0.017**
DST	0.38	0.22	0.69	0.65
ln(TMT)	0.88	0.61	0.46	0.57
g	0.78	0.43	0.47	0.49
MHVS	0.38	0.45	0.14	0.085

ET2DS			
Cognitive Test	rs1205	rs1800947	rs1130864
MR	0.58	0.36	0.52
LNS	0.59	0.70	0.32
VFT	0.95	0.76	0.38
DST	0.31	0.38	**0.012**
ln(TMT)	0.28	0.96	**0.030**
FACES	0.98	0.26	**0.017**
LM	0.87	0.68	0.28
g	0.69	0.41	0.092
MHVS	0.33	0.25	0.072

EAS			
Cognitive Test	rs1205	rs1130864	rs3093077
RAVENS	0.11	0.40	0.96
VFT	0.79	0.36	0.41
DST	0.24	0.60	0.95
LM	0.43	0.29	0.67
g	0.13	0.74	0.97
NART	0.75	0.78	0.78

LBC1936					
Cognitive Test	rs1205	rs1800947	rs1130864	rs3093077	rs3093068
MR	0.53	0.88	0.12	0.61	0.52
LNS	0.33	0.088	0.96	0.094	0.21
VFT	0.33	0.76	0.92	0.26	0.60
DST	0.58	0.41	0.70	0.90	0.77
LM	0.21	0.22	0.35	0.78	0.83
g	0.22	0.73	0.70	0.24	0.28
MHT-11	0.086	0.46	0.44	0.81	0.70

Table 9.9: ANCOVA p-values for the age and sex-adjusted associations between *CRP* SNPs and cognitive ability (p-values less than 0.05 highlighted in bold).

AAA Trial				
Cognitive Test	rs1205	rs1800947	rs1130864	rs1417938
AVLT	0.41	0.31	0.29	0.26
RAVENS	0.88	0.17	0.42	0.24
VFT	0.64	0.13	**0.017**	**0.014**
DST	0.57	0.15	0.92	0.95
ln(TMT)	0.60	0.49	0.17	0.20
g	0.83	0.27	0.44	0.48

ET2DS			
Cognitive Test	rs1205	rs1800947	rs1130864
MR	0.80	0.25	0.17
LNS	0.91	0.70	0.84
VFT	0.68	0.88	0.42
DST	0.13	0.44	0.083
ln(TMT)	0.26	0.59	0.17
FACES	0.82	0.41	**0.0010**
LM	0.87	0.70	0.76
g	0.42	0.41	0.38

EAS			
Cognitive Test	rs1205	rs1130864	rs3093077
RAVENS	0.085	0.37	0.85
VFT	0.65	0.14	0.73
DST	0.18	0.59	0.82
LM	0.51	0.21	0.78
g	0.12	0.51	0.87

LBC1936					
Cognitive Test	rs1205	rs1800947	rs1130864	rs3093077	rs3093068
MR	0.80	**0.017**	0.96	0.10	0.18
LNS	0.073	0.49	0.076	0.94	0.53
VFT	0.73	0.93	0.96	0.32	0.63
DST	0.92	0.52	0.56	0.29	0.46
LM	0.39	0.061	0.31	0.77	0.70
g	0.47	0.21	0.61	0.25	0.20

Table 9.10: ANCOVA p-values for the age, sex, and vocabulary-adjusted associations between *CRP* SNPs and estimated/actual lifetime cognitive change (p-values less than 0.05 highlighted in bold).

AAA Trial						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
AVLT	63.4 (16.52)	1793	-1.04	0.57	0.068	0.13
RAVENS	34.9 (9.28)	1745	-1.14	0.33	$\mathbf{4.7e^{-04}}$	$\mathbf{7.00e^{-04}}$
VFT	37.5 (12.73)	1837	-0.40	0.45	0.38	0.57
DST	40.3 (11.58)	1863	-1.57	0.37	$\mathbf{2.60e^{-05}}$	$\mathbf{7.30e^{-06}}$
ln(TMT)	4.6 (0.41)	1842	0.07	0.01	$\mathbf{1.70e^{-06}}$	$\mathbf{1.00e^{-06}}$
g	0.0004 (1.01)	1659	-0.14	0.04	$\mathbf{1.60e^{-04}}$	$\mathbf{7.90e^{-05}}$
MHVS	31.2 (4.70)	1795	-0.22	0.17	0.20	-
ET2DS						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
MR	12.8 (5.27)	1036	-0.41	0.23	0.075	0.053
LNS	9.7 (2.76)	1032	-0.03	0.12	0.79	0.87
VFT	37.0 (12.85)	1044	-0.07	0.58	0.91	0.99
DST	49.3 (14.80)	1041	-0.58	0.64	0.37	0.39
ln(TMT)	4.7 (0.41)	1036	0.05	0.02	**0.006**	**0.004**
FACES	65.8 (7.88)	1043	-0.29	0.34	0.40	0.44
LM	25.2 (8.20)	1034	-0.04	0.37	0.91	0.96
g	0.006 (1.00)	1005	-0.09	0.04	**0.048**	0.053
MHVS	30.9 (5.25)	1033	-0.08	0.24	0.73	-
LBC1936						
Cognitive Test	Mean (s.d.)	n	β	s.e.	p	Adjusted-p*
MR	13.5 (5.11)	1044	-0.17	0.25	0.51	0.43
LNS	11.0 (3.15)	1037	-0.27	0.16	0.087	0.66
VFT	42.5 (12.57)	1042	-0.57	0.62	0.36	0.82
DST	56.7 (12.92)	1043	-1.22	0.63	0.052	0.38
LM	71.6 (17.92)	1046	-1.71	0.87	0.051	0.63
g	0.01 (1.00)	1029	-0.10	0.05	**0.037**	0.73
MHT-11	49.0 (11.79)	989	-1.87	0.60	**0.0018**	-

Table 9.11: Age and sex (* and vocabulary)-adjusted regression of plasma fibrinogen levels against cognitive test scores in the three study cohorts (Unstandardised βs and standard errors – p-values less than 0.05 highlighted in bold).

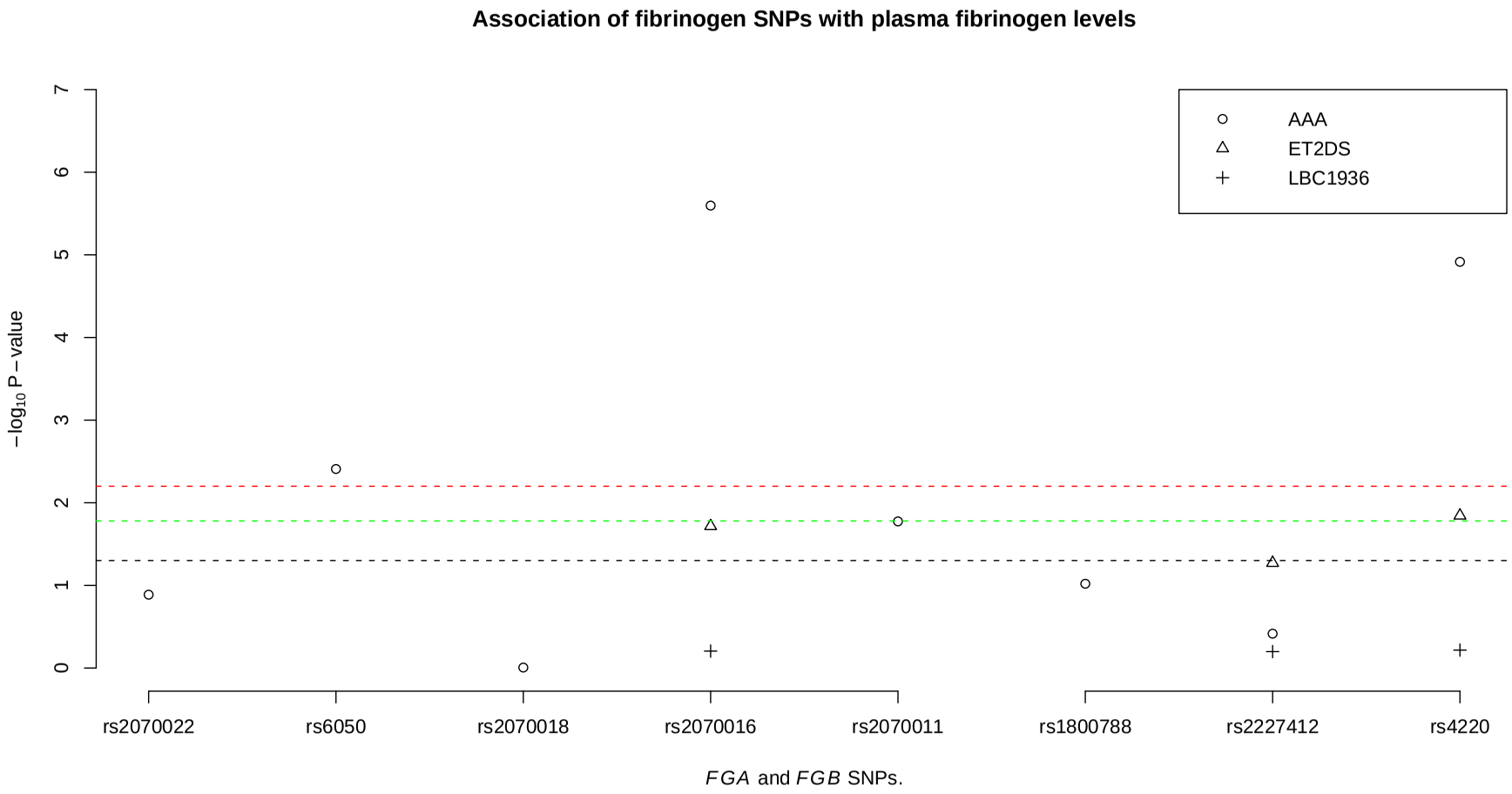

Figure 9.9: -$\log_{10}$ p-values for the age and sex-adjusted ANCOVA associations between *FGA* and *FGB* SNPs and plasma fibrinogen levels. The nominal significance threshold (p = 0.05) is indicated by a black line. Bonferroni adjusted thresholds are indicated by the red (AAA Trial), and green (ET2DS and LBC1936) lines.

AAA Trial

			FGA				*FGB*	
Cognitive Test	rs2070022	rs6050	rs2070016	rs2070018	rs2070011	rs4220	rs1800788	rs2227412
AVLT	0.07	0.85	0.47	0.76	0.77	0.70	0.81	0.32
RAVENS	0.43	0.96	0.53	0.75	0.85	0.31	0.90	0.30
VFT	**0.036**	0.24	0.72	0.52	0.20	0.083	0.66	0.11
DST	0.34	0.79	0.16	0.96	0.38	0.17	0.63	0.18
ln(TMT)	0.53	0.95	0.29	0.78	0.83	0.25	0.76	0.63
g	0.74	0.77	0.59	0.72	0.74	0.31	0.97	0.58
MHVS	0.38	0.22	0.18	0.94	0.75	0.95	0.78	0.32

ET2DS

			FGA				*FGB*	
Cognitive Test	rs2070022	rs6050	rs2070016	rs2070018	rs2070011	rs4220	rs1800788	rs2227412
MR			0.46			0.66		**0.024**
LNS			0.59			0.43		**0.022**
VFT			0.55			0.25		**0.0016**
DST			0.92			0.86		**8.00e^{-05}**
ln(TMT)			0.43			0.33		**0.016**
FACES			0.39			0.81		0.12
LM			0.85			0.46		0.38
g			0.89			0.45		**3.30e^{-04}**
MHVS			0.77			0.50		0.23

LBC1936

			FGA				*FGB*	
Cognitive Test	rs2070022	rs6050	rs2070016	rs2070018	rs2070011	rs4220	rs1800788	rs2227412
MR			0.37			0.20		0.49
LNS			**0.020**			0.45		0.93
VFT			0.87			0.74		0.29
DST			0.78			0.27		0.97
LM			0.31			0.095		0.29
g			0.22			0.075		0.84
MHT-11			0.53			0.16		0.55

Table 9.12: ANCOVA p-values for the age and sex-adjusted associations between *FGA* and *FGB* SNPs and cognitive ability (p-values less than 0.05 highlighted in bold).

AAA Trial

Cognitive Test	*FGA* rs2070022	rs6050	rs2070016	rs2070018	rs2070011	*FGB* rs4220	rs1800788	rs2227412
AVLT	0.13	0.68	0.16	0.82	0.83	0.22	0.79	0.73
RAVENS	0.17	0.53	0.85	0.74	0.94	0.28	0.87	0.066
VFT	0.065	0.065	0.59	0.54	0.13	**0.023**	0.52	0.17
DST	0.18	0.45	0.30	0.95	0.39	0.13	0.58	**0.039**
ln(TMT)	0.22	0.84	0.50	0.94	0.88	0.19	0.54	0.20
g	0.37	0.35	0.72	0.52	0.71	0.71	0.92	0.17

ET2DS

Cognitive Test	*FGA* rs2070022	rs6050	rs2070016	rs2070018	rs2070011	*FGB* rs4220	rs1800788	rs2227412
MR			0.75			0.70		0.61
LNS			0.50			0.35		**0.0027**
VFT			0.66			0.69		**1.6e^{-04}**
DST			0.39			0.54		0.062
ln(TMT)			0.30			0.74		0.055
FACES			0.91			0.41		0.45
LM			0.39			0.79		0.23
g			0.56			0.83		**4.3e^{-04}**

LBC1936

Cognitive Test	*FGA* rs2070022	rs6050	rs2070016	rs2070018	rs2070011	*FGB* rs4220	rs1800788	rs2227412
MR			0.50			0.27		0.61
LNS			0.92			0.79		0.13
VFT			0.81			0.51		0.96
DST			**0.016**			0.49		0.88
LM			0.65			0.12		0.49
g			0.22			0.14		0.92

Table 9.13: ANCOVA p-values for the age, sex, and vocabulary-adjusted associations between *FGA* and *FGB* SNPs and estimated/actual lifetime cognitive change (p-values less than 0.05 highlighted in bold).

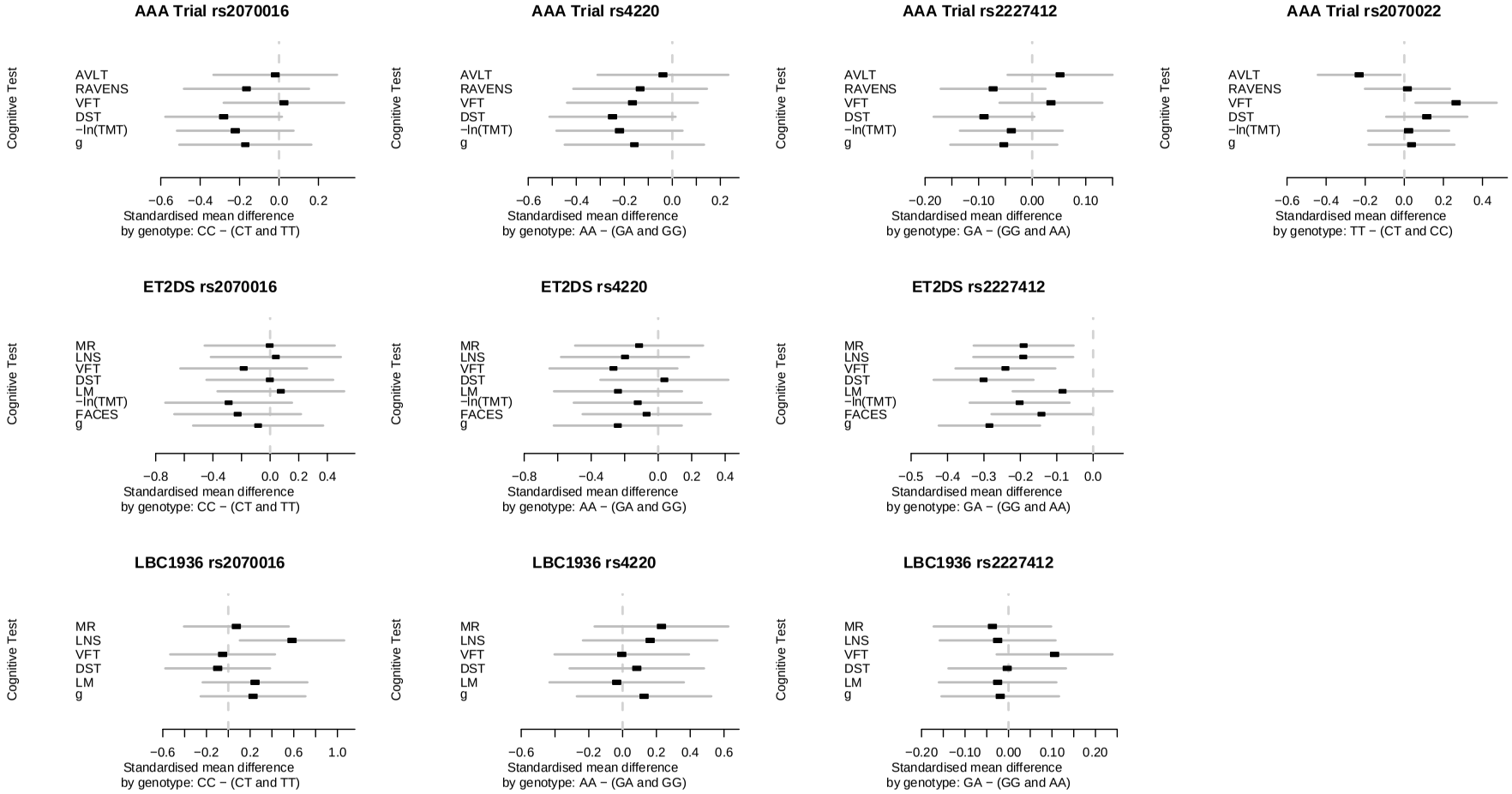

Figure 9.10: Plots of mean differences between standardised age and sex-adjusted cognitive scores for 'high risk' and 'low risk' *FGA* and *FGB* genotypes for poorer cognitive performance. The 'high risk' and 'low risk' genotypes were determined by informal comparison of the standardised age and sex-adjusted cognitive scores.

rs2070022

	CC		CT		TT			
Cohort	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	Cognitive risk genotype	t-test p-value
AAA Trial	-0.035 (0.96)	1161	0.067 (1.06)	577	0.031 (1.17)	85	TT	0.80

rs2070016

	CC		CT		TT			
Cohort	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	Cognitive risk genotype	t-test p-value
AAA Trial	0.629 (0.86)	40	0.109 (1.03)	456	-0.056 (0.98)	1333		$3.10e^{-05}$
ET2DS	-0.106 (0.58)	21	0.152 (1.05)	252	-0.050 (0.99)	725	CC	0.42
LBC1936	0.215 (0.77)	17	0.019 (0.87)	230	-0.010 (1.04)	779		0.26

rs4220

	AA		GA		GG			
Cohort	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	Cognitive risk genotype	t-test p-value
AAA Trial	0.388 (1.02)	51	0.132 (1.04)	506	-0.069 (0.97)	1260		0.008
ET2DS	0.001 (0.70)	28	0.145 (1.07)	283	-0.060 (0.98)	686	AA	0.99
LBC1936	0.118 (0.78)	25	0.039 (0.99)	264	-0.018 (1.01)	742		0.46

rs2227412

	AA		GA		GG			
Cohort	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n	Cognitive risk genotype	t-test p-value
AAA Trial	-0.022 (0.96)	1198	0.049 (1.05)	549	-0.012 (1.18)	70		0.18
ET2DS	-0.007 (0.98)	711	0.063 (1.07)	276	-0.391 (0.72)	31	GA	0.24
LBC1936	0.007 (0.98)	686	0.003 (1.04)	298	-0.164 (1.09)	33		0.96

Table 9.14: Standardised age and sex-adjusted plasma fibrinogen levels by fibrinogen SNP genotypes. Welch t-tests were used to formally test for differences in fibrinogen levels by the high and low risk genotypes for poorer cognitive ability.

AAA Trial							
Gene	SNP	n	Minor allele	Major allele	MAF	CEU MAF	HWE
TCF-1	rs1169292	2,009	T	C	0.31	NA	1.00
	rs1169301	2,026	T	C	0.29	NA	0.52
	rs2464196	2,017	T	C	0.29	0.30	0.33
IL-6	rs2069832	2,019	G	A	0.41	0.46	0.61
	rs2069840	2,006	C	G	0.37	0.29	0.89
	rs1800795	2,001	G	C	0.41	0.46	0.96
IL-1α	rs2856836	2,031	T	C	0.30	0.25	0.07
	rs3783546	2,024	G	C	0.31	0.29	0.08
	rs2856838	2,029	C	T	0.39	0.40	0.85
IL-1β	rs1143643	2,014	G	A	0.36	0.39	0.77
	rs1143634	2,021	C	T	0.23	0.21	0.85
F13A1	rs5985	2,017	G	T	0.27	0.21	0.26
ET2DS							
Gene	SNP	n	Minor allele	Major allele	MAF	CEU MAF	HWE
IL-1βC	rs554344	996	G	C	0.17	0.19	0.44
	rs580253	1,014	C	T	0.17	0.19	0.91
IL-6	rs8192284	1,017	A	C	0.40	0.35	0.95
TNF-α	rs1800630	1,014	C	A	0.15	0.15	0.06
TCF-1	rs2464196	1,018	G	A	0.31	0.30	0.24

Table 9.15: Summary of additional inflammatory SNPs in the AAA Trial and the ET2DS.

AAA Trial

	TCF-1			*IL-6*			*IL-1α*			*IL-1β*		*F13A1*
Cognitive test	rs1169292	rs1169301	rs2464196	rs2069832	rs2069840	rs1800795	rs2856836	rs3783546	rs2856838	rs1143643	rs1143634	rs5985
AVLT	0.89	0.49	0.69	0.70	0.70	0.87	0.24	0.89	0.48	0.9	0.46	0.60
RAVENS	0.67	0.89	0.88	0.41	0.60	0.52	0.92	0.36	0.30	0.89	0.54	0.20
VFT	0.40	0.36	0.35	0.96	0.82	0.97	0.68	0.56	0.61	0.75	0.86	0.83
DST	0.42	0.52	0.57	0.47	0.66	0.57	0.35	0.13	0.29	0.82	0.20	0.082
ln(TMT)	0.58	0.62	0.44	0.91	0.64	0.91	0.14	0.94	**0.044**	0.26	0.74	0.53
g	0.62	0.69	0.63	0.73	0.87	0.88	0.59	0.67	0.25	0.80	0.34	0.60

ET2DS

	IL-1βC		*IL-6*	*TNF-α*	*TCF-1*
Cognitive test	rs554344	rs580253	rs8192284	rs1800630	rs2464196
MR	0.57	0.46	0.89	0.61	0.49
LNS	0.66	0.67	0.47	0.55	0.17
VFT	0.83	0.75	0.76	0.17	0.18
DST	0.55	0.42	**0.032**	0.17	0.32
ln(TMT)	0.52	0.54	0.23	0.082	0.69
FACES	0.57	0.63	0.95	0.56	0.36
LM	0.99	0.82	0.30	**0.0057**	0.28
g	0.50	0.68	0.37	0.16	0.36

Table 9.16: ANCOVA p-values for the age and sex-adjusted associations between the additional inflammatory SNPs and cognitive ability (p-values less than 0.05 highlighted in bold).

Chapter 10

Discussion

In this chapter the study findings are discussed. Firstly, a commentary on the biomarker level-cognition associations are presented for both the AAA Trial and the ET2DS. Following this, the genetic analyses between the biomarker SNPs and cognition from the AAA Trial, the ET2DS and the replication cohorts are reviewed. Where possible, the results are compared to those from the literature. Furthermore, a critical evaluation of the pros and cons of the study methodology and statistical analyses are also provided. The chapter concludes with a summary of the importance and novelty of the work of this thesis in addition to giving suggestions for further work.

10.1 Association between plasma biomarker levels and cognitive ability

10.1.1 Markers of inflammation

AAA Trial

After five years of follow-up and following adjustment for age and sex, raised levels of plasma fibrinogen and CRP were associated with poorer general cognitive ability, non-verbal reasoning, executive function (CRP only), processing speed, and mental flexibility. All but one of these associations remained significant after adjustment for MHVS scores, suggesting an association with estimated lifetime cognitive decline. Further adjustment for covariates (smoking, cholesterol, diastolic blood pressure, and ABPI) made little difference to the results with the significant associations persisting in both the prospective and the estimated lifetime change models.

Contrasting the findings from the AAA Trial to those from the literature is

difficult due to the heterogeneity of the study populations, the different cognitive test batteries employed, and the varied methodological and analytical approaches taken. These differences are apparent in both the longitudinal and cross-sectional analyses.

Methodologically, the four key CRP papers reviewed in Section 2.8 varied as follows. The paper by Dik et al. was similar to the AAA Trial in that baseline inflammatory marker levels were modelled against cognitive change in four broad cognitive domains - including a g factor - over a three year period (Dik et al. 2005). In their analyses, change in cognition was measured as the raw difference between baseline and follow-up scores, and biomarker levels were split into tertiles for the main logistic regression analysis.

The paper by Schram et al. that investigated the Rotterdam and Leiden studies was relatively analogous to the AAA Trial in terms of study design (Schram et al. 2007). After measuring biomarker levels at baseline, a five year follow-up took place with cognitive assessment (in three domains) at both time-points. Cross-sectional and longitudinal analyses were performed with annual cognitive change being calculated by means of raw differences divided by time, in years, between the waves. Standardised effect sizes for the biomarkers were small and statistically significant only at the cross-sectional level for CRP. A longitudinal association was found between IL-6 and change in memory but only in the smaller Leiden group.

The third study by Weuve et al. focussed exclusively on a CRP-cognition relationship in women (Weuve et al. 2006). A comprehensive five test battery was administered five years after biomarker measures were taken. For the analysis, CRP was split into quintiles with mean cognitive scores being compared across groups. This yielded small but non-significant associations for raised CRP (highest versus lowest quintile) to associate with poorer cognition.

The final of the key studies, by Alley et al. differed substantially from the other three papers and also the AAA Trial in terms of its methodology and analysis (Alley et al. 2008). Growth curve modelling was used to analyse the CRP-cognition association using three waves of cognitive test data and baseline CRP levels. As with Weuve, there was no evidence to link CRP with cognitive ability or cognitive change. Moreover, the study may well have suffered from a practice effect with the three waves of cognitive testing all occurring within a seven year period.

The solitary key paper analysing plasma fibrinogen and cognition was by Rafnsson et al (Rafnsson et al. 2007). This paper analysed data from the Ed-

inburgh Artery Study (EAS), the background details of which were described in detail in Chapter 7. Given the crossover between the EAS and AAA Trial research groups, the methodology and analysis of the two studies were very similar. Similar to the key papers looking at CRP, the EAS showed small associations between biomarkers and cognition. The largest of these associations were for fibrinogen and IL-6 with the former associating with measures of processing speed and general ability, with five year decline in non-verbal reasoning, and with estimated lifetime decline of memory.

Given the methodological and analytical heterogeneity, it is difficult to compare these papers with the findings from the AAA Trial. However, the one main finding that can be drawn from all of the studies is that if associations do exist between inflammatory biomarkers and cognition then they are almost certainly going to be small. In the studies where null findings were reported, the point estimates of the associations were in the same direction as those from the positive reports i.e. increased biomarker levels associate with decreased cognitive ability.

Overall, the positive findings for both CRP and fibrinogen were most pronounced for tests associated with processing speed and mental flexibility. This possibly reflects the increased sensitivity of these tests to age-related cognitive decline compared with tests for alternative domains such as memory (Salthouse 2000). However, the Rafnsson paper did find plasma fibrinogen to associate with estimated lifetime decline in memory.

Why so many large studies are failing to replicate each others findings is unclear. In addition to study design differences, choice of potential confounders and time of CRP measurement may also be highly influential. Nevertheless, the genetic analyses of inflammatory biomarkers and cognition helped eliminate some of the need to worry about these factors.

In addition to the CRP-cognition associations, the potential interactive effect of $APO\epsilon$ on the relationship was investigated. Despite no significant associations being found between $APO\epsilon$ and cognition, the point estimates for the $\epsilon 4$ allele carriers were in the direction of poorer cognitive ability. Both this and the association between the $\epsilon 4$ allele and decreased CRP levels are consistent with the findings reported in the literature (März et al. 2004). The latter association is of particular interest in that it seems a counter-intuitive argument. That is, those with the worst risk profile for cognitive decline by $APO\epsilon$ status have the lowest levels of CRP, which are linked to the lowest risk profile group for cognitive decline. There was no evidence to suggest a relationship between $APO\epsilon$ status and fibrinogen level.

The reason for the association between $APO\epsilon$ allele status and circulating levels of CRP is unclear. The association could be related to inflammation, although a lack of associations with other biomarkers, such as fibrinogen, both in the AAA Trial and in the study by März et al. make this less likely.

Another possibility is that CRP production is affected by the mevalonate pathway in the liver. Those who possess the $APO\epsilon4$ allele absorb cholesterol more effectively than those without the allele (Kesäniemi et al. 1987). Subsequently, there is a down-regulation of liver-based cholesterol synthesis and LDL receptors, which increases the circulating levels of LDL. Moreover, this may also result in a lower production of plasma CRP. Support for this hypothesis comes from studies that have found statins that affect the rate-limiting step of the mevalonate pathway and the production of cholesterol also lower the synthesis of CRP (Rontu et al. 2006).

In the final stage of the $APO\epsilon$ analysis a model was built to investigate the evidence for a CRP–$APO\epsilon$ interaction with cognition. However, this failed to show any significant relationship between the interaction term and cognitive performance.

ET2DS

In the ET2DS, raised levels of plasma IL-6 and TNF-α, as well as a general inflammation factor were associated with poorer general cognitive ability. Domain specific associations were most notable for measures of non-verbal reasoning, processing speed, non-verbal memory, and mental flexibility. A significant association was also found between plasma fibrinogen levels and mental flexibility with trends or weakly significant associations for fibrinogen and CRP with non-verbal reasoning, and with g. After adjustment for vocabulary scores - which were used to estimate prior cognitive ability - and for cardiovascular and other health risk factors, significant associations persisted between IL-6, TNF-α, and the general inflammatory factor, and non-verbal reasoning, mental flexibility (excluding TNF-α but including fibrinogen), and g (excluding TNF-α and a trend observation for the general inflammatory factor). Furthermore, apart from fibrinogen, each of the markers associated significantly with the MHVS estimate of pre-morbid IQ.

These findings suggest a strong relationship between cognition and the upstream markers of inflammation, TNF-α and IL-6. IL-6 in particular showed consistent effects over the majority of the cognitive tests. There is also strong evidence for a general inflammation-cognition association with the two composite factors associating significantly in the MHVS-adjusted model with a trend

association in the fully adjusted model. However, despite all four markers loading strongly onto the general inflammation component it is possible that these results are being driven by the influence of the upstream markers.

Comparing the results of the ET2DS downstream markers (CRP and fibrinogen) with the findings from the AAA Trial yields a degree of similarity. Whilst the ET2DS associations were slightly smaller and less statistically significant, the overall pattern of results remains alike. That is, the greatest associations are presented for the measures of processing speed. Plainly, the design and subsequent analysis of the ET2DS data will improve upon collection of the four year follow-up cognitive data. It will be of great interest to see if the magnitude and significance of the associations match in what are two homogenous populations.

As with the attempted comparison of AAA Trial results with those from the literature, the evaluation of the upstream inflammatory markers is hampered by study heterogeneity. Of the key papers that assessed either or both of IL-6 and TNF-α the studies by Dik, Schram, Alley, and Rafnsson have already been summarised as part of the CRP and fibrinogen analysis. The remainder of the work identified was from papers where the study design did not match the criteria specified for the literature review. For example, the longitudinal studies by Yaffe et al. and Jordanova et al. suffered from using a single cognitive test and assessing a very small sample from an ethnic community, respectively (Jordanova et al. 2007, Yaffe et al. 2003). As with the downstream marker literature, these studies failed to apply a common analytical technique to their data. Both papers used odds ratios as their main outcome result with Yaffe et al. splitting the biomarker levels into tertiles and Jordanova et al. using a dichotomous split. There is a modicum of statistically significant evidence to relate the marker levels to cognitive dysfunction with the majority of evidence pointing towards small but consistently inverse associations.

Comparing the results of both the AAA Trial and the ET2DS with the literature it is clear that there are major inconsistencies in study methodology between different study groups. Whilst there are restrictions on when biomarker measurements are made and how long the cohort may be followed up for, there are some changes that could make a huge impact on generalising study results. For example, it is rare to find two studies that use the same battery of cognitive tests - there were no instances of this in the literature reviewed. It is understandable that different researchers will favour a different set of cognitive tests to measure the same underlying cognitive domain. However, such an approach severely hampers the possibilities for meta-analytic studies, which are sorely needed in this

field. The only instance where consistency was present was when the cognitive outcome assessed was the MMSE, which unfortunately, is too insensitive a test to measure age-related cognitive decline. In addition, a g-factor which has been derived from a large battery of different cognitive tests should provide a fairly reliable measure of cognition.

A second improvement, which is simple to implement, would be a consistent approach to the statistical handling of inflammatory biomarker variables. For variables such as CRP, where the plasma levels can be categorised into low, medium, and high risk of developing cardiovascular disease, it doesn't seem unreasonable that the same categories couldn't be used in the main statistical analysis. Alternatively, it would also be viable to treat CRP as a continuous variable (as in this thesis) and use a regression-type approach to relate actual differences or changes in CRP level to differences or changes in cognition. It is also critical for studies to translate the small effect sizes in to something that is manageable and meaningful to the reader. A common criticism for many of the published studies reviewed in this work is the presentation of statistically significant statistics minus a practical interpretation. If there is a causal association between inflammation and cognition then it is essential to have an understanding of the magnitude of elevation in biomarker level that results in increased risk of cognitive decline (the effect sizes uncovered in this thesis are discussed in the section directly following this). Whether such elevations are then clinically treatable is a question beyond the scope of this thesis but nonetheless, the identification of any potentially causal pathway widens the scope for possible upstream or downstream therapies.

Whilst several of the marker-cognition associations remained significant in the estimated lifetime change (MHVS-adjusted) models, a weakening of both the magnitude and significance of the findings was observed. This coupled with the association between the markers and the MHVS scores cannot rule out the possibility of some contribution from reverse causation.

Explicitly, vocabulary IQ may predict late-life inflammation and attenuate the inflammation-cognition associations. A similar finding was made by Luciano et al. who investigated CRP and fibrinogen levels with late-life cognitive ability and lifetime cognitive decline (Luciano et al. 2009). In their study (which used the full cognitive data from the LBC1936), IQ from age 11 rendered contemporaneous correlations between cognition and CRP and fibrinogen at age 70 non-significant.

The association of pre-morbid IQ with late-life inflammatory risk factors might occur via reverse causation or through both cognitive ability test scores and levels of inflammatory markers being indicators of some more basic bodily system

integrity i.e. confounding. Some alternative mechanisms that may explain the association include: a direct effect for CRP, with increased levels being found in neuritic plaques and neurofibrillary tangles in the brains of Alzheimer's patients (Schmidt et al. 2002); mediation through vascular disease (Ravaglia et al. 2005); and an association with fibrinogen independent of its inflammatory properties (van Oijen et al. 2005).

Another possible reverse causation pathway can be annotated through socioeconomic status. Lower socioeconomic status in adult life is a strong predictor of raised inflammatory marker levels, including CRP and fibrinogen (Pollitt et al. 2007, Tabassum et al. 2008). These associations weaken when comparing the biomarker levels with childhood socioeconomic status (Gimeno et al. 2007). Adjustment for adult cardiovascular disease risk factors also strongly attenuate the associations between CRP and early life SES.

Effect sizes

In the AAA Trial the maximum standardised effect size found was –0.095 for CRP using the DST to assess age and sex-adjusted cognition. This implies that for every standard deviation change in CRP levels the DST scores change by –0.095 standard deviations. As the standard deviation on the DST was approximately 11 (Table 8.2) the effect size can be roughly interpreted as a 1.0 point decrease on DST performance for a three-fold increase in CRP level. The magnitude of the increase in CRP level was calculated by taking the anti-log of the standard deviation ($e^{1.1}$) of the transformed CRP levels.

Compared to the AAA Trial results and other studies of inflammation and cognition, the magnitudes of the associations found in the ET2DS are similarly modest. the lack of significant findings between CRP and cognition is in disagreement with some, but not all, of the evidence presented in large, prospective studies (Alley et al. 2008, Dik et al. 2005, Schram et al. 2007, Weuve et al. 2006, Yaffe et al. 2003). Given the lack of studies investigating inflammation and cognition in persons with Type 2 diabetes, it is possible that the inflammation-cognition interaction differs in such populations.

Although these effect sizes are small, if inflammation does lie on the causal pathway of cognitive decline then small changes in the levels of these proteins may be greatly magnified through downstream processes in cognitive ageing.

10.1.2 Markers of rheology

AAA Trial

Of the rheological factors tested, baseline plasma viscosity was associated with: late-life general cognitive ability, processing speed, and mental flexibility; estimated lifetime general cognitive decline; and five year decline in mental flexibility. These results are consistent with findings in men participating in the Caerphilly Study (Elwood et al. 2001). Since plasma fibrinogen is a major determinant of plasma viscosity it is not surprising that the findings for both biomarkers were similar (the correlation between the marker levels was 0.454). After adjusting for fibrinogen, plasma viscosity remained significantly associated with scores on the TMT. This could mean that altered rheology, over and above any effect of fibrinogen molecules themselves, accounts for the association between plasma viscosity and cognitive ability. Contrary to the Caerphilly Study, no associations were observed between haematocrit and cognitive ability. While further large prospective studies of haematocrit and cognitive function and decline are required, it is possible that our findings reflect a more important role for plasma viscosity than for haematocrit or whole blood viscosity in determining blood flow in the cerebral microcirculation (Lowe 2001). The magnitude of the plasma viscosity associations are in line with those for CRP and fibrinogen – the maximum standardised β observed was 0.089.

10.1.3 Study strengths

AAA Trial

The main strengths of the AAA Trial included the application of a comprehensive cognitive battery; cognitive change measurements being taken over a five year period (which is at least as long as the follow-up period from previous studies by Schram et al. and Rafnsson et al.); and the assessment of four inter-related measures of inflammation and rheology in a large population-based sample of over 2,000 persons. The AAA Trial is also one of the first studies to analyse plasma viscosity and haematocrit with cognition in a mixed gender population. Therefore, the positive findings for plasma viscosity (including the estimated lifetime change models) are extremely encouraging and warrant further investigation.

Moreover, assessing participants with no history of and no prevalent cardiovascular disease at baseline enabled the measurement of biomarker-cognition associations in a section of the population with minimal evidence of vascular disease. The dampening of the potential confounding affect of CVD on both biomarker levels and cognitive function makes this a unique cohort. Nevertheless, despite these measures to stratify the cohort, there was still large variability in some of the biomarker measurements, most notably CRP. This resulted in genetic markers

of the biomarkers being used in place of the plasma levels as markers of lifetime exposure to inflammation.

ET2DS

The strengths of the ET2DS were the application of a seven test cognitive battery and the assessment of four inter-related measures of inflammation in a large population-based sample of over 1,000 persons. The novel approach of calculating a general inflammation factor enabled a test of the importance of overall systemic inflammation. In conjunction with the analyses of the specific upstream and downstream markers of inflammation, this helped in identifying if and where inflammation is associating with cognitive function. To account for the possibility of the inflammation-cognition relationship being mediated or confounded, comprehensive adjustments were made for cardiovascular risk factors, lifestyle factors, depression, and duration of diabetes. These analyses were amongst the most thorough of those reviewed in the literature for covariate assessment and adjustment.

Furthermore, this was one of the first studies to assess an inflammation-cognition association in persons with Type 2 diabetes. Given that diabetics are at greater risk of developing cognitive decline and that they decline at a faster rate than the normal population, such models provide a somewhat accelerated analysis of cognitive change. This may well become visible in the models of four year cognitive change, which will be calculated after the follow-up analysis in 2010.

10.1.4 Study limitations

AAA Trial

One of the main limitations of the AAA Trial was the potential affect of aspirin on the inflammation-cognition results. However, a recent analysis of the unblinded Trial data found no evidence for an association between aspirin and cognitive ability (Price et al. 2008c). Other limitations of the Trial included the small number of persons in the cognitive subset. Whilst the study was well powered to detect small associations between the baseline marker levels and follow-up cognitive scores, this was not the case in the subset, which was under one quarter of the total population. Limited power in the subset may help to explain differences between the subset analysis and the estimated cognitive decline analysis. Furthermore, a five year follow-up in a relatively young and healthy cohort is going to increase the chance of error and bias due to a practice effect on the

cognitive tests. As already commented, this may have been the underlying cause of the observed association between increased CRP levels and increased memory performance over five years. Other potential flaws of the AAA Trial methodology include the use of single, peripheral measurements of biomarkers. In theory, this may have resulted in regression bias, diluting the associations observed (Schram et al. 2007). Although the AAA Trial cohort was population-based and community-dwelling, bias may have resulted from selective non-response and withdrawal from the trial. However, it is likely that persons with poorer cognitive function were less likely to participate. Indeed, those with missing values at follow-up tended to have worse scores at baseline testing. In totality, the limitations of the study suggest that, if anything, the observed associations are likely to be underestimating the true relationship between the markers and cognitive ability or decline.

ET2DS

The main limitation of the ET2DS is its cross-sectional design. This was partially accounted for by adjusting for a vocabulary measure (MHVS) to gain an insight into the estimated lifetime cognitive change. Clearly, it would be ideal to have an actual pre-morbid measure of cognition, as in the LBC1936. However, the MHVS is a well-validated and robust estimate of peak cognitive ability with vocabulary-based skills varying little across a lifetime.

An actual measure of cognitive change will be possible after participants attend the 4 year follow-up clinic where they will be cognitively tested using the same seven test battery. In parallel with the AAA Trial analysis, the use of a single, peripheral measurement of the biomarkers may again have caused regression dilution bias and an underestimation of the associations found (Emberson et al. 2004). Furthermore, as markers of inflammation are sensitive to acute illness and are influenced by several factors prevalent in old age, it may be questionable whether a single, late-life measurement accurately reflects the lifetime risk of exposure to inflammation.

Whilst the novelty of examining the inflammation-cognition association in persons with Type 2 diabetes was a study strength, it also limited the possibilities for comparisons with other inflammation-cognition papers and the AAA Trial results.

10.1.5 Summary

The results from both the AAA Trial and the ET2DS provide a degree of evidence for a relationship between inflammation, rheology and cognitive ability in old age. Although effect sizes for the associations were small, they were consistent with results from previous studies (Dik et al. 2005, Rafnsson et al. 2007, Schram et al. 2007, Weuve et al. 2006). It is possible that the associations are not causal but the result of other conditions that underlie both cognition and inflammation/rheology. One potential confounding, or mediating factor is cardiovascular disease, which has been shown to be associated with both inflammation (Libby et al. 2002) and cognitive ability (Breteler et al. 1994). However, when both sets of modelling were repeated with cardiovascular risk factors as covariates most of the significant associations remained. Together with the asymptomatic study population in the AAA Trial, this suggests that the relationship between inflammation and cognition is at least partially independent of cardiovascular disease. In addition, as the fibrinogen and CRP levels correlated negatively and strongly with the MHVS scores, there is also a possibility of reverse causation affecting the interpretation of the results. It may be that poorer cognitive ability in earlier life is linked to conditions affecting inflammation in later life, whether directly or through associations with lifestyle and socio-economic status.

To help explain the physiological relationship between inflammation, rheology, and cognitive decline and to determine whether they are causal, it would be useful to consider the associations between cognitive ability and genetic polymorphisms affecting biomarker levels. Indeed such an approach was taken and discussed in detail in Chapter 9. A causal role for inflammatory markers in cognition is certainly plausible, either with or without vascular pathology as an intermediary. Increased plasma viscosity may also have a direct effect on cognitive function by decreasing cerebral blood flow (Elwood et al. 2001, Lowe 2001). If blood viscosity within the low-normal range and maintained brain performance by rehydration is beneficial to the acutely challenged, hypo-perfused brain in older patients, then it may be possible to extend the potential link between blood viscosity and brain function to chronic cognitive decline in the general population (Lowe 2001).

In conclusion, it has been shown that raised circulating inflammatory and rheological biomarker levels are contemporaneously and prospectively associated with poorer late-life cognitive ability even after adjustment for pre-morbid IQ. These findings are particularly notable for the upstream inflammatory marker IL-6, the downstream inflammatory/rheological marker fibrinogen, and the rheological marker plasma viscosity. Future longitudinal and genetic studies examining

these markers are required to indicate the reliability of the findings and whether the associations being observed are causal.

10.2 Genetic associations

10.2.1 *CRP*

Combining data from four large-scale population-based studies it appears unlikely that CRP has a direct and causal effect on cognitive ability. However, significant associations were observed between *CRP* SNPs and plasma CRP levels. Moreover, plasma CRP levels also associated with some of the measures of late-life cognition and consistently across all four cohorts with estimated or actual prior cognitive ability. Upon adjusting the plasma CRP-cognition models for vocabulary scores, the previously significant associations were attenuated in three of the studies and considerably weakened in the AAA Trial. The strength of the LBC1936 study design with cognition assessed at both age 11 and age 70 adds substantial weight to the reverse causation hypothesis proposed. It is entirely possible that decreased cognitive ability in early life leads to an increased risk of late-life inflammation via its association with health knowledge, lifestyle, and socio-economic status (Beier & Ackerman 2003).

The null findings from the main analysis of the cognitive test scores by *CRP* SNP genotypes are not altogether surprising. Previous Mendelian randomisation studies of the *CRP* gene have failed to directly implicate it in a variety of conditions (Brunner et al. 2008, Davey Smith et al. 2004, Kivimäki et al. 2008, Timpson et al. 2005). It is possible, but unlikely, that a direct *CRP* SNP cognition association does exist and that our results can be explained by a failure to genotype the causal SNP or a SNP in high LD with the causal variant. Analysing some of the strongest SNP predictors of CRP levels (Verzilli et al. 2008) enables us to place a reasonable degree of confidence in our results. In all likelihood, these data point towards CRP being a clinical correlate but not a causal factor for determining cognitive ability and in the development of cognitive decline.

10.2.2 Fibrinogen

Combining data from three large-scale population-based studies, significant associations were shown between plasma fibrinogen levels and cognition, between fibrinogen SNPs and plasma fibrinogen levels, and between fibrinogen SNPs and cognition. To my knowledge, this is the first study to have examined the relation-

ship between fibrinogen genes and cognition. Whilst no single result replicated across the three studies, the findings do suggest a potentially causal role for fibrinogen in cognitive ageing.

The initial set of analyses showed elevated plasma fibrinogen levels to associate with poorer cognitive function. Adjustments for an estimate or measure of pre-morbid cognitive ability made little difference to these results, implying a relationship between raised fibrinogen levels and estimated or actual lifetime cognitive decline. However, the weakly significant associations in the LBC1936 did disappear after adjustment for age-11 scores on the MHT.

Modelling SNPs from the *FGA* and *FGB* genes against age and sex-adjusted plasma fibrinogen levels showed rs4220 and rs2070016 to be the best predictors of plasma fibrinogen levels. However, given that only three SNPs were typed on more than one occasion, this conclusion is based largely upon the associations in the AAA Trial. By contrast, a previous study found no association of rs4220 and rs2070016 with fibrinogen levels, but a significant finding for rs2070011 after a correction for multiple testing (Jacquemin et al. 2008).

After rejecting a Mendelian randomisation approach due to the potentially large confounding effects on late-life fibrinogen levels, the main analysis modelled directly the association between fibrinogen SNPs and cognitive ability. Taking this approach, very strong evidence was found for associations between rs2227412 and cognition, even after the most stringent correction for multiple testing (Bonferroni adjustment). These associations were strongest for the general intelligence and mental flexibility/processing speed domains. For the rs2227412 SNP, carriers of the heterozygous GA alleles were found to be at greatest risk of poorer cognitive ability and greater lifetime cognitive decline. Weakly significant associations were also found between cognition and rs2070016, rs2070022 and rs4220.

Furthermore, the genotypes that were found to be linked to impaired cognition also tended to be associated with higher levels of fibrinogen. Of the significant SNPs, rs2227412 (an intronic SNP) and rs4220 (a non-synonymous coding SNP) lie in the *FGB* gene, which is involved in fibrinogen synthesis, whilst rs2070016 (an intronic SNP) is in the *FGA* gene, which helps determine clot structure (Lim et al. 2003).

10.2.3 Other Genes

Despite a few weakly significant results, the predominant evidence was for no association between the *TCF-1*, *IL-1α*, *IL-1β*, *F13A1*, and *TNF-α* genes and cognition. A single association was significant at the $p < 0.01$ level: rs1800630

(*TNF-α*) and verbal declarative memory in the ET2DS. This association was retained at the $p < 0.05$ threshold in the estimated lifetime decline model.

10.2.4 Study strengths

The strengths of these analyses include their incorporation of four large-scale, elderly population-based cohorts. All studies were based in Scotland and were ethnically homogenous. In addition to the estimates of pre-morbid IQ, the LBC1936 had an age-11 IQ score, which was adjusted for to gain an insight into actual lifetime cognitive decline. The four cohorts provided the opportunity for a large and comprehensive analysis of the association between late-life CRP and fibrinogen levels and cognitive ability. To my knowledge, this is the first study to have looked at variants in the genes that encode CRP and fibrinogen and cognitive ability. For both markers it was decided to model the SNP-cognition associations directly as opposed to taking a Mendelian randomisation-type approach. The latter randomises the CRP (fibrinogen) levels to *CRP* (fibrinogen) SNP genotypes before modelling against cognition. However, as CRP (fibrinogen) levels are extremely sensitive to acute illness and influenced by several factors prevalent in old age, the approach taken centred around the *CRP* (fibrinogen) SNPs being better indicators of lifetime exposure to plasma CRP (fibrinogen) than late-life plasma CRP (fibrinogen) levels. Despite slightly different cognitive batteries being administered in each of the cohorts, there was considerable overlap among the tests, which allowed for close comparisons to be made with regard to specific cognitive domains.

10.2.5 Study limitations

Some of the study weaknesses include the cross-sectional nature of the ET2DS data although this should have no effect upon the SNP-cognition associations. In addition, future studies would ideally take an early-midlife measure of the biomarkers, instead of relying upon SNPs as being proxy measures of lifetime exposure to CRP. Such data were available in the Honolulu-Asia Aging Study, which showed midlife CRP levels to predict dementia 25 years later (Schmidt et al. 2002). The similarity in age and location of our cohorts means that caution should be taken over whether the results could be generalised to other elderly populations, though that expectation seems reasonable. Finally, the AAA Trial, the EAS, and the LBC1936 were all far healthier cohorts than the ET2DS. As mentioned previously, persons with Type 2 diabetes are at greater risk of develop-

ing cognitive decline and also decline at a faster rate. Whether the associations seen between rs2227412 and cognition are exclusive to diabetics or if they are only seen after a certain degree of cognitive ageing is unclear.

10.2.6 Summary

Fibrinogen

The genetic analyses showed associations between both fibrinogen levels and fibrinogen genes with cognitive ability. Despite a lack of consistent evidence across all three studies, the results provide a degree of support for a causal association. Notably, the strongest SNP-cognition associations were found in the cross-sectional, diabetic cohort. Although similar in age to the other cohorts, this group's cognitive scores were significantly lower. It is thus possible that the relationship of fibrinogen genotypes with cognition only becomes apparent after a certain degree of cognitive decline. Clearly, replication and further study are required to confirm or refute this claim and also to determine the pathways through which fibrinogen may operate.

CRP

By contrast to the fibrinogen analyses, there was no evidence for an association between *CRP* SNPs and cognitive ability. However, a consistent finding of associations between plasma CRP and a prior intelligence estimate or measure does suggest a possible reverse causation mechanism with poorer cognition in early life resulting in both raised CRP and independently, poorer cognition in later life.

Other genes

Similar to the findings for the *CRP* SNPs, null results were also observed for the other genetic markers investigated. Perhaps there is further scope for investigating the *TNF-α* gene and the rs1800630 SNP although a thorough literature review of the variants in this and the other genes assessed is required before proceeding. The SNPs typed in the AAA Trial and the ET2DS have provided at best, a brief snapshot of these genes' variation. Although the results favoured null associations, it would be a somewhat bold move to exclude them completely as causal factors on the cognitive decline pathway.

Causal pathways and mechanisms

The results of these genetic analyses support the evidence for an association

between elevated fibrinogen levels and cognitive dysfunction and decline (Elwood et al. 2001, Lowe 2001, Rafnsson et al. 2007). Raised fibrinogen levels have also been associated with risk of both vascular dementia and Alzheimer's disease (van Oijen et al. 2005), and cross-sectionally with ischemic stroke and vascular dementia (Stott et al. 2001). However, other studies have failed to replicate these findings (Mari et al. 1996, Sevush et al. 1998). Additionally, associations have been observed between fibrinogen haplotypes but not plasma levels and cerebral small vessel disease (van Oijen et al. 2008), which is a risk factor for stroke and dementia (Vermeer et al. 2003). The potential role of fibrinogen in cognitive dysfunction is uncertain although current opinion leans towards its haemostatic and rheological, as opposed to inflammatory properties (Lowe 2001, Rafnsson et al. 2007, van Oijen et al. 2005).

The study by van Oijen et al. investigating the relationship between the markers CRP and fibrinogen with dementia found only the latter to be a significant predictor. Similarly, the results of this thesis showed significant associations between fibrinogen SNPs and cognitive ability using data from the AAA Trial, ET2DS, and LBC1936 cohorts. Combining this information with the null *CRP* SNP–cognition associations suggests that more focus should be placed on the haemostatic and rheological characteristics of fibrinogen as opposed to its inflammatory properties and its inflammatory correlates. Indeed, the correlation between fibrinogen and CRP levels is typically quite strong (0.40 in the study by van Oijen et al., 0.44–0.58 in the four studies analysed here) so perhaps the previously reported CRP-cognition associations are merely using CRP as a proxy marker for fibrinogen. To elaborate on its non-inflammatory properties, elevated levels of fibrinogen increase blood clotting, which may subsequently reduce cerebral blood flow, causing brain damage (Lowe 2001).

10.3 Conclusions and future directions

The primary aim of this thesis was to identify the evidence for associations between biomarkers of inflammation and cognitive ability and decline in old age. These aims were addressed using a two-fold approach. Firstly, plasma levels of the biomarkers (CRP, fibrinogen, plasma viscosity, haematocrit, TNF-α, and IL-6) were modelled against late-life cognitive ability and estimated lifetime cognitive decline in two large-scale, community-dwelling cohorts – the AAA Trial and the ET2DS. The second step of the analysis took a genetic approach, using SNP information from the genes that encode the biomarkers to determine the evidence

for a causal association between inflammation and impaired cognitive ability and cognitive decline.

The initial biomarker cognition analyses showed strongly significant prospective associations between late-life CRP, fibrinogen, and plasma viscosity levels and cognitive ability after five years of follow-up. These associations were most pronounced for the cognitive tests that measured processing speed and also the general intelligence factor. In addition, the associations persisted after adjustment for vocabulary scores, which implies a relationship between inflammation and lifetime cognitive decline as well as with late-life cognitive ability. Despite being cross-sectional in design, the ET2DS data showed evidence for associations between fibrinogen, TNF-α, and most strongly with IL-6 and cognition. Again, the significant results were retained after adjustment for the vocabulary-based estimate of pre-morbid IQ. Whilst most studies in the literature had not used a pre-morbid measure of IQ to estimate lifetime cognitive change, the small magnitudes of the late-life cross-sectional and prospective associations were in line with those previously presented.

The genetic analysis used data from two replication cohorts, the EAS and the LBC1936 in addition to the AAA Trial and the ET2DS. The ET2DS analysis showed significant associations between the rs2227412 *FGB* SNP and cognitive ability. These findings were not replicated across the other cohorts. Whether this is a false positive finding, a diabetes-specific association, or a result that is more pronounced as cognitive performance decreases is unclear. Whilst the cohorts were very well matched for age, the ET2DS scored consistently and significantly lower on the cognitive tests. For the other marker gene SNPs (*CRP*, *TCF-1*, *IL-1*α, *IL-1*β, *F13A1*, and *TNF-*α) null or weakly significant associations were found.

The biggest strength of this thesis is that it is one of the first times inflammatory biomarker SNPs have been assessed in relation to cognitive ability. Whilst this limits the ability to compare the results with other studies, the novel research has generated some exciting results and laid the foundations for further work in this field.

Other novelties of this thesis include: the analysis of the inflammation cognition associations in persons with Type 2 diabetes; the derivation of an overall inflammatory factor that was modelled against the cognitive scores in the ET2DS; the study of the rheological variable, plasma viscosity, with cognition in a community-dwelling population of both men and women; the assessment of late-life inflammatory biomarkers with 59-year cognitive decline in the LBC1936 repli-

cation cohort; and the use of a Bayesian regression model to perform a Mendelian randomisation analysis.

In terms of recommendations for future studies, it would be ideal for studies to have serial and longitudinal measures of both inflammatory biomarkers and cognitive ability across the human lifespan. Clearly, this would be an expensive and time-consuming exercise. In the meantime, I would recommend future studies to include a crystallised measure of intelligence – such as the MHVS or NART – as part of their cognitive assessment. Not only would this allow for an estimate of lifetime cognitive decline but it would also add to the body of growing evidence to suggest a reverse causation mechanism between late-life biomarker–cognition associations through early life cognitive ability. The results of the analysis in this thesis also point towards the importance of rheological and haemostatic factors in addition to inflammation in predicting late-life cognitive ability. In particular, the associations between plasma viscosity, plasma fibrinogen, and the *FGA* and *FGB* genes with cognition and cognitive decline warrant replication and further investigation.

Closing remark

The results of this thesis have identified a potentially causal risk factor in the process of cognitive ageing. Whilst, replication of the associations between the fibrinogen SNPs and cognition were not consistent over three studies, the combination of evidence indicates something over and above a false positive finding. Notably, the heterozygous genotype for the rs2227412 *FGB* SNP associated with increased fibrinogen levels, poorer cognitive performance, and increased cognitive decline in a cohort of 1,066 persons with Type 2 diabetes. To date, the majority of inflammation-cognition associations have focussed on the acute-phase protein CRP. The main outcomes from this thesis suggest that its close correlate, fibrinogen, is an equally, if not more important factor in the complex process of cognitive ageing.

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Appendix A

AAA Trial: $APO\epsilon$–cognition associations

```
### AVLT ###

lm(formula = fAVLT ~ Age + Sex + APOE, data = AAA)

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 100.97284    4.62311  21.841   <2e-16 ***
Age          -0.52492    0.06048  -8.679   <2e-16 ***
SexM         -7.73929    0.87143  -8.881   <2e-16 ***
APOEe2e3      1.85050    2.63195   0.703    0.482
APOEe2e4     -1.23080    3.55942  -0.346    0.730
APOEe3e3     -0.27416    2.42319  -0.113    0.910
APOEe3e4     -0.69777    2.51362  -0.278    0.781
APOEe4e4     -3.62035    3.75895  -0.963    0.336
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

### RAVENS ###

lm(formula = fRAVENS ~ Age + Sex + APOE, data = AAA)

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 63.35087    2.58461  24.511  < 2e-16 ***
```

```
Age         -0.43507    0.03404 -12.780  < 2e-16 ***
SexM         1.65709    0.48174   3.440 0.000597 ***
APOEe2e3     1.08932    1.44103   0.756 0.449805
APOEe2e4    -0.98802    1.95303  -0.506 0.613005
APOEe3e3     0.49080    1.32249   0.371 0.710599
APOEe3e4    -0.04059    1.37600  -0.030 0.976468
APOEe4e4    -2.34450    2.04319  -1.147 0.251363
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

### VFT ###

lm(formula = fVFT ~ fAge + Sex + APOE, data = AAA)

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 45.19938    3.67556  12.297   <2e-16 ***
fAge        -0.10930    0.04837  -2.260   0.0240 *
SexM        -0.58830    0.69684  -0.844   0.3987
APOEe2e3     0.55757    2.06981   0.269   0.7877
APOEe2e4    -0.19963    2.75584  -0.072   0.9423
APOEe3e3     0.05874    1.89907   0.031   0.9753
APOEe3e4    -0.77910    1.97300  -0.395   0.6930
APOEe4e4     1.94960    3.03687   0.642   0.5210
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

### DST ###

lm(formula = fDST ~ fAge + Sex + APOE, data = AAA)

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 83.09313    3.00751  27.629  < 2e-16 ***
fAge        -0.63158    0.03932 -16.062  < 2e-16 ***
SexM        -4.40919    0.56763  -7.768 1.38e-14 ***
APOEe2e3     1.91114    1.71171   1.117    0.264
```

```
APOEe2e4     1.78532    2.27392   0.785    0.432
APOEe3e3     0.99043    1.57463   0.629    0.529
APOEe3e4     0.30485    1.63404   0.187    0.852
APOEe4e4    -2.14914    2.45146  -0.877    0.381
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

### TMT ###

lm(formula = log(fTMT) ~ fAge + Sex + APOE, data = AAA)

Residuals:
     Min       1Q   Median       3Q      Max
-1.05805 -0.27184 -0.03239  0.23173  2.08397

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  3.296244   0.110924  29.716  < 2e-16 ***
fAge         0.018896   0.001455  12.989  < 2e-16 ***
SexM         0.083966   0.020947   4.009 6.38e-05 ***
APOEe2e3    -0.054276   0.062954  -0.862    0.389
APOEe2e4     0.095416   0.084144   1.134    0.257
APOEe3e3     0.026141   0.057878   0.452    0.652
APOEe3e4     0.041574   0.060101   0.692    0.489
APOEe4e4     0.041150   0.090052   0.457    0.648
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1

### g ###

lm(formula = follow-up.g ~ Age + Sex + APOE, data = AAA)

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  3.406544   0.284548  11.972  < 2e-16 ***
fAge        -0.050255   0.003744 -13.423  < 2e-16 ***
SexM        -0.270226   0.053028  -5.096 3.91e-07 ***
```

```
APOEe2e3     0.186092   0.159237   1.169    0.243
APOEe2e4    -0.104824   0.214190  -0.489    0.625
APOEe3e3     0.053004   0.146581   0.362    0.718
APOEe3e4    -0.003060   0.152422  -0.020    0.984
APOEe4e4    -0.185932   0.226567  -0.821    0.412
---
Signif. codes:  0 *** 0.001 ** 0.01 * 0.05 . 0.1   1
```

Appendix B

JAGS code for Bayesian Mendelian randomisation

```
## Remove all objects from the current R environment
rm(list=ls())

## Load the genetics library
library(genetics)

## Load the snpMatrix library
library(snpMatrix)

#################################################################
## AAA Trial dataset - CRP
#################################################################

## Read the AAA Trial data from a text file
data <- read.table("AAA.txt", header=T)

## Convert the four CRP SNP data into genotypes
genotypes <- data[,21:24]

## Should check that pairs of alleles are ordered alphabetically
## Factor levels are ordered alphabetically

## Re-writes the genotypes as integers with either 0, 1, or 2
## copies of the first (alphabetically) allele and prints the
```

```
## Hardy-Weinberg p-value for each of the SNPs.

for(col in 1:dim(genotypes)[2]) {
  cat(dimnames(genotypes)[[2]][col], table(genotypes[, col]))
  genotypes[, col] <- as.integer(factor(genotypes[, col])) - 1
  cat(" HWE p-value",
  HWE.chisq(as.genotype.allele.count(genotypes[, col]))$p.value, "\n")
}

## Choose SNPs and remove those out of Hardy-Weinberg equilibrium
g <- as.matrix( genotypes[, dimnames(genotypes)[[2]]])

## The mean of the genotypes -- a number between 0 and 2
g.bar <- apply(g, 2, mean, na.rm=TRUE)

## The total number of SNPs
T <- dim(g)[2]

## The number of cases
N <- dim(data)[1]

## The outcome variable, general intelligence
y <- data$follow.g - 1

## Age at follow-up
age <- data$fAge

## Age centred about zero
age <- age - mean(age, na.rm=TRUE)

## Sex, re-written as integers
sex <- as.integer(data$Sex)

## Mean sex i.e. proportion of men in the study
sex.bar <- mean(sex, na.rm=TRUE)

## The intermediate variable, log of baseline CRP, which is standardised
```

```
x <- log(data$bCRP)
x.bar <- mean(x, na.rm=TRUE)
x.sd <- sd(x, na.rm=TRUE)
x <- x - x.bar
x <- x / x.sd

## Plausible measurement precision for x
tau.xmeas <- 4

## The variance of the ratio of causal:crude evidence for an
## association between CRP and g was set to 25, which corresponds
## to a precision of 1/25 = 0.04
tau.theta <- 0.04

## Writes the list of variables to the file crpdata.R
dump(c("N", "y", "x", "age", "sex", "sex.bar", "T", "g", "g.bar",
       "tau.xmeas", "tau.theta"),
     file="crpdata.R")

## Prints the number of genotypes typed for each SNP
nogenotypes <- logical(N)
for(i in 1:N) {
 nogenotypes[i] <- !sum(as.integer(!is.na(genotypes[i, ])))
}
cat("Number genotyped", dim(genotypes[!nogenotypes, ])[1], "\n")


## Standard regression analyses
## CRP and 'g' on genotype
for(locus in (1:dim(genotypes)[2])) {
  cat("\nRegression of x and y on", dimnames(genotypes)[[2]][locus], "\n")
  print(summary.glm(glm(x ~ age + sex + genotypes[, locus],
                        family="gaussian"))$coefficients[4, ])
  print(summary.glm(glm(y ~ age + sex + genotypes[, locus],
                        family="gaussian"))$coefficients[4, ])
}
```

```
## Regression of 'g' on CRP
cat("\nRegression of y on x\n")
print(summary.glm(glm(y ~ age + sex + x,
                      family="gaussian"))$coefficients[4, ])


#################################################################

## The model - built in JAGS

## Observed data: Continuous outcome y ('g'), intermediate phenotype x
## (plasma CRP), SNP genotype g (CRP SNPs).
## Unmeasured confounder c (age and sex) has effects on both x and y.

model{
  ## Coefficients for regression of x on g
  ## Diffuse Gaussian priors for regression coefficients
  ## Diffuse Gamma prior on tau.x, the precision of plasma CRP
  ## Beta prior on locus provides weight towards heterozygotes
  beta0.x ~ dnorm(0, 0.001);
  for(locus in 1:T) {
    beta.gx[locus] ~ dnorm(0, 0.001);
    phi[locus] ~ dbeta(0.5, 0.5);
  }
  beta.sex.x ~ dnorm(0, 0.001);
  beta.age.x ~ dnorm(0, 0.001);
  tau.x ~ dgamma(0.01, 0.01);

  ## Coefficients for regression of y on resid.x -- diffuse Gaussian priors
  beta0.y ~ dnorm(0, 0.001);
  beta.ry ~ dnorm(0, 0.001);
  beta.sex.y ~ dnorm(0, 0.001);
  beta.age.y ~ dnorm(0, 0.001);
  theta ~ dnorm(0, tau.theta);

  ## Beta prior on allele freq
  phi.ns ~ dbeta(0.5, 0.5);
```

```
## Beta prior on sex ratio
phi.sex ~ dbeta(1, 1);

## Gaussian prior on age with Gamma prior for precision
mu.age ~ dnorm(0, 0.001);
tau.age ~ dgamma(0.001, 0.001);

## Gamma prior for precision of 'g'
tau.y ~ dgamma(0.001, 0.001);

## Each genotype allele is Binomially distributed
for(i        in 1:N) {
  for(locus in 1:T) {
    g[i, locus] ~ dbin(phi[locus], 2);
  }

  ## Sex follows a Bernoulli distribution
  sex[i] ~ dbern(phi.sex);

  ## Age follows a Normal distribution
  age[i] ~ dnorm(mu.age, tau.age);

  ## A beta weight for the genotypes, which combines information
  ## from all SNPs typed
  beta.gxg[i] <- sum(beta.gx * (g[i, ] - g.bar));

  ## Effect of genotype and confounder on intermediate phenotype
  mu.x[i] <- beta0.x +
             beta.sex.x * (sex[i] - sex.bar) + beta.age.x * age[i] +
             beta.gxg[i];

  ## True value of intermediate phenotype
  xlatent[i] ~ dnorm(mu.x[i], tau.x);

  ## xmeas is measurement precision
  x[i] ~ dnorm(xlatent[i], tau.xmeas);
```

```
    ## Random effect on x includes confounder
    resid.x[i] <- x[i] - mu.x[i];

    ## Regression of y on resid.x under H0
    mu.y[i] <- beta0.y  +
               beta.sex.y * (sex[i] - sex.bar) + beta.age.y * age[i] +
               beta.ry * (theta * mu.x[i] + resid.x[i]);
    y[i] ~ dnorm(mu.y[i], tau.y);
  }
}

#################################################################

## Commands to run the model in JAGS and notification of the parameters
## to be monitored and stored from the analysis

model clear
model in crp.bug
data in crpdata.R
compile, nchains(2)
parameters in init1.txt, chain(1)
parameters in init2.txt, chain(2)
initialize
samplers to samplers.txt
adapt 100
monitor beta.gx
monitor tau.x
monitor beta.ry
monitor tau.y
monitor theta
update 1000
monitors to monitors.txt
coda *
exit

#################################################################
```

```
rm(list=ls())
library(rjags)
library(coda)

## A function to calculate the log likelihood function for theta.

loglikFromSamplesN <- function(mu=0, sigmasq, x.samples, bwspec=0.25) {

  ## prior N(mu, sigmasq), posterior samples x.samples
  ## log likelihood  = log (posterior weighted by inverse of prior density)
  ## invprior <- exp(0.5* (x.samples - mu)^2 / sigmasq) *
  ##             sqrt(2 * pi * sigmasq)

  invprior <- 1/ dnorm(x.samples, mean=mu, sd=sqrt(sigmasq))
  invsum <- 1 / sum(invprior)
  invprior <- invsum * invprior
  d <- density(x.samples, weights=invprior, bw=bwspec)
  x <- d[[1]]
  logL <- log(d[[2]])

  ## Scale logL to have value zero at first positive value of x
  ## FIXME - use interpolation

  logL <- logL - logL[match(TRUE, x > 0)]
  return(cbind(x, logL))
}

###############################################################

source(file="crpdata.R")

## Generate initial values from two chains to reduce probability of
## model converging to local optima
theta <- 0
beta.ry <- 0.5
tau.x <- 0.8
```

```
dump(c("theta", "tau.x"), file="init1.txt")
theta <- 1
beta.ry <- 0
tau.x <- 0.4
dump(c("theta", "tau.x"), file="init2.txt")

## Run JAGS
t0 <- Sys.time()
system("jags instrumental.jmd")
cat("Time elapsed", Sys.time() - t0, "\n")

## Collate the output from the MCMC
s.instrumental <- mcmc.list(read.coda("CODAchain1.txt", "CODAindex.txt"),
                            read.coda("CODAchain2.txt", "CODAindex.txt"))

## Read the MCMC output into the file 's.instrumental.R'
dput(s.instrumental, file="s.instrumental.R")

################################################################

s.instrumental <- dget("s.instrumental.R")

## Prior variance
sigmasq <- 25

theta.samples <- data.frame(s.instrumental[[1]])$theta

## Log likelihood function for theta
z <- loglikFromSamplesN(0, sigmasq, theta.samples, 0.25)

## Plot diagnostics
par(las=1, cex=2.5)
pdf("traceplots.pdf")
plot(s.instrumental)
dev.off()
pdf("Gelmandiagnostics.pdf")
gelman.plot(s.instrumental)
```

```
dev.off()
pdf("autocorrelations.pdf")
autocorr.plot(s.instrumental)
dev.off()

## Calculate and plot likelihood function
sigmasq <- 1 / tau.theta # prior variance
theta.samples <- data.frame(s.instrumental[[1]])$theta
z <- loglikFromSamplesN(0, sigmasq, theta.samples, 0.25)

pdf("thetalik.pdf")
plot(z[, 1], z[, 2], type="l",
     xlab="Ratio causal / crude effect of \nintermediate phenotype on outcome",
     xlim=c(-1, +2),  ylab="Log-likelihood (nats)", ylim=c(-2, 2), main="",
     cex=2.5, axes=FALSE)
axis(side=1, pos=0)
axis(side=2)
dev.off()

#################################################################
```

Appendix C

Supplementary tables for fibrinogen genetic analysis

AAA Trial

	rs2070022					
	CC		CT		TT	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
AVLT	-0.004 (0.98)	1254	0.040 (1.05)	607	-0.220 (0.93)	89
RAVENS	0.021 (0.99)	1204	-0.044 (1.02)	601	0.015 (1.01)	85
VFT	-0.023 (0.99)	1276	0.009 (1.00)	628	0.253 (1.07)	93
DST	0.010 (0.98)	1295	-0.037 (1.05)	639	0.110 (0.96)	93
ln(TMT)	-0.017 (1.00)	1287	0.037 (1.01)	625	-0.021 (0.87)	94
g	0.010 (0.98)	1146	-0.026 (1.04)	567	0.035 (0.96)	83

	rs2070016					
	CC		CT		TT	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
AVLT	-0.019 (1.00)	40	-0.047 (1.02)	495	0.017 (0.99)	1422
RAVENS	-0.162 (0.84)	39	0.021 (1.01)	474	-0.003 (1.00)	1384
VFT	0.025 (1.05)	42	0.030 (1.07)	502	-0.011 (0.98)	1458
DST	-0.275 (1.01)	45	-0.011 (1.00)	506	0.012 (1.00)	1482
ln(TMT)	0.217 (0.91)	45	0.016 (1.05)	506	-0.012 (0.98)	1461
g	-0.168 (1.02)	35	-0.005 (1.02)	453	0.006 (0.99)	1314

	rs4220					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
AVLT	-0.038 (1.07)	53	-0.028 (1.01)	551	0.013 (0.99)	1338
RAVENS	-0.130 (0.87)	51	0.048 (1.02)	528	-0.014 (1.00)	1303
VFT	-0.161 (0.86)	53	0.071 (1.06)	561	-0.023 (0.98)	1373
DST	-0.242 (1.02)	57	0.017 (1.01)	567	0.003 (0.99)	1394
ln(TMT)	0.213 (0.92)	57	-0.019 (1.05)	561	-0.001 (0.98)	1379
g	-0.154 (1.04)	47	0.045 (1.03)	504	-0.012 (0.99)	1236

	rs2227412					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
AVLT	-0.009 (0.98)	1291	0.037 (1.04)	581	-0.139 (0.94)	71
RAVENS	0.026 (0.99)	1246	-0.051 (1.02)	570	-0.041 (1.00)	67
VFT	-0.023 (1.01)	1315	0.025 (0.98)	601	0.210 (1.08)	74
DST	0.026 (0.99)	1335	-0.063 (1.03)	610	0.053 (0.98)	74
ln(TMT)	-0.015 (1.02)	1324	0.027 (0.99)	599	0.048 (0.81)	75
g	0.017 (0.99)	1181	-0.037 (1.03)	544	-0.0003 (0.95)	65

Table C.1: AAA Trial: Standardised age and sex-adjusted cognitive test scores by fibrinogen genotype.

ET2DS

	rs2070016					
	CC		CT		TT	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	-0.003 (0.99)	19	0.068 (1.01)	241	-0.023 (1.00)	725
LNS	0.039 (0.73)	19	0.054 (0.96)	254	-0.020 (1.02)	718
VFT	-0.181 (0.79)	20	0.043 (0.94)	255	-0.010 (1.02)	727
DST	-0.002 (1.24)	20	0.022 (0.94)	253	-0.007 (1.01)	727
ln(TMT)	0.284 (1.06)	20	0.004 (0.97)	253	-0.009 (1.01)	722
FACES	-0.222 (0.83)	20	0.055 (1.02)	253	-0.013 (1.00)	728
LM	0.073 (0.96)	20	-0.027 (1.01)	252	0.007 (1.00)	720
g	-0.084 (0.92)	19	0.019 (0.98)	247	-0.005 (1.01)	699

	rs4220					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	-0.111 (0.93)	27	0.038 (0.99)	280	-0.011 (1.01)	686
LNS	-0.192 (0.83)	27	0.047 (1.00)	282	-0.012 (1.01)	680
VFT	-0.260 (0.79)	27	0.054 (0.97)	285	-0.012 (1.02)	689
DST	0.036 (0.99)	27	-0.027 (0.95)	284	0.010 (1.02)	687
ln(TMT)	0.118 (0.88)	27	0.065 (0.99)	283	-0.031 (1.01)	683
FACES	-0.067 (1.11)	27	-0.025 (0.99)	284	0.013 (1.00)	689
LM	-0.233 (0.96)	27	-0.005 (0.99)	281	0.011 (1.01)	683
g	-0.234 (0.77)	27	-0.007 (0.95)	274	0.012 (1.03)	661

	rs2227412					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	0.055 (0.99)	705	-0.138 (1.03)	279	-0.019 (0.93)	30
LNS	0.049 (1.00)	702	-0.140 (0.98)	277	0.133 (1.02)	31
VFT	0.074 (0.99)	710	-0.175 (1.00)	281	-0.112 (1.04)	31
DST	0.077 (0.97)	711	-0.219 (1.03)	277	0.204 (1.17)	31
ln(TMT)	-0.053 (0.99)	707	0.147 (1.01)	277	-0.112 (1.06)	30
FACES	0.042 (0.97)	709	-0.103 (1.07)	281	-0.015 (1.01)	31
LM	0.018 (1.01)	703	-0.061 (0.98)	278	0.146 (1.04)	31
g	0.075 (0.97)	686	-0.207 (1.04)	268	0.143 (1.04)	29

Table C.2: ET2DS: Standardised age and sex-adjusted cognitive test scores by fibrinogen genotype.

LBC1936

	rs2070016					
	CC		CT		TT	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	0.072 (0.91)	17	-0.080 (1.02)	237	0.022 (1.00)	791
LNS	0.575 (1.12)	17	-0.093 (1.07)	235	0.015 (0.97)	786
VFT	-0.051 (0.89)	17	-0.026 (1.00)	237	0.009 (1.00)	791
DST	-0.095 (0.88)	17	-0.032 (0.99)	237	0.012 (1.01)	790
LM	0.241 (0.74)	17	-0.070 (0.99)	236	0.016 (1.01)	794
g	0.223 (0.83)	17	-0.089 (1.03)	235	0.022 (0.99)	778

	rs4220					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	0.225 (0.80)	25	-0.077 (1.04)	274	0.020 (0.99)	754
LNS	0.159 (0.91)	25	-0.055 (1.04)	272	0.015 (0.99)	749
VFT	-0.005 (1.05)	25	-0.040 (1.01)	274	0.015 (0.99)	754
DST	0.082 (0.83)	25	-0.084 (0.98)	273	0.028 (1.01)	754
LM	-0.034 (0.80)	25	-0.111 (1.01)	274	0.042 (1.00)	756
g	0.124 (0.74)	25	-0.117 (1.03)	271	0.039 (1.00)	742

	rs2227412					
	AA		GA		GG	
Cognitive Test	mean (s.d.)	n	mean (s.d.)	n	mean (s.d.)	n
MR	0.019 (1.00)	704	-0.026 (1.00)	300	-0.166 (1.04)	35
LNS	0.009 (1.02)	699	-0.018 (0.96)	299	-0.019 (1.01)	34
VFT	-0.033 (0.99)	702	0.075 (1.00)	302	0.019 (1.11)	35
DST	0.003 (1.00)	704	-0.002 (0.99)	299	-0.041 (1.08)	35
LM	0.020 (0.99)	704	-0.017 (1.02)	302	-0.246 (0.97)	35
g	0.010 (0.99)	694	-0.013 (1.01)	296	-0.082 (1.04)	34

Table C.3: LBC1936: Standardised age and sex-adjusted cognitive test scores by fibrinogen genotype.

Appendix D

Publications

Published

Luciano, M., Marioni, R. E., Gow, A. J., Starr, J. M., and Deary, I. J. (2009), 'Reverse causation in the association between C-reactive protein and fibrinogen levels and cognitive abilities in an aging sample', *Psychosom Med.* **71**, 404-409.

In Press

Marioni, R. E., Stewart, M. C., Murray, G. D., Deary, I. J., Fowkes, F. G. R., Lowe, G. D. O., Rumley, A., and Price, J.F. 'Peripheral levels of fibrinogen, C-reactive protein, and plasma viscosity predict future cognitive decline in non-demented individuals', *Psychosom Med.*

Submitted

Marioni, R. E., Deary, I. J., Murray, G. D., Lowe, G. D. O., Strachan, M. W. J., Luciano, M., Houlihan, L. M., Gow, A. J., Harris, S. E., Stewart, M. C., Rumley, A., Fowkes, F. G. R., and Price, J. F. 'Genetic evidence for a causal association between fibrinogen and both late-life cognitive ability and estimated lifetime cognitive decline'

Marioni, R. E., Deary, I. J., Murray, G. D., Lowe, G. D. O., Rafnsson, S. B., Strachan, M. W. J., Luciano, M., Houlihan, L. M., Gow, A. J., Harris, S. E., Stewart, M. C., Rumley, A., Fowkes, F. G. R., and Price, J. F. 'Genetic variants of C-reactive protein are not associated with late-life cognitive ability in four Scottish samples'

Deary, I. J., Corley, J., Gow, A. J., Harris, S. E., Houlihan, L. M., Marioni, R. E., Penke, L., Rafnsson, S. B., and Starr, J. 'Age-associated cognitive decline'

In Preparation

Marioni, R. E., Strachan, M. W. J., Reynolds, R., Lowe, G. D. O., Mitchell, R. J., Fowkes, F. G. R., Frier, B., Lee, A., Butcher, I., Rumley, A., Murray, G. D., Deary, I. J., and Price, J. F. 'Peripheral levels of upstream in flammatory markers are associated with cognitive ability in an elderly population with Type 2 diabetes'