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Systemic Inflammation and Late - Life Cognitive Ability

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Declaration

I, Markéta Keller, hereby declare that this thesis is my own composition. It has not been submitted for any other degrees or professional qualification and all sources of information have been acknowledged.

The data collection at baseline for the Edinburgh Type 2 Diabetes Study was completed when my project commenced. The clinical and cognitive follow up datasets were result of team work, to which I have made a substantial contribution in terms of data collection, data cleaning, management and preparation. I have conducted all the presented analyses of the prospective data. The genetic association analyses required collaborative work with other research centres. I have not been involved in the process of data collection but I was fully responsible for data management, preparation and data analyses as required for this project.

Signed:

Date: 31. 01. 2014

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Abstract

Objectives – Cognitive ageing is an inevitable part of human life. Research from disciplines such as epidemiology, medicine and neuroscience implicate a wide range of determinants in the pathophysiological processes that lead to clinical symptoms of neurodegeneration. Markers of systemic inflammation are postulated to play an important role in mechanisms underlying a neuro-pathological cascade, either directly, through neuro-inflammatory processes, or through the mediating effect of diseases that are associated with cognitive deficits, such as cardiovascular disease and variation and disruption to cerebral blood flow. This may be particularly important in people with type 2 diabetes, where the increased prevalence of vascular events and glycaemic upset along with elevated levels of various circulating biomarkers, have been implicated in accelerated cognitive decline. Increasingly, evidence suggests a contribution of vascular disease state in the development of Alzheimer's disease in which inflammation could be a significant factor.

Determining the direction of association between individual markers of inflammation and altered cognitive performance is important in order to understand the possible role of inflammation in the development of cognitive decline and to inform the development of preventive clinical interventions. Therefore investigating these risk factors in relation to the trajectory of age related cognitive decline is crucial; in this respect, longitudinal evidence, detecting change in cognitive performance over a defined period of time, is most appropriate. To date, the majority of evidence is inconclusive, predominantly due to methodological obstacles embedded in the prospective design of cognitive ageing studies and in the investigation of a complex

disease state, such as insufficient follow up period and restricted cognitive assessment.

Since associations reported from modelling late life cognitive change in epidemiological studies may be the result of confounding variables, such as gender, vascular risk factors/disease, education attainment and social status, investigating the causal nature of inflammatory mediators in cognitive decline, has proved more problematic. Additionally, even a casual association may be due to 'reverse causation'. One method of unravelling such associations is through the use of genetic association, where the exposure variable of interest (such as genetic variants affecting plasma biomarker levels) is modelled against the outcome, thereby overcoming some of the problems of confounding and reverse causation inherent in non-genetic epidemiological studies.

Aim – The primary aim of this thesis was to test for associations of baseline measures of acute-phase proteins (fibrinogen and C-reactive protein) and central pro-inflammatory cytokines (interleukin – 6 and tumour necrosis –α) with four-year change and estimated life-time change in cognitive ability in older people with type 2 diabetes. The second aim was to explore the association between fibrinogen-related SNPs (SNPs shown previously to be associated with altered plasma fibrinogen levels) and cognitive ability in the general population.

Methods –Data from the Edinburgh Type 2 Diabetes Study (the ET2DS), a prospective epidemiological study of older people with type 2 diabetes were available, including that collected at a baseline clinic (2006-07) on 1066 participants,

mean age 67.9 years (SD 4.2). For the present study, follow up cognitive assessment was carried out after four-years (2010-11) at which cognitive data was collected on 828 survivors. Cognitive ability at both time points was assessed using the same, comprehensive, seven neuropsychological tests battery, including measures of fluid as well as crystallised intelligence (vocabulary test). Principal component analysis was conducted to derive a general cognitive factor 'g' and a general inflammatory factor, derived from individual cognitive scores and from baseline measures of four inflammatory markers (fibrinogen and C-reactive protein, interleukin – 6 and tumour necrosis –α), respectively.

Genotype and cognitive data were collected from seven, well-established population-based cohorts with clearly defined sampling frames and data collection procedures. Five cohorts comprised of community-dwelling elderly people, living in central Scotland (AAA Trial, n = 2061, EAS, n = 534; ET2DS, n = 1045; LBC 21, n = 517; LBC 36, n=1005) and two large were cohorts based in England (ELSA; n = 5458; and Whitehall II; n = 3400). In total, genotype and cognitive data were available for 14033 participants, age range between 60 to 80 years. In all studies cognition was assessed on three cognitive domains: memory, executive functioning and information processing. Compatibility of cognitive data allowed for calculation of a general cognitive factor 'g' that was comparable between all cohorts. The instrument variables consisted of 61 fibrinogen-related polymorphisms within 13 different loci. These were identified through a detailed literature search as well as through search of relevant, genetic databases.

Results – in the ET2DS, the age and sex-adjusted analyses revealed statistically significant associations between raised plasma inflammatory markers and poorer 'g' at follow-up; this was observed for all biomarkers, with the strongest associations detected for IL-6 and the general inflammation factor (p values <0.001). These findings persisted in linear regression models of baseline biomarker levels with four-year cognitive change as well as estimated life-time change – here the general inflammatory factor and plasma IL-6 levels were the strongest predictors. Adjustment for conventional vascular risk factors and cardiovascular disease attenuated the associations of cognitive decline with fibrinogen, CRP and TNF-α; associations were largely attenuated in analyses assessing IL-6 and the general inflammation factor and tended to remain statistically significant.

Meta-analysis was conducted in order to explore associations between pre-selected fibrinogen-related SNPs and impairment in general cognitive ability as indexed by 'g'. The analysis identified five plasma fibrinogen-related SNPs that were significantly associated with impaired 'g' at the nominal threshold level of $p < 0.05$. These were: rs2070016 (*FGB gene*); rs2070016 (*FGA gene*); rs1800497 (*ANKK1 gene*); rs4251961 (*IL1RN gene*) and rs1130864 (*CRP gene*).

Discussion – the results of the ET2DS indicate that in an elderly diabetic population, there is a significant relationship between baseline levels of circulating inflammatory markers and four-year cognitive change as well as estimated life-time cognitive decline. These associations were generally independent of common cardiovascular risk factors and events, suggesting a possible pathway where cytokine-induced activation of glial cells may be responsible for the consequent neuro-inflammatory

processes resulting in declined cognitive ability. The lack of some associations may be due to a relatively short follow up period. The main strength of the ET2DS was the availability of prospective cognitive data, the large sample size and the use of a comprehensive cognitive battery, including a vocabulary test for crystallised intelligence and thus calculation of estimated life-time cognitive change.

Genetic association analysis indicated a significant association between five pre-selected SNPs each located within different genes (in general, genes associated with inflammation), and impaired general cognitive ability. This provides some support for a causal role of inflammation in age-related general cognitive impairment. One of the major strength was the use of a large dataset and the applied methodological approach. Meta-analysis was conducted on raw, prospectively generated data, allowing determination of the cognitive phenotype variable.

The main outcomes of this thesis suggest that systemic inflammation may indeed be involved in aetiology of age-related cognitive decline, possibly via neuro-inflammation. Further epidemiological investigation should involve a measurement of biomarkers trajectories in modelling cognitive change. Use of a stronger genetic instrument for inflammatory biomarkers, modeled against cognitive decline rather than cognitive ability as in the current study could further advance knowledge of the bio-pathological mechanisms underlying age-related cognitive decline. Results could ultimately inform subsequent investigations in the form of a randomised control trial, testing an evidence-based anti-inflammatory clinical intervention in diabetic populations as well as the general populations.

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AAA	The Aspirin for Asymptomatic Atherosclerosis Trial
A-H4	the Alice Heim 4-1
AD	Alzheimer's disease
ACRI	Age Related Cognitive Impairment
COPD	Chronic Obstructive Pulmonary Disease
CRP	C - reactive protein
CVD	Cardio vascular disorder
DST	Digit Symbol Test
EAS	Edinburgh Artery Study
EEG	Electroencephalogram
ELSA	English Longitudinal Study of Ageing
ET2DS	The Edinburgh Type 2 Diabetes Study
'g'	General Cognitive (intelligence) factor
ICD - 10	International Classification of Disease - 10
IL-1	Interleukin 1
IL-6	Interleukin 6
GIF	General Inflammatory Factor
IM	Inflammatory Markers

MCI	Mild Cognitive Impairment
MRI	Magnetic Resonance Imaging
Mmol/l	mini-mol per litre concentration
LBC	Lothian Birth Cohort
LD	Linkage disequilibrium
LM	Logical Memory
LNS	Letter Number Sequencing
MHVS	Mill Hill Vocabulary Scale
MHT	Moray House Test (only linked to LBC cohorts)
MR	Raven's Matrices
NART	National Adult Reading Test
PET	Positron emission tomography
SIMD	Scottish Index of Multiple Deprivation
T2DM	Type 2 Diabetes Mellitus
TIA	Transient Ischaemic Attack
TMT – B	Trial Making Test – B
TNF-α	Tumour Necrosis Factor alpha
VaD	Vascular Dementia
WH II	Whitehall

Chapter 1: Cognitive Ageing, Systemic Inflammation, and Genetics

Ageing, specifically cognitive ageing, is an extremely complex and dynamic process with a great deal of heterogeneity in the rate of age related cognitive decline, reflecting the multifactorial nature of cognitive ability and is thus considered as a life-long trait. In light of the demographic changes resulting in the growth older of the older population, cognitive ageing and consequences of neuro-degeneration are increasingly becoming a major public health concern. This is particularly important as along with a growing proportion of the ageing population, there is also an increasingly higher prevalence of type 2 diabetes; epidemiological observations implicate type 2 diabetes as one of the major risk factors in development of age-related cognitive dysfunction. To complicate matters further, brain-related neuro-degeneration is a complex, gradual process. It has now been well established that cerebral damage might occur well before a clinical manifestation of cognitive impairment. Identifying preclinical, potentially modifiable markers that contribute to patho-physiological mechanisms resulting in altered age-related cognitive change is therefore an urgent matter in order to develop effective clinical interventions.

The primary aim of this thesis was to investigate the relationship of elevated markers of systemic inflammation and change in cognitive performance in older people with type 2 diabetes. The use of prospective data and vocabulary-based estimator of pre-morbid cognitive ability resulted in a possibility of modelling a four-year as well as

life-time change in cognitive performance. A genetic association study was conducted on data collated from seven population-based studies, the aim of which to determine the associations of pre-selected single nucleotide polymorphisms (SNPs) with cognitive functioning.

This chapter provides a review of some of the key details and background information. It commences with an introduction to cognitive functioning, including general intelligence, followed by an overview of the main neuro-biological processes and pathologies, age related changes in cognitive ability, including mild cognitive impairment and main types of dementia. This chapter will discuss the concept of cognitive reserve and concludes with main evidence with regards to prevalence of age-related cognitive dysfunction. The second part of this chapter consists of two main sub-sections; first, information and facts relevant to systemic inflammation are discussed. Four circulating inflammatory markers are discussed in more detail in terms of their biological properties. Epidemiological evidence supporting the inter-relationship between inflammation and vascular risk and cardiovascular disease and between inflammation and type 2 diabetes conclude this section. The third part provides an overview of T2DM, defining the disease, its risk factors and current prevalence of this condition. The following section is an introduction to genetics, describing DNA stricture as well as it components, and main issues in genetic association studies. It concludes with information of genetic determination of plasma fibrinogen levels and relevant biological mechanisms involved in disturbed plasma fibrinogen levels.

Many studies have examined the role of systemic inflammation in age-related cognitive impairment and decline. A literature review on epidemiological studies of inflammatory circulating biomarkers in cognitive decline is presented in Chapter 2. The literature review only included studies in the English language. Only prospective studies, recruiting dementia-free subjects and with a sufficient follow-up period cognitive assessment were included. Animal studies or basic biological studies were not included. Studies on genetic epidemiology were only included if the above criteria were met. It concludes with the summary of the main findings and description of the aims of the presented thesis.

The following two chapters introduce the studies and populations that were analysed. Chapter 3 addresses the Edinburgh Type 2 Diabetes Study (ET2DS), Chapter 4 commences with an overview of the seven collaborating cohorts, details about particular cohort description, including methods of cognitive assessments. The second part of this chapter provides detail of method that was used to identify the pre-selected, fibrinogen-related SNPs that were required for the genetic association analysis. Chapter 5 presents results of the ET2DS longitudinal analysis, and in Chapter 6, the results of the genetic association study are described. A discussion of these findings are presented in Chapter 7; including summary and interpretation of the ET2DS, followed by summary and interpretation of the genetic meta-analyses findings and evaluation of strengths and limitations of this evidence. It concludes with the overall summary and the implications of this work and recommendations for future research.

1.1 Cognitive Functioning, Cognitive Impairment and Cognitive Decline

The changes in mental abilities that are observed as people grow older are commonly referred to as cognitive ageing. The brain undergoes age-related patho-physiological changes that are clinically manifested in decline of one or more cognitive domains. The neurology of cognitive ageing is a complex and complicated process. The focus of this section is on defining cognitive function and age-related cognitive changes, describing main neuro-patho-physiological changes. It also provides background information of cognitive ageing research. In the literature, there is a considerable overlap in terminology referring to age-related cognitive processes. In this thesis, the term *cognitive impairment* refers to 'one-off' measures, i.e., assessment of cognitive ability. *Cognitive decline*, on the other side, refers to a change of cognitive ability over a defined period of time

1.1.1 Definition

Cognition, or cognitive functioning, is defined as a mental activity with a multifactorial property, encompassing functions such as an acquisition, memory/storage of information, thinking and selection, visual-spatial functions, information processing, conceptualisation, language production and comprehension and executive functioning[1,2].

Interest in brain function dates back approximately 5000 years to a medical document from ancient Egypt that described observation of behavioural changes as a result of head injury[3]; nevertheless, despite the work of Aristotle (who proposed a direct functional link between heart and brain function[4]), there was a little interest and understanding of mental (cognitive) processes until the 19th century, when intelligence and cognition, its determinants, mechanisms and functionality through the human life became a of scientific enquiry. Although some advocate the notion of *holism* which claims that even though some lesions may have a local effect, the brain functioning must always involve multiple structures[5], generally. most current research is guided by the doctrine of *localisation*[4] where specific location/structure of brain correspond with specific cognitive function. The majority of work following this doctrine is based on observation and studies of patients with localised brain lesions, accompanied by sets of clinical symptoms and patterns of behaviour[6,7]. Approach of localisation of particular cognitive domain/function along with advanced development in technology and scanning (such as MRI, PET, EEG, etc.),

has allowed for understanding of increasing specific experimental measures of intelligence and cognitive functioning, i.e. neuropsychological assessments[8].

1.1.2 Domains of Intellectual Abilities, the General Cognitive Factor '*g*'

Understanding of human cognition, both functional and neuro-cognitive has advanced insight of many facets and patterns of intellectual ageing. Different domains of cognition can be conceptualized and assessed via validated neuropsychological tests. Cattell-Horn theory of intelligence[9,10] proposed multifactorial, multidimensional and multi-directional nature of intellectual ageing trajectory[11,12]. In this explanation of human cognitive functioning, two broad categories of intelligence were identified, each presenting different profile in terms of onset, speed and pattern of decline; *crystalized* and *fluid* intelligence[13]. This is commonly referred to as the 'Dual component theory of intellectual development'[14] . Crystallized intelligence reflects experienced and culture-based general knowledge and is linked to cognitive abilities such as general knowledge and vocabulary ability[15]. Typically, it shows very little age-associated decline, remaining relatively stable across a life-span[16]. As such, validated (usually vocabulary-based) assessment of crystallized intelligence provides an estimate of a person's peak cognitive ability, i.e., cognitive proficiency prior to the onset of age-associated neuro-pathological processes[17]; this provides comparative data for longitudinal design where the change in cognitive performance can be thus determined. Fluid intelligence, on the other

side, is affected by biology-based processes accompanying brain ageing. As such it is susceptible to neurological damage and there is a great degree of heterogeneity between individuals[18]. Determination of the speed and pattern of decline is conducted by a set of standardized tests that are sensitive to cognitive domains such as memory (range of sub-types), reasoning, speed of information processing and executive functioning[7,19]. Furthermore, evidence suggests a strong inter-correlation between decline on individual tests; this suggests the presence of the general intelligence component[19]. Since it was first proposed by Spearman[20], it has received a great deal of support from a great deal of epidemiological evidence[2] as well as experimental support, including evidence for specific neuronal function associated with performance on a diverse set of cognitive tests[21]. One of the most compelling pieces of evidence was proposed by John Carroll; The Three Stratum Theory[22,23]. Factor analysis of approximately 460 data sets resulted in the identification of three layers of cognitive abilities: narrow, broad and general. The narrow stratum consists of 69 cognitive abilities that are grouped into 7 categories within the broad stratum. These domains are: crystallized and fluid intelligence; general memory and learning; broad visual & audio perception; retrieval ability; cognitive speediness and information processing speed[22]. Consistent with the 'Dual Component Theory of Intellectual Development' outlined above, Carroll also identified differences in pattern and speed of decline between crystallized and fluid abilities.

1.1.3 Neuro-Biological Process and Pathological Determinants of Cognitive Ageing

Commonly, cognitive ageing is associated with later life brain-related biological processes. Whereas some brain structures remain relatively intact until much later in a person's life, some specific structures and volume brain alternations can commence as early as in third decade of person's life. The location, degree and speed of anatomical degeneration are closely linked to brain development in two ways: the phylogenetic (evolutionary) and ontogenetic (life-time) development.

The brain consists of multi-layered structures: phylogenetically the oldest is the subcortical structure; followed by relatively young cortex, further subdivided into two hemispheres. The evolutionary youngest subdivision is the neocortex[24]. From this point of view, the brain is dominated by one grand theme – the gradual transition from hard-wired structures to evolutionary younger open-ended structures. In terms of ageing, the younger cortical subdivisions suffer most from the detrimental effects of ageing. In particular, in the neocortex, the hetero-modal association cortex (the region that receives multiple sensory input requiring integrated cognitive activities[25]) including the infero-temporal, infero-parietal and particularly the (evolutionary) youngest prefrontal cortex are most affected. In contrast, the oldest parts receiving raw sensory information and the motor cortex are least affected, remaining relatively intact till the final stages of person's life[1]. Similar principle applies when we look at the brain anatomy processes associated with the ontogenetic development and decay – those parts of the brain that are developing latest in one person's life are most

affected by age-related changes; in this respect, it is the frontal lobe, especially the prefrontal cortex that generally doesn't reach the fully functioning development until the age of 16-18[26]. In an otherwise healthy brain, age-associated anatomical changes have been attributed to numerous factors, such as oxidative stress[27], prolonged inflammation[28], age-related vascular events[29] and stress-related corticosteroid levels[30].

On average, the weight and volume of the brain decreases about 2% every decade of the adult life. This brain atrophy is linked to the progressively increasing size of the ventricles whilst sulci become more prominent. The exact mechanism of this is yet to be determined, nevertheless, a few mechanisms have been proposed. It has been argued that decrease in dendritic synapses and loss of synaptic plasticity might be responsible for brain shrinkage. *Synaptic plasticity* is a term referring to the ability of synapses to adapt to overall neuronal activity. Synapses are either strengthened by combined pre- and postsynaptic neuronal activity or they are weakened by non-coincidental neuronal firing. Research has found that synaptic plasticity is facilitated by specific ion channels[31]. Further, there is an evidence for a key role of several kinds of neurotransmitters in this process[31]. Moreover, neurotransmitter levels relate to age related decline in cerebral dopamine receptor density, with a central role implied in modulating responses to contextual stimuli and regulating attention[31]. Generally, there is an 'anterior-to-posterior gradient' with the pre-frontal cortex being the most, and the occipital lobe being the least affected[30]. Furthermore, age-related brain atrophy occurs faster in cortical thickness and grey matter rather than white matter[16]. For example, the natural ageing process accounts for approximately

36% of reduction in the nucleus accumbens (mood regulation), 37% in the thalamus (sight, hearing, sleep-awake cycle) and 33% in the hippocampus (episodic memory)[7,31]; these are some of the most notable age-related changes, affecting person's ability to carry out the activities of daily living as they once used to.

In terms of the white matter age related anatomical changes, hyper-intensities (small focal lesions), usually reflect vascular illness as well as demyelination of pathways. Chronology of myelination is a great marker for both development and decline; pathways that require the longest time to myelinate are most susceptible to effects of ageing[31]. Again, it is the prefrontal cortex, and, within that, the dorsolateral subdivision in particular[32]. This region plays a vital role in information transfer between cortical areas, a process that is crucial for higher and/or comprehensive cognitive proficiency[16,33,34]. However, there is no linear/causal relationship between white matter lesions and cognitive decline. Indeed, the damage to white matter integrity can be seen as have a threshold nature: up to a certain level it is benign, and beyond that it affects speed of cognitive decline.

In summary, the exact evidence of association between age-anatomical brain changes is yet to be determined; age related anatomical changes account for 25-100% of variance in cognitive ability[15] between young and aged individuals, with a decline in some cognitive abilities commencing in a third decade of life, others maintained until middle of sixth decade and some relatively unchanged in the eight decade of person's life.[35]

1.1.4 Age-related changes in cognitive ability

Age-related cognitive impairment is commonly subcategorised into 'normal' (non-pathological) or 'pathological'. With respect to the former one, changes in cognitive performance are anticipated and within the norm for the person's age and education.

This is distinguished from the pathological cognitive impairment and/or decline where the decreased ability in cognitive function that is greater than expected for the sex and age matched population norm and where it presents with clinical manifestation of cognitive impairment/dementia[15].

As an alternative to the distinction between normative and pathological ageing, it can be argued that since decline in cognitive performance is always due to a set of neuro-pathological changes, as described in section 1.3, cognitive ageing should be viewed as a continuum where the degree of damage is manifested in tandem with clinical symptoms and that this might be, in certain circumstances, accelerated by a whole array of risk factors and events. For example, one of the most prominent risk for declined performance in cognitive functioning is the presence of systemic inflammation; research indicates that decline of both cognitive ability and the immune system might commence as early as the 3rd decade of life[36], thus suggesting an inter-related effect on cognitive performance that is pathology-loaded. This approach better accounts for the gradual shift from mild cognitive changes to a detectable subclinical impairment (i.e. mild cognitive impairment) and, in some percentage of the population, to a clinical profile of frank dementia. Furthermore,

there is a great of heterogeneity between individuals with respect to the speed and trajectory of domain specific decline. Some remain relatively stable[37] and some might even show improvement[1], including emotional stability[37]. Lastly, the presence of Alzheimer's Disease neuropathological facets was detected in brains of otherwise clinically asymptomatic individuals[38]. Therefore, classification of 'normal' brain ageing might be too narrow and potentially misleading.

Typically, memory functioning is one of the first domains to decline; specifically, it is the delayed recall of verbal information, working memory, and short term recall[39]. Most memories are formed and stored in the neocortex; certain memories require the support of few subcortical (non-neocortical) structures and these are extremely vulnerable to decay and neuro illness[1]. Greater quantitative changes usually occur in episodic memory than semantic memory (episodic memory or autobiographical memory refers to memory of events that have occurred in our personal past. Semantic memory can be seen as a sort of impersonal mental dictionary[7])[31]. In episodic memory, it is the ability to recall that deteriorates faster than recognition/familiarity[40]. Furthermore, performance on task assessing executive functioning is often detected at earlier stage of ageing[26]. Given the high, superior role of the frontal lobe functioning, particularly its importance managing and organising the complexity of individual cognitive functions, it can be reasoned that it can mediate domain specific decline[33]. Moreover, there is also a strong possibility that domain specific decline occurs as a result of a general decline in information processing speed[41]. This might also (partially) explain why when we get older we require more time and effort to encode and utilise new information. However,

evidence accounting for an exact bio-pathological mechanism is so far inconclusive, currently under ongoing investigation.

1.1.5 Mild Cognitive impairment Cognitive Decline and Dementia

The meaning of the word dementia is "the loss of mind" [1]; term that refers to a gradual process, affecting certain faculties progressively faster than others[26] and that results in notable disturbance to intellectual and personality functioning and communication. Therefore, cognitive ageing should be perceived as gradual continuous processes with certain distinct, measurable points.

Mild Cognitive Impairment

On the continuum of the ageing process, MCI is considered an immediate or transitional state of cognitive impairment. Typically, an fMRI scan can detect brain pathology in a form of neurofibrillary tangles (NFT) in temporal or temporal-parietal lobes, which corresponds with clinical symptoms including objective impairment in memory function greater than expected for an individual's age and education (amnestic MCI)[42]. Other cognitive faculties remain intact, i.e., presentation is according to expectations of matched age and education person. The clinical criteria and clinical profile associated with dementia symptoms and measures are not satisfied. Also based on a neuro-psychological profile other types of subclinical

impairment are classified; i.e., non-amnestic MCI. It is, however, the amnestic MCI that shows the highest conversion rate to dementia, particularly into Alzheimer's Disease[43]. In fact, the presence of apolipoprotein ApoE ε4 allele, associated with sporadic AD[44], is also significantly higher in the amnestic subtype of MCI suggesting a possible explanation for this relationship[45].

Figure 1 below demonstrates the progressive stages of neuro-pathological changes; compared to control, some changes are detectable in MCI patients (the three middle images), whereas other regions and consequently other cognitive faculties remain relatively preserved; a scan of a brain at AD at the far right shows spread neuropathological changes in the form of massive reduction in hippocampal volume. Also, changes in fronto-temporal structures might indicate early onset of fronto-temporal dementia[31]

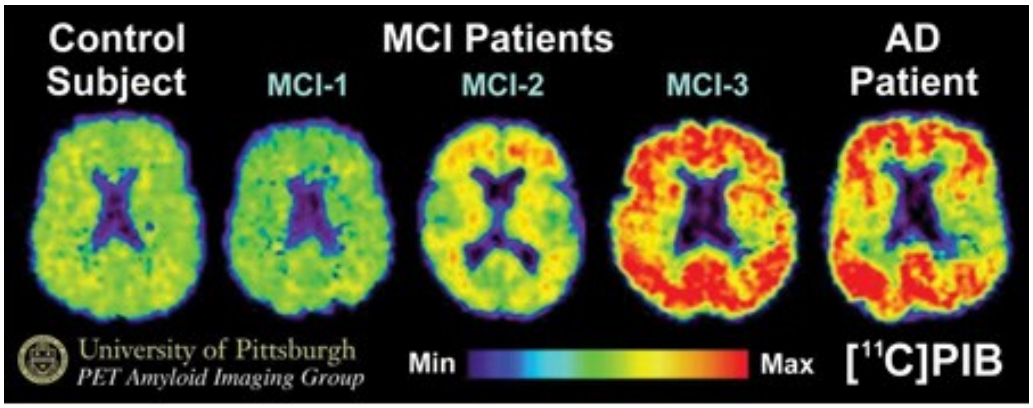


Figure 1: Progress of brain pathology from healthy functioning to detectable AD pathology

Cognitive Decline and Dementia

Currently, there are approximately 50 dementia aetiologies proposed[15], with differing degree of severity of associated deficits and differing clinical profiles. Furthermore, the duration of this condition also differs between types, ranging from chronic to gradually more progressive to a final, detrimental stage; always it is represented by a cluster of dementia-type specific symptoms. With ongoing progress, the affected person experiences gradually increasing difficulties related to the affected cognitive faculties. Disturbances in mood[46], behaviour[47] are also extremely common in dementia diagnosis.

Among the identified types of dementia, the most common causes are Alzheimer's disease (AD) and vascular dementia (Vad). Biologically, the precise mechanism of AD is yet to be fully determined; definite diagnosis can only confirmed by post-mortem examination[15]. Nevertheless, a number of brain pathologies are shared in AD patients; neurofibrillary tangles (NFT) and β - amyloid plaques. Intra-cellular NFT consist of a hyper-phosphorylated form of micro-tubule, associated protein tau. Hyperphosphorylation (among other processes) is crucial to the molecular pathogenesis of neurofibrillary degeneration of AD. Also, abnormal filaments are seen in soma and proximal dendrites of specific neurons[48]. Once a cell is affected, it basics mechanism is disturbed, which leads to a cell death, leaving the tangle behind[31]. Over production β - amyloid protein leads and disturbance to its natural function of down regulation of excitatory synaptic transition and plasticity; as a result an extra-cellular deposit of β - amyloid plaques are formed by a dense core of surrounded by degenerating axons and dendrites, activated microglia and reactive

astrocytes. Once the degenerating tissue has been destroyed, only a core of β-amyloid remains[31,49]. Clinically, standardised diagnostic criteria include significant memory impairment accompanied by impairment in at least one other domain, most typically aphasia, apraxia, agnosia and executive functioning[50]

In terms of VaD, most commonly it is caused by a range of vascular lesions; cerebral small vessel diseases, associated with lacunar infarct[51] and damage to myelin in a form of white matter lesions[52]. Large vessel VaD is usually associated with trombo-embolic, either localised (single infarct dementia) or infarctions spread throughout the brain (multi-infarct dementia[53]. The former commonly leads to a progressive deterioration of the frontal lobe, linked to decline in executive functioning[53]. This multi-pathological origin of VaD complicates a definite diagnosis; rather a set of clinical criteria are commonly applied. At present, clinical diagnosis of VaD requires detection of significant cognitive impairment (as described in AD diagnosis), along with confirmation of relevant vascular profile.

Definite diagnosis of each type of dementia can be further complicated by the possible co-existence of symptoms[54]. Furthermore contrary to the previous view distinguishing between aetiologies of AD and VaD, there is increasing evidence of the vascular nature in development of AD[55]. Lastly, clinical diagnostic criteria rely heavily on research evidence assessing a change in cognition over time associated with a whole range of inter-related, multifactorial determinants as well as protective factors. Both cross-sectional and longitudinal designs have their pros and cons, resulting in inconclusive evidence. This is discussed in section 1.5.2 below.

1.1.6 Cognitive Reserve

For decades it was assumed that cognitive deterioration, accompanied by specific clinical symptomatology was an inevitable and irreversible attribute of the overall ageing process. In other words, if one lives long enough, s/he will exhibit age-specific symptoms and consequent difficulties. Such people were commonly described as 'senile'[7]. Nevertheless, in recent years growing evidence suggests an alternative hypothesis that postulates a certain degree of resilience to age-related neuro-pathologies.

There is a great degree of heterogeneity between individuals in terms of pattern and speed of the trajectory of cognitive decline. Within the 'reserve hypothesis' there is a large degree of individual variation in the extent of how much the cognitive functioning, defined by results of psychometric testing and observed ability to cope with activities of daily living, can be protected against the influence of age-related brain neuro-pathological changes[56]. There is a wide-ranging spectrum of putative indices for cognitive reserve.

By definition, the concept of *cognitive reserve* maintains that aspects of brain structure and function, such as neural plasticity and efficiency of the neural network, buffer the effects of either age-related neuropathological changes, such as formation of β- amyloid plaques and neurofibrillary tangles, vascular damage, etc[38]. There are currently two possible explanations for the concept *cognitive reserve*: neuro-protective and neuro-compensation. The former one postulates that the neural

reserve, defined as the brain‘s capacity to buffer the effects of insults, is closely linked with neural regeneration and functional brain re-organization that can prevent cognitive ageing and/or preserve cognitive function despite chronological age[57]. The explanation based on the neuro-compensation mechanism is centered around the hypothesis that the ageing brain may compensate structural losses in functional areas by recruiting previously unrelated parts of the brain to take over cognitive function roles[34]. For example, hippocampal atrophy is significantly correlated with stronger function of the pre-frontal lobe and this has been explained as a possible compensation mechanism[58].

Epidemiological evidence indicates the role of childhood intellectual performance[59], educational attainment and occupational status[60] plays a role in preserving cognitive proficiency. Pre-morbid intellectual functioning is considered as a chief factor; in a longitudinal study of ageing, 43.4% of variance in old-age cognitive ability (in a middle 70s) is determined by an early life (11 years of age) cognitive performance[61]. Valenzuela found that educational level was significantly associated with the preservation of cognitive skills but only in combination with occupational complexity and social engagement[56].

Interestingly, recent systematic literature review indicate that high level of cognitive reserve, i.e. high pre-morbid intellectual ability was positively correlated with far faster decline after proposed diagnosis of AD[38]. This is possibly due to a concise constellation of strongly connected neurons in people with rich factual and procedural knowledge. It is reasoned that the advanced cognitive faculty enables an ability of formation and recognition of cognitive patterns, commonly referred to as

'*cognitive templates*'[1,38]. Neurofibrillary tangles, one of the most typical attribute of AD, occurs in varying quantities in the brains of non-demented subjects[62]. Substantial amount of studies reported autopsy detection of neurofibrillary tangles in individuals who were diagnosed with MCI who had not progressed to frank dementia as defined by Diagnostic Statistical Manual-IV[63,64]. It is reasoned that in subjects with advanced pre-morbid cognitive ability, greater pathological alternation and damage is required prior to manifestation of clinical symptoms of dementia[1] and prior a formal neuropsychological and clinical assessment. Therefore in these subjects, frank dementia is diagnosed at higher level of severity when the rate of decline is steeper. At this stage of dementia it is also less likely that clinical intervention can effectively manage inevitable consequences of dementia[65,66].

The exact mechanism of the *cognitive reserve* concept is yet to be established. Attempt to explain how, in the face of normative age-associated structural brain changes, individuals vary greatly in the process of cognitive ageing is imperative – identification of modifiable risk factors for accelerated age-related cognitive decline as well as protective factors that might reduce the otherwise inevitable age-related cognitive impairment and decline will provide a major platform for targeted clinical intervention and consequent positive outcome.

1.1.7 Cognitive epidemiology - Prevalence of age related cognitive impairment and cognitive decline – overview

Life expectancy has changed radically over the last century. Whereas in 1901, it was 49 years of age for women and 45 years of age for men, in the 2005 healthy life expectancy at 65 years of age is 15 years for women and 13 years for men[67]. However, approximately 15% people of 65 have been classified as disabled, of those 62% need care at some point on daily basis and 21 % on a continuous care basis[68]. In Scotland specifically, the majority of over 65 years of age have been diagnosed with two or more conditions and the majority of over 75s with three conditions with a multi-level and multi-factorial relationship between them all; either directly or indirectly contributing to accelerated cognitive decline and/or dementia. In particular, those diagnosed with dementia can also suffer from any combination of the following conditions: coronary heart disease: hypertension heart failure; stroke/TIA; T2DM; COPD; clinical depression or other detrimental conditions[69].

However, the difficulties in distinguishing between age-related cognitive changes and accelerated cognitive decline and the inconsistencies in defying mild cognitive impairment (MCI) and dementia present a severe issue in establishing prevalence and incidence of these conditions. In particular, a unique criterion for diagnosis of MCI has not yet been postulated; rather, diagnosis incorporates a set of clinical judgments. The subtle changes to the diagnostic criteria have been showed to modify estimates

of prevalence and incidence of MCI and/or dementia. Estimates of MCI in population of 65 years of age was fluctuating between 3% up to 19%, estimates for cognitive decline ranged from 21% in 60 [70] and older to 27% in 65 and above years of age [71]. The incidence rates of MCI have ranged greatly from 8 to 58 per 1000 per year in the general elderly population[43], depending on diagnostic criteria, sample structure, follow-up and cognitive measures. Furthermore, annual conversion rates from MCI to dementia have been found to be high in clinic samples ranging from 10% to 15% but are often substantially lower in population-based studies (i.e., 3.8%-6.3%)[73,74].

Similarly, establishing the precise number of incidents of AD and VaD, and distinguishing them from other dementia types, is complicated by shared aetiologies as well as the possibility of both conditions co-existing.[54,68]. Despite the use of different methodologies for case identification and definition between studies, increases in the prevalence and incidence of dementia with age have been consistently reported. In terms of world-wide prevalence of dementia, whereas at 2001, there were 24 million dementia sufferers, at 2010 it leaped to 35 million with an estimated increase to 65 million in 2030 and 113 million in 2050[75]. A similar trend is observed in the UK; currently dementia affects approximately 700 000 people above the age of 65 years and it is expected to double by the year 2051[76]. Specifically, data in the UK indicate that prevalence of dementia of all types ranges from 1.3% to 2.9% in 65-74 years of age, 5.9%-112.2% in 75-84 years of age and from 20.3%to 32.5% in 85 and above years of age[76]. An age-related trend in dementia prevalence was observed for both sexes. Further analyses demonstrated the

AD was the dominant type of dementia, accounting for 62% of all cases (and was more common in women), while VaD and mixed dementia, accounting for nearly one third (27%) of all cases (and was more common in men). Similarly, among participants in the Cardiovascular Health Study, a steady increase in the incidence of dementia with age was noted in both men and women[77].

1.2 Risk Factors – Determinants

In the previous chapter a number of determinants and risk factors have been mentioned that might play a role in age-related cognitive decline. To date, research has identified the relationship of increased levels of circulating inflammatory makers in relation to cardiovascular co-morbidity and type 2 diabetes that themselves are significant risk factors for age-related cognitive impairment and cognitive decline. The focus of the following sections will be on an overview of biological mechanisms of inflammatory and haemostatic markers. Epidemiological evidence of association between elevated systemic inflammatory markers and cardiovascular disease and type 2 diabetes will be discussed. The last part will focus on type 2 diabetes, to define the disease, its diagnosis and relevant risk factors. This section will conclude with discussing evidence of relationship between prevalence of type 2 diabetes and age-related cognitive decline.

1.2.1 Inflammatory and Acute-Phase Response Markers

Inflammation is an integral first line of defence response to injury or a disease-causing stimulus with the primary attempt to restore and maintain homeostasis after injury[78]. Although inflammation is essentially beneficial, an excessive or prolonged inflammation can cause harm. Most of the body's defence elements are located in the blood; inflammation is the means by which body defence cells and chemicals leave the blood and enter the tissue around the injured or infected site[79].

Principally, inflammation can be subdivided into acute and chronic phases, each manifesting with specific components and processes. In terms of an *acute inflammation*, it can be subdivided into three interrelated components: vascular dilatation (vasodilatation), endothelial activation and neutrophil activation[80]. Visually, the characteristic signs of inflammation are redness, heat and swelling; indeed, the literal meaning of the word inflammation is "setting on fire"[81].

Vasodilatation is the earliest inflammatory response, followed by relaxation of the vascular smooth muscle. This results in an increased permeability and blood flow (i.e., hyperaemia). Hyperaemia causes redness and the entry of the fluid into the tissues (erythema and oedema, respectively)[82]. Acute inflammation can present with varied outcomes, such as resolution, where normality is restored, scaring (fibrosis) and abscess formation or progressing into chronic inflammation. Therefore, typically *chronic inflammation* is prolonged type processes, caused either by persistent inflammatory stimulus or ineffective inflammatory mechanism[82].

On a microscopic level, it involves responses in the immune system by a number of different chemical substances, called *acute phase protein* and *cytokines*, which are elevated and released by the rather complex immune system[83]. Specifically, sentinel cells, e.g. macrophages that are derived from blood monocytes, detect threats from infection or other environmental factors, such as food or chemicals, due to pattern recognition receptors[81]. Activated microglia produce the potent pro-inflammatory cytokines interleukin-6 and TNF-alpha[84]. These inflammatory cytokines are the main mediators of a hepatic acute-phase response, causing an elevation of acute-phase proteins, including plasma fibrinogen and C-reactive protein. An acute phase

response is accompanied with systemic and metabolic changes, specifically increased blood viscosity, platelet number and activity[83] in order to restore body haemostasis and to remove the cause of disturbance to the system[83,85]. Therefore on a molecular level, these inflammatory markers mutually affect production and regulation of other cytokines and acute phase reactants. Specifically relevant to this study, IL-6 is known to inhibit an expression of TNF-alpha[86], which plays a key role in the hepatic production of CRP[87] and, apart from a host of other acute-phase reactant, also influences the production and synthesis of plasma fibrinogen[88]. Furthermore, IL-6 on its own[88] and together with TNF-alpha have been shown to stimulate both fibrinogen and CRP production[81]. This biologically tight and mutually compatible mechanism provided rationale to assess associations between cognitive scores and these four markers individually as well as in the form of general inflammatory factors.

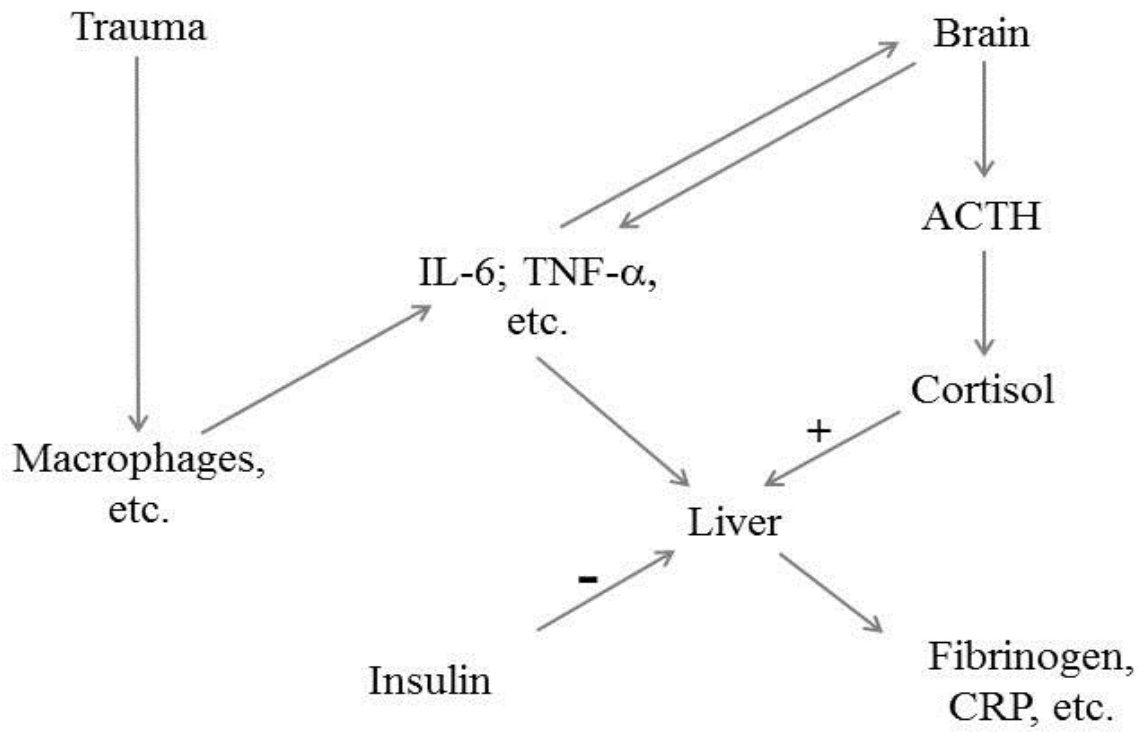


Figure 2: Innate Immune System

A bi-directional relationship between the brain and the immune system allows for a an interactive coordination where the brain is informed about peripheral process by the immune system, acting as a diffuse sense organ[89]. At the same time, the brain itself is immunologically active, actively communicating with the immune system, forming an integrated connection between brain and body, thus systemic inflammation influences brain function. Typically, elevated levels of cytokines are associated with *sickness behaviour*[90]. This term commonly used to explain a bodily strategy that is triggered by mechanisms of brain actions to elevated levels of cytokines (figure 2). Pro-inflammatory cytokines, such as IL-1, IL-6, TNF - α,

produced by activated microglial cells of the innate immune system, play a pivotal role in the acute-phase response. Sickness behaviour is also characterised by activation of hypothalamic-pituitary-adrenal (HPA) axis[91]. As a response to psychological stress, activation of the sympathetic nervous system as well as activation of the HPA axis leads to release of stress-specific hormones, such as adrenocorticotrophic and glucocorticoids[92,93]. Cytokine IL-6 is particularly known to affect activation of the HAP axis during an individual's exposure to a psychosocial stress[85]. The term sickness behaviour therefore encompasses psychological and biological components of the bodily response to pathogen-associated processes.

The next part of this chapter will provide an overview of the cytokines Interleukin-6, TNF-alpha and acute-phase protein fibrinogen and C-reactive protein. The following sections will then discuss epidemiological evidence addressing the role of systemic inflammation in cardiovascular disease and in T2DM.

1.2.2 Interleukin - 6

Interleukin 6 (IL-6) is a pleiotropic cytokine known to influence a number of cellular processes, such as a cell growth and regulation as well as activating inflammatory cells to induce a process of inflammation. It is produced by several cell types, including activated monocytes and macrophages, T cell lymphocytes, endothelial cells and adipose cells[94,95]. As well as TNF-alpha, its primary tissue of origin is the adipose tissue and typically it circulates at s low level; IL-6 has a short half-life (2-4 hours) and a greater within-person variability[82].

IL-6 expression, specifically its anti-inflammatory properties, are mediated as a reaction to stimuli of other inflammatory markers, such as pro-inflammatory TNF-alpha, IL-1, IL-4, for example[88,96]. It plays a fundamental role in regulation of the immune responses, making it a crucial component in inflammatory cascade process.

IL-6 can induce both pro and anti-inflammatory response, and it has both, inflammatory and haemostatic properties. IL-6 has a role in up-regulating a number of haemostatic variables that includes fibrinogen as well as an inflammatory role that leads to an increased endothelial cell adhesiveness[85,97].

Due to its pleiotropic effects, IL-6 affects a majority of peripheral-to-brain immune responses by modulating neural, neuroendocrine and behavioural systems[98]. Animal studies provide experimental evidence of an IL-6 altering role linked to the brain function due to its ability to cross the blood-brain barrier (BBB) [99]. The blood-brain barrier is selectively permeable with some substances passing through. IL-6 is one such a marker. This cytokine is able to by-pass the BBB and bind to the receptors on macrophages, activate them consequently modulate the BBB permeability. As a result, the compromised BBB disruption is the hallmark of neuroinflammatory disease state, linked to an activation of glial cells. Glial cells form approximately two-thirds of the brain cells, the remaining one third is formed by neurons. The main function of the glial cells is providing support and nourishment to neurons. There are two types: *astrocytes* that directly participate in neural computations by modulating the work of neurons and *oligodendrocytes* that wrap around axons, forming a fatty protective coating (myelin). Myelin facilitates signal transmission along the axon, enhancing and improving transmission of info within the large, co-ordinated

neuronal network. Therefore the role of fully functioning glial cells is vital for a healthy brain function[1,31]. During a chronic inflammation state, all glial cells remain active for an extended period during which the production of mediators are sustained; this leads to neuronal death and particular molecular and cellular mechanisms that are known to underlie cognitive function, such as synaptic plasticity, long term potentiation, neuro-genesis and memory consolidation. Animal experimental studies also show that IL6 can enter the blood with the reabsorption of the cerebral-spinal fluid[99]. It was demonstrated that whereas mice with an over-expressed IL 6 showed 63% reduction in cognitive ability and those mice that did not express IL-6 improved their cognition (maze learning and reduced amnesic effect), suggesting that due to its inflammatory property, IL-6 is a powerful mediator of cognitive ability[100].

1.2.3 Tumor Necrosis Factor – α

Tumour Necrosis Factor – alpha (TNF-alpha) owes its name to its ability to destroy malignant tumour cells in mice, as described by Carswell[101]. The TNF-alpha primary role is pro-inflammatory with its a vital role in maintaining immunity. As a potent mediator of pro-inflammatory process, it plays an important role of a whole range of (patho) physiological mechanisms: an induction of cell death leading to a cell removal is one of the key role of TNF-alpha. The production of TNF-alpha predominantly occurs in mononuclear cells and activated by a tissue injury and tumour cells[102]. Although its synthesis and production also happens in the glial cells

in the brain, unlike IL-6, it lacks the ability to penetrate the blood-brain barrier[99]. Nevertheless, increased concentrations of TNF-alpha are known to have an injurious, damaging effect on neurons[103].

Along with IL-1 and endotoxin, TNF-alpha induce in vivo IL-6 production and is a powerful responder to bacterial infection[104]; along with IL-6, it is directly involved in a hepatic activation of the acute phase response[81]. TNF-alpha increases vascular permeability, and is directly linked to metabolic and structural damage in vascular endothelial cells[105].

1.2.4 Fibrinogen

Fibrinogen was first named by Rudolf Virchow (1821-1902), who identified three components necessary for thrombogenesis: i) endothelial dysfunction ii) blood constituents and iii) blood flow[106]. Fibrinogen is synthesized in the liver through hepatocytes[107]. Alternative production is also possible in a bone marrow cells (megakaryocytes) that are mainly responsible for the production of blood platelets, required for normal blood clotting[108,109]. It has a biological half-life of approximately 100 hours[108]. Fibrinogen is a complex, multifunctional and large (340 kD[110]) plasma glycoprotein, composed of two identical halves[109]. Each consist of three pairs of polypeptide chains, two alpha, two beta and two gamma that are bound together by disulphide bonds[107].

Fibrinogen is the main coagulation protein in plasma, less susceptible to acute changes than C-Reactive protein; fibrinogen circulates in plasma in concentrations between 1.5 g/L to 4.5g/L 8. Hepatic synthesis and production of fibrinogen is regulated by a number of cytokines and other molecules, mainly by IL-6, where evidence supports that IL-6 production is dependent on prior stimulation by fibrin degradation products. Additionally, fibrinogen production is also stimulated by IL-1 and TNF-α, whereas the synthesis is suppressed by IL-4, IL-10 and IL-13 [109].

As forming a part of the process of haemostasis, haemocoagulation is formed by the cascade of enzymatic reactions with the key aim to transform fibrinogen into insoluble fibrin. This process comprised of two pathways, the intrinsic and extrinsic, eventually combined into a single pathway. The final product of the cascade fibrin follows by combining aggregated platelets into a final product is the clot – the thrombus. Particular substances called coagulation factors are mainly plasmatic proteins, synthesised in the liver. Traditionally they are marked by Roman numerals; allocation of the letter 'a' indicates that this factor has been activated. Following from the stage of the common pathway, thrombin (factor IIa) has a catalytic influence which results in separation of several monomer-type peptides, resulting in fibrin that, through polymeric reaction with other monomers leads to formation of fibrin mesh. An activation of the factor IIIa along with positively charged calcium ions Ca^{2+} must occur to stabilise and strengthen the fibrin mesh[107,111].

1.2.5 C-Reactive Proteins

The first detection of C-reactive protein (CRP) as an inflammatory marker was in 1930 in the sera of patients who were acutely ill with pneumococcal pneumonia. It was named because its reactivity with polysaccharide 'C' of streptococcus pneumonia[79]. CRP is a calcium-binding pentameric protein. It consists of five identical non-glycosilated polypeptides 23-kd subunits. It is an acute phase reactant. Hepatic production occurs within a few hours after the injury or in the presence of inflammation. As plasma fibrinogen, CRP activation occurs under activation of IL-6; IL-1 and TNF-alpha also paly role in this[112]. Normal, physiological CRP level is considered to be a value <10mg/L with healthy individual generally having <1 mg/L. In comparison to other inflammatory markers, CRP has a longer half-life (19-20 hours), therefore it also has a greater stability over a longer time period. Levels of CRP remain increased throughout the whole acute phase response until normal tissue function is restored [79].

There are two main functional roles of CRP. It is able to modulate the function of phagocytic cells and it plays an activation role in the classic complementary pathway[82]. Additionally, CRP participates in an induction of inflammatory cytokines and tissue factors in monocytes and it also activates endothelial cells in order to produce adhesion molecules VCAM-1, ICAM-1and E-selection[82].

1.2.6 Markers of Inflammation and Cardio-Vascular Comorbidity

Elevated inflammatory mediators have been associated with coronary heart disease (obstructed blood supply to the heart), with cerebrovascular disease (obstructed blood supply to the brain) and with peripheral artery disease (obstructed blood supply to the lower limbs)[82]. These atherosclerotic conditions are collectively referred to as 'macrovascular disease'. Large-vessel atherosclerosis often leads to cerebral hypofusion that may result in artery-to-artery embolism that can also be associated with higher risk of cerebrovascular event. There is evidence of coronary artery disease in patients who suffered from transient ischaemic attacks (TIA) and cerebral stroke[113].

There is convincing evidence from clinical as well as biological studies that in the presence of macrophages, inflammation leads to the localised recruitment of neutrophils, monocytes and the presence activated macrophages in the cap of the atherosclerotic plaque[85]. Atherosclerosis is a systemic disease that affects the majority of the vascular system. Lesions in arteries supplying blood to the brain, i.e. carotid arteries and extra-cranial arteries are of greater clinical importance. The pathogenesis of atherosclerosis is a complex, cascade process that includes development of chronic inflammatory processes. The haemodynamic damage to the artery leads to dysfunction of the endothelium along with activations of cytokines, adhesion molecules and growth factors that are all involved in this mechanism[114].

The selection of factors contribution to endothelial dysfunction is rather complex. The process includes haemodynamic issues, formation of reactive forms of oxygen and nitrogen, elevated artery pressure, genetic variants coding for inflammatory and respiratory responses, elevated homocysteine levels, hypertension, diabetes, hyperlipidaemia and smoking. As a response to the endothelial damage, normal (physiological) arterial properties are altered. Responding to leucocytes and palates increased levels, adhesive endothelial cells are affected and there is an increase in walls' permeability. Consequently, endothelium adopts pro-coagulant (instead of anti-coagulant) properties and cytokines and adhesion molecules are elevated, which results in further damage that eventually leads to focal necrosis[115]. Endothelial dysfunction can be therefore seen as a vascular expression of inflammatory mechanisms[82].

Contrary to the previous assumption that atherosclerosis is caused by cholesterol diseases and consequently obstructing arteries[116], the predominant role of inflammation and haemostasis in the aetiology of atherosclerosis is now generally acknowledged and accepted[117]. In fact, approximately half of the people who suffered from stroke or myocardial infarction had a normal, physiological levels of plasma cholesterol[118].

The infusion of IL-6 and TNF-alpha induces systemic inflammatory response as well as activation of coagulation. In laboratory experiment with mice, blocking the TNF-alpha did not affect coagulation, unlike blocking the IL-6 expression[119], suggesting the central role of IL-6 in the mechanism of activation of the coagulation cascade. Through an activation of tissue factor, it induces fibrin deposition. Fibrinogen

promotes atherosclerosis due to its pro-coagulant as well as pro-inflammatory properties[110]. It plays a key role in the clotting cascade through to conversion to fibrin that stabilizes blood clots fibrinogen, which induces platelet aggregation, forming a clot, which reflects the thrombotic potential of blood[107]. During the development of the atherosclerosis, the disease is often asymptomatic but there is an increased risk of atherosclerotic plaque rupture and activation of the coagulation system, due to a complex mechanism. Specifically, fibrin is known to moderate endothelial cells. It is an adsorptive surface for LDL cholesterol; its breakdown during the process called fibrinolysis results in aggregation of lipids and plaque growth. When the plaque eventually ruptures, it results in an inflammatory response that, in turn, initiates the atherothrombotic cycle[107].

Cytokines IL-6 and TNF-alpha and acute-phase proteins fibrinogen and CRP have been identified as prominent, contributing factors in the development of both stable and unstable atherosclerotic diseases[120,121]. In cognitively healthy, community-dwelling subjects, systemic inflammation was significantly correlated with hypertension, including those on prescribed anti-hypertensive medication[122]. Significant correlation between fibrinogen, IL-6, CRP and TNF-alpha and angina was detected, with CRP and TNF-alpha recognised as the strongest predictor of coronary event[123]. IL-6, TNF-alpha and CRP interfere directly as well as indirectly with the endothelial function,TNF-alpha and IL-1 were found in atherosclerotic lesions[124] and also directly linked to hypertension[102] .

Elevated plasma fibrinogen levels have been described as one of the major risk associated with cardiovascular disease[125]. Increased risk of stroke incidence has also

been supported in relation to elevated plasma fibrinogen levels[126]. A comprehensive review of studies that investigated the role of plasma fibrinogen and cardiovascular co-morbidities was conducted (Fibrinogen Studies Collaboration[127]). Findings from a meta-analysis of 154211 adults, collated from 31 prospective studies with a minimum of a 1 year of follow-up time period. This report summarised that increased levels of plasma fibrinogen were significantly associated with 6944 cases of non-fatal myocardial infarctions or strokes and 13210 cases of death. In terms of evidence gathered from genetic association studies investigating relationship of fibrinogen-related gene variants with incidence of CVD[128-130], the current results are largely inconclusive, suggesting the need for larger, epidemiological studies.

In terms of CRP and its role in the process of atherosclerosis, CRP has been suggested to be a valuable diagnostic marker; elevated levels of plasma CRP are known to precede clinical symptoms of atherosclerosis[131]. Evidence of a direct role of an increased CRP levels in development of atherosclerosis is conflicting[132]. CRP elevation was associated with a 10-year risk of development of coronary heart disease in middle aged adults, independently of presence of the conventional vascular risk factors[133]. Findings from the Edinburgh Artery Study, population-based cohort of men and women aged 55 to 74 years, reported significant association between hsCRP levels and increased severity of peripheral arterial disease[134]. However, in vivo mice models provide inconclusive results of a causal role of CRP in development of vascular disease[135]. It therefore seems that the role of CRP in development of atherosclerosis is indirect, related to vascular damage along with other inflammatory mediators, such as IL-6 and TNF-alpha. In terms of the IL-6 and

TNF-alpha, evidence shows an involvement of these cytokines in the development of cardiovascular condition. In a large study of middle aged men and women, after adjustment for conventional cardiovascular events and risk factors, the risk ratios for the highest tertile of these markers and myocardial infarction was 1.27; 95% CI, 1.10 to 1.48; for congestive heart failure 1.72; 95% CI, 1.40 to 2.12 and for stroke it was 1.45; 95% CI, 1.12 to 1.86[120]. Nevertheless, findings from the Rotterdam Study found no significant association between IL-6 and carotid IMT or carotid plaques[136]. In general, studies tend to report reasonably consistent evidence of significant associations between IL-6 and CVD, independently of adjustment for major vascular risk factor[82]. The role of TNF-alpha appears to be mainly in terms of activation of expression of IL-6 (along with IL-1)[85] and thus inducing the inflammatory cascade (described above), rather than a direct role in aetiology of atherosclerosis. Over-expression of TNF-alpha has been detected in the adipose tissues of obese adults and is associated with reduced activity of the insulin receptors[137].

In summary, epidemiological evidence suggests significant association between elevated inflammatory markers and increased risk of atherosclerotic disease. The relationship between inflammation and atherosclerosis can be seen as bi-directional with multiple and multifactorial role of these markers in inflammation as well as coagulation processes. In recent years, increasing evidence from prospective studies has been reported; however, further large-scale studies are required.

1.2.7 Markers of Inflammation and Type 2 Diabetes

The relationship between low-grade inflammation and development of type 2 diabetes was first proposed in 1987[81] and since then it has received an increasing attention. It has been proposed that inflammation induced insulin resistance and impaired insulin secretion might be an integral part of the pathogenesis leading to diabetes. T2DM and insulin resistance are closely correlated (approximately 90% of diabetic population[138]), which is the preceding signs of the disease.

It is well acknowledged that there are a number of possible mechanisms in which inflammation, cytokines in particularly, contribute to insulin resistance, impaired insulin secretion dyslipidemia and progressive atherosclerosis[82]. This suggests a possible ongoing cytokine mediated acute-phase response that is closely involved in the pathogenesis of the T2DM. Prospective evidence suggests that elevated acute-phase proteins, such as plasma fibrinogen and CRP are significantly associated with development of insulin resistance in T2DM[112]. This would suggest that the consequence of atherosclerosis is not only inflammation but also insulin resistance.

The role of both, obesity and atherosclerosis has been implied in this relationship. Obesity, particularly truncal obesity, is strongly related to increased levels of inflammatory markers, in particular plasma CRP levels[85]. However, the increased levels of cytokines, most notable IL-6 and TNF-alpha activate the hepatic production of acute-phase protein, resulting in increased levels of circulating inflammatory markers. Acute phase response is strongly associated with development of

atherosclerosis. Interestingly, not all people with increased levels of inflammatory markers and T2DM present with sub-clinical vascular co-morbidity[139]. It can therefore be questioned whether atherosclerosis develops as a result of activated inflammatory marker or whether both conditions (atherosclerosis and T2DM) develop in parallel, whilst sharing the activation of the innate immune system. In the majority of studies investigating the relationship between markers of inflammation and T2DM, detected associations remain after adjustment for body mass index[140,141], which supports the relationship and provides a convincing evidence for the role of increased levels of inflammatory marker in this process. Nevertheless, the exact mechanism of the relationship between obesity and the activated innate immune system in the diabetic population is yet to be determined. Another alternative explanation postulates that the possible mechanism is linked to a chronic hyperglycaemia that is involved in increased concentrations of glycation end products [142]. These are known to activate macrophages, increase oxidative stress and activate the whole innate immune system, as presented in the Figure 2, above.

Although these associations shows a strong evidence of the relationship between elevated inflammation and incidence of T2DM, it is yet to be established whether inflammation really lies on the causal pathway between these conditions. One possible approach is to select genetic variants known to control the synthesis and production of a particular inflammatory marker (or group of markers). Gene variants can then model the exposure variable against the defined outcome (Mendelian randomisation). A number of studies have attempted to adopt this approach but

majority of this evidence produced conflicting or null findings. Nevertheless, the development of more sophisticated technology may yield some interesting evidence.

1.2.8 Type 2 Diabetes Mellitus - Definition, Clinical Manifestation and Diagnosis and Risk Factors

Definition, Clinical Manifestation and Diagnosis

Type 2 diabetes mellitus (T2DM) is a life-long, chronic, progressive, complex metabolic disorder with multiple aetiologies. In terms of classification and diagnosis, criteria for diagnosis of diabetes has been revised number times, with the current generally accepted classification is presented in the American Diabetes Association (ADA[143]) and the World Health Organization (WHO[144]). Whole range of diabetes presentations, as well as relevant factors (e.g. age of onset and occurrence of obesity) are covered.

The most common types of diabetes with distinct diagnostic criteria: insulin dependent, type 1 diabetes and non-insulin dependent type 2 Diabetes[138]. Other types might develop as a response to medication (iatrogenic diabetes), due to illness of the pancreas or due to genetic coding[145]. Approximately 90% of diagnosed cases follow into the type 2 diabetes sub-category[143]. This thesis is primarily concerned with the role of inflammatory markers in accelerated cognitive decline in type 2 diabetes sufferers, which will be the main theme of the following subsection. This type of diabetes is referred to as *non-insulin dependent diabetes mellitus* (NIDDM)[138]. Type 2 diabetes is common in older patients, typically accompanied by a wide range of comorbidities.

In healthy organism, preproinsulin is secreted from the β– cells of the pancreas and when blood level glucose rise, it is subsequently converted to insulin. Insulin (the main hormone for the regulation of blood glucose levels) stimulates glucose transport into muscle and adipose cells and reduces glucose production in the liver and overall reducing the glucose levels in the blood; this process is defined as the glucose metabolism [146]. In the early stages of the type 2 diabetes the insulin production remains intact for most people and so the sufferer is able to produce insulin. But due to the metabolic alternation in the glucose homeostasis, the person's body insulin dependent tissues fail to respond sufficiently, leading to impaired transition of glucose for energy[138,147]. Consequently, increased plasma glucose (chronic hyperglycaemia) with disturbances of carbohydrate, fat and protein metabolism occurs as a result of insulin resistance leading to a bodily response termed gluconeogenesis (glucose synthesis and failure to metabolise glucose) and a consequent chronic hyperglycemia[148].

Main clinical manifestation is comprised of a group of specific symptoms, such as thirst whilst passing a large volume of urine (polyuria), possible sustained ketosis (elevated levels of ketone, which is a blood compound that the body uses as an alternative to glucose in order to sustain required energy levels). Unintentional weight loss also occurs, especially in T2DM. If a patient presents with a prolonged occurrence of one or more of these symptoms, random testing is required to confirm or exclude a possible diabetes diagnosis[138].

The diabetes specific diagnostic criteria might vary between institutions[149]. The World Health Organisation (WHO) defines the fasting plasma glucose levels above

7.0 mmol/l or plasma glucose above 11.1 mmol/l two hours following an oral glucose tolerance test (OGTT). According to the American Diabetes Association a single reading of fasting glucose levels above 7.0 mmol/L (no caloric intake for at least eight hours), a casual reading of above 11.1 mmol/L (without regard to time since last meal), or a reading of above 11.1 mmol/L in an oral glucose tolerance test (OGTT) during which the effect of an ingested glucose solution on fasted blood levels is tested, is sufficient for diagnosis. Recently, both ADA and the WHO extended their criteria to accept glycated haemoglobin levels (HbA1c), which reflect average blood glucose levels over two to three months, of above 6.5% as indicative of diabetes[145].

In the fasting plasma glucose (FPG) after an overnight fasting, at least 8 hours, the patient is asked to take 75 g of glucose (diluted in approx. 250 ml of water) with the concentration of blood glucose being measured 2 hours after. Impaired glucose tolerance (IGT) is defined by fasting plasma glucose levels that fall below the diagnostic criteria for diabetes (<7 mmol/l) but the two-hour post OGTT results are 7.8 mmol/l to 11.1 mmol/l. Impaired fasting glucose (IFG) is proposed as fasting glucose levels between 6.1 and 6.9 mmol/l, with two- hour post-OGTT below 7.8 mmol/l if measured[145]. For the purposes of epidemiological studies, the WHO recommends applying the 2 hour repeated glucose measure; in this way standardization of the measure is sustained, reducing possible bias in interpretations[150].

Epidemiology of type 2 diabetes, risk factors

Approximately one third of individuals with IGT or IFG remain pre-diabetic, one third revert to normoglycaemia, and one third progress to diabetes[151].

In terms of a worldwide estimate, there is a progressively growing trend in the prevalence of type 2 diabetes. Whereas in 1935, 15 million people worldwide were estimated to be suffering from a form of diabetes (1% of the world population of 2 billion), this number exploded to 220 million in 2010 (over 3% of a world population approaching 7 billion) and it is expected to rise to 336 million (4.4%) of people by the year of 2030[152,153]. Changes in diagnostic criteria since 1965 and improved case ascertainment partly account for this development. However, actual increases in fasting plasma glucose levels in the Western world are undeniable[154 155,156]. According to the Department of Health, in the UK, it is estimated 4% of population has diabetes, of these T2DM sufferers form approximately 85-95% of the overall diabetes population[157,158]

There are a number of issues with establishing incidence, prevalence and thus estimating further trend in those. In particular, these are hindered by methodological issues linked to differing diagnostic criteria as well as due to variability of age and other factors in populations. Furthermore, since the diseases, especially T2DM can remain asymptomatic for a number of years it may go unnoticed/undiagnosed or masked by other comorbidities; cases of no symptoms at all during the whole duration of the condition have been described[138]

Despite the fact that precise biochemical pathological processes leading to diagnosis of T2DM are yet to be fully determined, multiple risk factors associated with insulin insensitivity hence been proposed, are broadly divided between genetic and environmental. From the genetic point of view, evidence suggests a possible monogenetic determinant of T2DM[159]. However, incidence of multi-genetic (different combination of several gene defects) origin has also been documented, although comparatively this is rather rare[160]. In general, the majority of T2DM cases are due to polygenetic (simultaneous action of several genes) influence, showing a clear familial aggregation. Though not in a typical Mendelian fashion, having one or more parent or sibling with T2DM is an identified risk factor[161]. Nevertheless, despite progressively more convincing evidence of genetic determinants of diabetes onset, complex inter-relationship between genotype and environmental factors is a probable pathway in the explanation of this condition.

In particular, an advanced age is a strong risk factor; generally, the most affected proportion of population is formed by these above 65 years of age. Although T2DM is less likely to be diagnosed at an age of 40 and above, increasingly lower age ranges have been documented and thus presenting an additional NHS and other resources cost[157]. Comparatively, in developing countries, onset of T2DM is generally lower, approximately 45-64 years of age[155]. Worldwide, there is a trend in gender that more women than men are affected, most likely due to differences in life expectancy[155]. However, this figure is not age or ethnic specific and only represents the overall sum. In this respect, ethnicity was also shown to be an associated risk factor with a higher prevalence being observed in the South-East Asian population,

followed by the United States in terms of African Americans, Mexican Americans and Native Americans[138,157,162]. In a recent years a growing trend has been observed among younger ethnic populations, in particularly South Asian and African Caribbean[157,163]. In general, there has been a rise in prevalence of T2DM in younger populations. Since physical exercise tends to increase insulin sensitivity[164], it is reasoned that the decreased physical activity and co-occurring increased rates of obesity among children and young adults are the key factors are responsible for the increasing prevalence of T2DM in this age group[165].

In terms of additional environmental factors, primary concerns are smoking, lack of physical activity, dietary factors, ethnicity and inter-connectivity[138,166]. A cluster of risk factors, such as obesity, central/abdominal obesity in particular, dyslipidaemia, hypertension, hyper coagulation, micro-albuminuria are commonly referred to as 'metabolic syndrome'[167].

1.2.9 Association with Cognition

One of the most pronounced risk factors for age-related cognitive impairment and age-related cognitive decline is the presence of type 2 diabetes. Epidemiological evidence indicates that, in comparison to the non-diabetic, age and sex matched population, people with T2DM are at 1.5-2.5 fold greater risk of dementia. In particular, there is a 2-fold increased risk of vascular dementia and up to 2.3-fold greater risk of dementia of the Alzheimer's type[168]. The reason for this is thought to be multifactorial; nevertheless, the exact biological mechanism yet to be determined.

Both hypoglycaemia and chronic hyperglycaemia have been implicated in the development of cognitive deficits in the diabetic population. Also, Draelos[169] reports overall impairment in cognitive function in patients with insulin-dependent diabetes mellitus during hyperglycaemia and hypoglycaemia events.

In terms of the hypoglycemic events, the majority of contemporary evidence is based on retrospective results and cross-sectional data. This evidence reports the relationship between incidence of (severe) hypoglycaemia and impaired cognition. However, the direction of this relationship cannot be assessed in cross-sectional design, leaving a scope for speculation about reverse causation, in that person with reduced cognitive ability might be at higher risk of (severe) hypoglycaemia. Also, the majority of studies focused on type 1 diabetes or they report inconclusive evidence with varying results[169]. Recently, we have showed a significant relationship between severe hypoglycemic events (i.e. self-reported incidence of hypoglycemia that required external help[170]) and impaired general cognitive ability at baseline as well as accelerated decline detected at four-year follow up measures. At the four-year follow up, prevalence of severe hypoglycemia was also linked to a significantly higher risk of declined performance in tests assessing non-verbal reasoning, working memory, attention, mental flexibility and executive functioning[171].

Recently published reviews by Cukierman et al[168] and Kodl and Seaquist[172] concisely summarised the evidence of association between T2DM and cognitive decline. Convincing evidence for the relationship between T2DM and deficits in domain-specific cognitive dysfunction was detected, in particular memory, executive functioning and psychomotor speed. A negative association was observed in visual

retention, attention and motor functioning[172]. Cukierman et al reported an overall greater risk as well as decline in information speed processing and attention and a greater risk of future dementia. Examination of evidence from longitudinal studies fully support these findings. Furthermore, one identified study reported a significant relationship between T2DM and vascular dementia in a population-based study[173]. Recent findings from the Epidemiology of Vascular Ageing (EVA) Study suggests that ageing individuals with diabetes were two times more like to suffer from overall four-year cognitive decline, independently of conventional cognition-related confounding factors, such as smoking, alcohol and relevant co-morbidities[174]. Brain-imaging studies provide further support suggesting that diabetes-related brain pathology result in impairment of the majority of cognitive functioning, with memory performance being the least affected[175].

In a recent study, demographic analysis of 232 participants from The Austrian Stroke Prevention Study demonstrated that the presence of metabolic syndrome was associated with less physical activity, more CHD, higher frequency of each component of metabolic syndrome, higher levels of C-reactive protein and also with a lower educational attainment. Perhaps not surprisingly, compared to age and sex matched counterparts, those with the metabolic syndrome performed worse on all cognitive tests. The discrepancy between these groups was most visible at memory test and executive functioning tests; difference on psychomotor skills reached a marginal level of significance[176]. These findings are supported by results based on large data set of 1969 ageing men and women that reported the metabolic syndrome significantly predicted mild cognitive impairment. This relationship was modified by

elevated levels of plasma CRP, increasing the risk for the non-amnestic mild cognitive impairment 2.31 times[177]. Another large study, comprising of 959 randomly selected older adults, reported a significant relationship between metabolic syndrome and prevalence of Alzheimer's disease, irrespective of major risk factors, including APOE, ε4[178] (which itself is a major risk factor for development of Alzheimer's Disease).

Further evidence suggests that older people with good glycaemic control are less likely to experience the same impact on their cognitive ability as older people with poor glycaemic control. However, this has only been observed in relatively younger subjects. Beyond the age of 70 years, interaction between T2DM and cardiovascular conditions likely results in accelerated cognitive decline, possibly converting into frank dementia of either Alzheimer or Vascular type[138,179].

Epidemiological observations provide a robust evidence of the type 2 diabetes and cognitive decline relationship. The exact biological mechanism, however, is yet to be determined, which has proven methodologically complicated[173]. Evidence of a direct relationship between microvascular association with cognitive impairment and decline is scare, mainly due to difficulties in assessment of microvascular status[180] . Furthermore, there is a lack of longitudinal data on a reasonably large samples, many studies use only limited selection of neuropsychological tests that might allow for a comprehensive assessment of the whole range of cognitive domains. Lastly, there is a great inconsistency in selection of covariates, with age and sex, being the most common. However, as discussed above, diabetes is a complex condition, almost always happening in association with comorbid conditions, particularly cerebro-

vascular, cardiovascular disease and/or elevated systemic inflammatory status, each of which can act as a mediator or as independent risk factor for ACRI in people with diabetes[53].

1.3 Chapter Summary

Systemic inflammatory responses are complex, encompassing a wide range of mechanisms and pathways. Among the main inflammatory-related changes are vascular and metabolic changes. Inflammation causes endothelial dysfunction and through a cascade of circular mechanisms leads to development of atherosclerosis. Inflammation also affects lipid metabolism, which is associated with insulin resistance; as such, insulin resistance can be seen as a metabolic expression of inflammation[82]. Epidemiological evidence is largely supporting the role between inflammation, cardiovascular disease and T2DM. However, there are some conflicting or inconsistent results, suggesting the need for further investigation. Such investigations are methodologically complicated due to the multi-directional relationship between each aspect; distinguishing what is causal, confounding, mediating or co-existing factor is complicated due to the lack of risk specificity. Therefore further support of causality is warranted. Genetic association studies have been proposed as one of the possible approach. The next section will focus on issues relevant to genetics, genetic association studies and it will also focus specifically on genetic variants related to increased levels of plasma fibrinogen.

1.4 Genetic Epidemiology

1.4.1 Genetics – basic principles

Genetics (from the Greek 'ghenno = procreate[181]) is a science that is concerned with inheritance and variability between species. The primary aim of genetics is to determine mechanisms of inheritance and differences and similarities in genetic profiles between generations by examining the contribution of particular gene (genes) to completion of a particular trait, or group of traits, commonly referred to as *phenotype*. All living organisms are made of cells within which most chemical reactions and processes take place. The genetic information is essential for the maintenance of the existing cell and the production of new cells. Genetics is therefore the essential biological discipline, arguably one of the most important sciences.

There are approximately 25 x10^{15} cells in one human organism. The control center of the cell is the nucleus; this is where the genetic information is stored in the form of DNA[182]. Each cell contains forty-six chromosomes, coming in twenty-three pairs (with the exception of sex cells). Chromosomes are formed by thin strands of deoxyribonucleic acid (DNA); each chromosome contains hundreds of genes. Genes are the segments of DNA with the primary function as hereditary units. They are further transcribed into proteins that carry out a wide range of functions essential for

bodily mechanism. An alternative pair (or group) of genes that is positioned in a specific loci of the chromosome is called allele[183].

The next subsection of this chapter describes the basic principles of genetics.

1.4.2 DNA, Gene, Chromosome, Allele, Haplotype, SNP

The structure of the DNA (deoxyribonucleic acid) was discovered and first described by James Watson and Francis H. C. Crick in 1953 and since then provided the basis of further scientific activities[184].

DNA is the essential blueprint of inheritance; it is the primary carrier of the genetic information in all living organisms (with the exception of few viruses where RNA plays this role[183]). The DNA molecule is organized in the form of a double-stranded helix that consist of 5-carbon sugars (deoxyribose) and two groups of simple units called nucleotides, each composed of nitrogen bases. In DNA, these represent purine: *adenine* (A) and *guanine* (G) and pyrimidine: *cytosine* (C) and *thymine* (T). Interaction between nucleobases always occurs according to the complementarity rule, i.e., only two specific bases are attached, one purine and one pyrimidine (i.e. *adenine* & *thymine* and *cytosine* & *guanine*). The two strands in the double helix are positioned in opposite directions. As the two polymer strands align, bind and twist, they give the DNA its classical double helical shape. Human DNA is comprised of approximately 3 billion base-pairs[184].

Chromosome, gene

DNA (in eukaryotes) is organised into 46 *chromosomes*; 22 pairs of autosomal chromosomes and two sex chromosomes. A g*ene*, from the molecular genetic point of view, is a specific sequence of nucleic acids which determines the structure and function of the gene. It is located on a fixed point of the chromosome, called the *locus*. Genes can be defined as units of specifically stored, inheritance information for specific proteins, corresponding to a range of biological mechanisms and traits (figure 3).

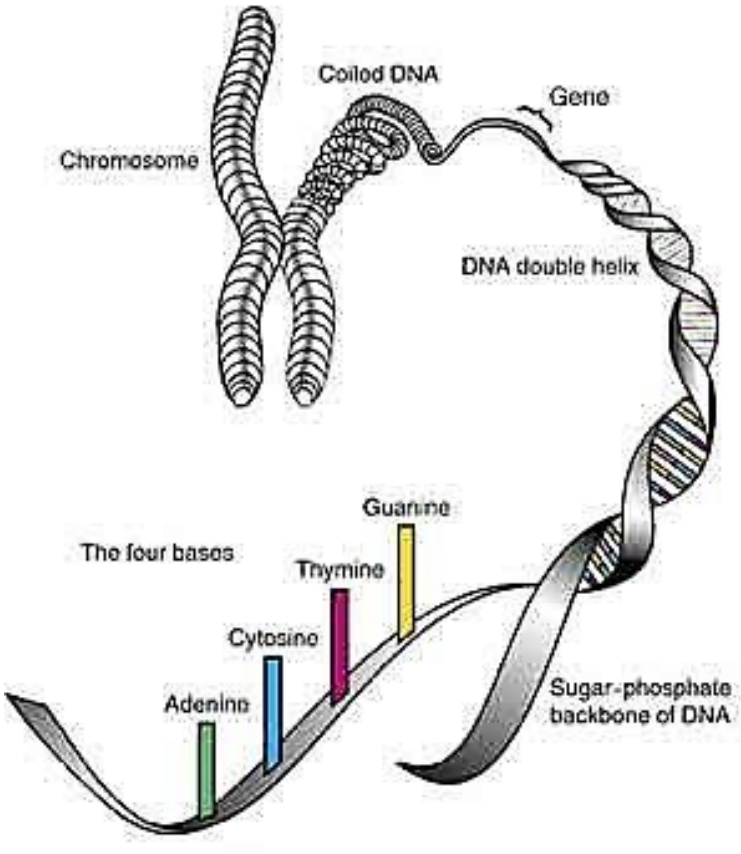


Figure 3: Structure of the gene

Furthermore, genes can also contain 'non-coding' regions that are involved in determining its expression in the process called *transcription*, which uses information from both coding and non-coding parts of the gene. The part of the gene that eventually transcribed into a protein, is called the exon, the non-translated regions are called introns; the length of these varies between genes.

Allele

The specific form of gene, occurring in a specific locus, is called the *allele* (allomorphic gene). The combined information from both alleles represents the person's *genotype*. Alleles are crucial in determining the final form of the individual's genetic trait. Various combinations of alleles also result in variability of phenotypic presentations. Inside of a diploid cell, there are always two alleles for one gene; this can be in the form of a homozygous individual (individual has two same alleles for a trait) or a heterozygote (two different and distinct alleles for the same trait).

If one allele is dominant , an allele is producing the same phenotypic effect regardless of homozygous or heterozygous inheritance. Complete dominance occurs when the dominant allele (commonly referred to as 'A') masks fully the function of the recessive allele (commonly referred to as 'a'); the recessive allele i.e., an allele that is masked by the dominant allele, does not produce a phenotypic effect if inherited heterozygously, however, it affects the phenotype if inherited homozygously.

Genetic variability is primarily determined through genotype frequency. From this, we can calculate the *allele frequency*; this represents a contribution of specific allele or genotype within specific loci. Allele frequency is occasionally mistaken for gene frequency – this would be misleading as whereas alleles differ in frequency, genes do not[183].

Haplotypes

The human genome, of a total length of three billion base-pairs, is made of haplotype 'blocks'; group of alleles on the same chromosome, within which alleles at different loci are strongly associated. This group is commonly referred to as '*haplotype*' (a combination of the phrase 'haploid genotype'). The average block length for Europeans is 25kb. Blocks occur as the results of constraints on population size during the history of our species. Within a block, two-four common haplotypes typically account for >90% of all allelic variations.

1.4.3 Genetic variation

As mentioned above, a gene, as a hereditary unit, is a stretch of DNA sequence that encodes information in a combination of nucleotide bases. This information is subsequently expressed as another class of bio-polymers, called proteins. Depending on the mutation location and whether the protein structure is altered, genetic variants can have, to a smaller or bigger degree, an effect on health.

Genetic variation happens through a several different types of mutation altering the structure of the genes. Two individuals share 99.9% of the DNA sequence with the 0.01% defining variability between individuals. The *phenotype variability* is based on the *genotype variability* that is determined by the role of numerous gene variants. i.e., *polymorphism*. Polymorphism refers to a situation where two alleles determine a specific trait. Whereas polymorphic alleles are frequent, meaning that the frequency exceeds 1% any lower frequency is referred to as a mutation. The smallest possible change/variance in DNA sequence is replacement of one nucleotide, a so called the *Single nucleotide polymorphism(s) (SNP, plural SNPs).*

The 'coding' part forms only a small proportion of the DNA sequence whereas the remaining part of the genome is formed by non-coding and extra-gene sequences. The manifestation of DNA sequence in phenotype presentation is also determined by degeneration of genetic coding when during the process called *translation,* in the coding sequence, the substitution of one nucleotide is not into amino-acid, determined by the messenger RNA (mRNA). This means that in the sequence of nucleotides there is a substantial amount of polymorphisms that might not affect phenotype. One way to detect this is through methodology linked to molecular genetics.

In terms of medical genetics, genetic methodology and public health, the most important polymorphisms is the single nucleotide polymorphisms[182].

The frequency of SNPs in one genome is approximately one in every 1000 base-pairs (bp). Most SNPs are bi-allelic, meaning they consist of two alleles[185]. Individual

SNPs affect the structure and role of proteins only to a degree; this depends on the type of mutation[186]. Even a relatively small change, i.e., of one nucleotide, can result in a wide spectrum of different effects. However, SNPs in the coding regions form approximately 5% of genetic diversity the remaining part of genetic variability is therefore in extra-gene and non-coding regions. Recently, contrary to previous views, there is an increasing body of evidence indicating the role of non-coding sequences in human biology[187].

Commonly, detection of individual SNP is carried out via polymerase chain reaction - PCR. Increasingly common is the use of DNA microarrays in SNP analysis, which allows for detection and identification of a very large number of SNPs at one time (on the order of hundreds of thousands/one million).

1.4.4 Genetic Epidemiology

There is an increasing amount of evidence providing understanding of biological/pathological mechanisms of complex conditions; this includes an identification of a wide range of modifiable risk factors, determinants, protective factors and the multi-directional relationship between them. However, traditional epidemiological studies suffer from the effect of confounding and/or mediating variables which constrains the interpretation of their findings. Further, bias can occur in measurement of predictor as well as outcome or exposure variables; in particular, measurement of exposure may be affected by the disease onset and elimination of selection bias is often impossible, particularly in case-control studies.

Epidemiological studies, despite using the same or similar methodological approaches, often provide inconsistent, or even conflicting results and identification of a robust causal factor/determinant for a given disease is not possible.

Extensive documentation of the human genome allows for an alternative approach that can at least partially resolve these limitations by the use of an *instrumental variable* in the form of genetic variant(s) that can be modelled against the defined outcome. [188]. This approach is part of the discipline known as *genetic epidemiology*. Primarily, the approach does not differ from the traditional epidemiological approach – once the potentially causative genetic determinants of the predictor(s) are identified, they can be modelled directly or via an intermediate phenotype against the outcome of interest.

Genetic epidemiology is "the study of the joint action of genes and environmental factors in causing disease in human populations and their pattern of inheritance" (Thomas, 2004, p. 3[189]). Genetic association studies can either focus on an individual gene or gene variants candidate or on examination of mutations through the whole genome. Genetic association studies aim to map and investigate regions of linkage disequilibrium containing the causal variant(s). As these investigations identify a smaller region of the genome, they have a greater power to detect smaller effects[189]. This methodology has a number of advantages. In correctly designed studies, there is a limited chance of bias in measurement of exposure (i.e., the genotype) and the selection bias is usually less serious as it can be dealt with by controlling for population stratification. In the case of haplotypes, the use of tagging SNPs can deal with this issue sufficiently.

1.4.5 Causal Inference in Genetic Epidemiology

Genetic association studies are one of the most popular approaches to the identification of genetic propensity of organisms to a specific trait. Primarily, the aim is to test the strength of a correlation between genetic variation and a specific condition to identify either candidate genes or a genome region that might contribute to the diseases status or increased prevalence of such conditions[190]. The frequency of SNPs is most widely investigated in candidate association studies; alternatively, with the HapMap (discussed below), it is now possible to detect and/or test novel associations through genome wide association studies (GWAS) where a large number of individuals are examined for a commonality of genetic variants associated with a specific trait[182]. The candidate gene study can take the form of a case-control study with a comparison of allele frequency of candidate gene(s) prevalence. Linkage studies, which are family-based, aim to identify particular regions of genome that contain a non-random inheritance pattern. Typically, these regions are very large with millions of base-pairs. In comparison, association studies focus on narrower region of the genome; there are more suitable for fine-mapping due to a greater power to detect effects smaller than the linkage investigation[183].

To date, candidate gene studies for a wide range of diseases[191] as well as GWAS[192] have detected direct associations and thus true causal SNPs for increased disease vulnerability. However, as with any other research method, genetic association studies suffer from a number of limitations that differ from conventional epidemiological studies that can lead to false interpretation of the study results[193].

Specifically to GWAS, testing a large selection of genetic variations, the risk of type I error is considerably high; this can be addressed by multi-stage replication studies that might eliminate the risk of missing (potentially important) SNPs that did not reach the significance level in the original genome-wide association study[194]. Further problem affecting GWAS include SNPs is in linkage disequilibrium (LD) to the true causal SNP resulting in indirect association, population stratification presenting a potential confounding variable with the risk of false-positive results, and interaction between genes (epistasis)[192,195]. The following section of this chapter will discussed this in greater details.

1.4.5.1 The HapMap

The International HapMap Project was designed to cover the 'entire' human genome through using commercial DNA microarray-based methods, identify common haplotypic variants, and this facilitate examination and identification of genetic variance in common disease[196,197] The project was completed in 2000 with the first study published in 2003. By 2005, HapMap completed a haplotype map of the human genome and determined allele frequencies for about 1.3 million SNPs (The International HapMap Consortium, 2005[198]); this led to documentation of regions that are difficult to study as well as it identified linkage disequilibrium patterns[199]. Subsequently, by 2007, a further 2.1 million SNPs (25-35%) were identified[200]. Currently, at the time of writing this thesis, almost all mapping information for 10 million SNPs has been completed (http://www.genome.gov/11511175).

1.4.6 Methodological issues of genetic-association study design and in meta-analysis of genetic association studies

There are number of limitations and methodological obstacles that impact on interpretation of gene-association study results, particularly when the aim is to determine causal relationship in a complex condition, such as age-related cognitive changes.

1.4.6.1 Linkage Disequilibrium (LD)

Linkage disequilibrium (LD) essentially describes the non-independence of alleles at two or more loci (making up a haplotype).

Non-random associations are caused by physical proximity (linkage) in a way that the two alleles happen to occur together in the same gamete. This happens more often than expected from their allele frequencies. As every new mutation occurs in a haplotype, it becomes more frequent with that particular background. The magnitude of LD depends on the amount of recombination between the loci that breaks down the haplotype over time. The LD will persist for many generations between tightly linked loci. Linkage equilibrium is attained gradually through random mating.

LD can also be caused by population *stratification* where the sample is a mixture of sub-populations with different allele frequencies or by *admixture* where the population is comprised of individuals of mixed ancestry – containing blocks of their

genome from different populations. Causal factors/reasons that disturb HWE (see below) seem to lie on the same causal pathway for the LD.

The genome consists of blocks of high LD, separated by hotspots of recombination; in the genome of non-Africans, the LD typically extends for ~100 kb. Only a few different haplotypes can commonly be found within each block. Only three to four markers can 'tag' all the present variations. LD mapping is therefore important as it is one of the possible ways to map the entire genome, which has over 10 million common variants.

1.4.6.2 Hardy-Weinberg Equilibrium (HWE)

Allele and genotype frequencies may be related according to Hardy-Weinberg law, i.e., the ordered genotype frequencies in offspring are the product of the allele frequencies in the parental generations through a random mating. Non-sex cells always carry two alleles; paternal and maternal. Assuming that the population is large, mating is arbitrary, and that no mutations or migration occurs and there is an average fertility, then the probability of an individual with allele AA is p[A] times p[A]; the same rule applies to probability of individuals with two alleles AA, Aa, aa. Therefore the expected frequencies of the 4 ordered genotypes are:

p^2 [AA] = p[A] x p[A]

pq [Aa] = qp[aA] = p[A] x q[a]

q^2 [aa] = q[a] x q[a]

This equation allows for a calculation allele frequency in future generations, that is:

$p^2 + 2pq/2 = p\ (p+q) = p$

$a^2 + 2pq/2 = q\ (q+p) = q$

Therefore estimated frequency of both alleles remains the same across generations. As a typical example of the Hardy-Weinberg law is the prevalence of blood group with a negative Rh in European population where a prevalence of just under 17% prevalence remains constant[201,202]. However, conditions necessary to satisfy the HW law are very seldom possible; possible explanations for deviation of frequencies from HWE include non-random mating, allele frequencies change from generation to generation as the population evolves, through mutations due to arbitrary environmental interactions, genotyping error or random fluctuations in allele frequency (*gene drift)*[189]. Whether the sample, i.e., the estimates of genotype frequencies within a chosen population, follows the HWE law can be checked mathematically (goodness of fit, chi-square) or through software developed specifically for this purpose (e.g., www.wbiomed.curtin.edu.au/genepop).

1.4.6.3 Epistasis

Epistasis, the interaction between different genes, is one of the major obstacles in gene association studies. It was first described in 1909 by William Bateson who referred to the complexity of the mapping relationship between genotype and phenotype, using the example of hair colour, specifically dominance of alleles within the same pair, i.e., the same locus[203]. He noted that regardless of the genotype (i.e., b/b, b/B or B/B) individuals with any of the G copies had a one hair colour[203]. This

effect of gene interaction, in particular interaction between alleles at different genes, can occur at the same step or at different stages of the same biological pathway.

In the case of a simple, 'Mendelian', disorder it is less difficult to identify specific loci. However, in complex traits, such as diabetes, asthma, multiple sclerosis, etc. this is particularly complicated due to factors such as increasing numbers of identified loci and contributing alleles, and, to an extent, contributing environmental factors. Additionally, detection is complicated by masking effects of one gene on another gene, reducing the impact of the causal genetic variance[195].

1.4.6.4 Heterogeneity

By definition, heterogeneity refers to detectable systematic differences between studies, predominantly occurring due to study design, measurement of phenotype, differences in effect sizes or any combination. In meta-analysis, this is a particular issue as it can lead to underestimation of meta-analysis results[204]. When results from collated studies present with different study characteristics and/or different effects, the use of a random effects model, rather than a more rigid fixed effects model, is recommended. In meta-analysis, random effect models operate on the assumption that there are substantial differences between individual studies' magnitude of effect and thus statistical heterogeneity is assumed.

1.4.6.5 Population stratification

Bias can occur as a result of population heterogeneity when the study population is formed by a mixture of individuals with varied genotypes, and different populations present with a specific disease caused by non-genetic factors[205]. Differences in allele frequency due to systematic ancestry differences presents a major issue as it can produce false associations. A possible way to address population stratification is to perform a principal component analysis to model ancestry differences[206] as well as infer genetic ancestry[207], controlling for ethnicity[208].

1.4.6.6 Age

DNA methylation is a process that involves addition of methyl group to the cytosine and adenine nucleotides [182] and that is also associated with the ageing process[209]. Recent evidence suggests that age-dependent methylation changes play a role in autoimmunity and cancer incidence. Also, in some tissues, age-related demethylation (decreased levels of methylated cytosines) can be responsible for chromosomal instability and rearrangement with the consequence of increased risk of neoplasia[210].

1.4.7 Fibrinogen

Fibrinogen gene, location, normal function and gene variants

Fibrinogen is a plasma glycoprotein, produced by the liver. It consists of three units, alpha, beta and gamma that are coded by three different, corresponding genes: *FGA*[1], *FGB*[2] and *FGG*[3, 211]. Molecular location of the genes is on the short arm of the chromosome 4; cytogenetic location 4q23-q33, covering an area of 50kb, arranged in order *FGG-FGA-FGB* .

[1]http://ghr.nlm.nih.gov/gene/FGA; [2]http://ghr.nlm.nih.gov/gene/FGB; [3]http://ghr.nlm.nih.gov/gene/FGG

The *FGA, FGB* and *FGG* genes function is to instruct a production of the fibrinogen Aα, fibrinogen Bβ, and fibrinogen γ (gamma) individual chains of the fibrinogen protein that, combined together, forms the functional fibrinogen[212]

The fibrinogen protein is a fundamental element in the formation of blood clots, a process called coagulation. Additionally, the beta gene is known to be involved in fibrinogen synthesis[213]. Mutations in either of the fibrinogen genes may results in numerous bleeding conditions, such as congenital afibrinogenemia (excessive bleeding), hypofibrinogenemia (decreased fibrinogen in the blood), dysfibrinogenemia (abnormal functioning of fibrinogen) or combination of these two, hypodysfibrinogenemia (abnormally functioning low levels of fibrinogen)[214].

Circulating fibrinogen levels can be partially accounted for by SNPs across these three fibrinogen genes, aalthough only limited evidence supports this explanation for variability in plasma fibrinogen levels; only 20 to 51% of variations in circulating fibrinogen levels can be explained through heritability[109] and even large, GWAS investigations, such as the Framingham Heart Study[215] could not detect such association. Instead, locations on chromosome 2 and chromosome 10 were linked to the highest LOD (*logarithm of odds score;* a statistical test applied in genetic linage analyses to test the probability if the two loci are not linked[183]). The discrepancy may be due to an increased frequency of alleles that are associated with elevated plasma fibrinogen levels, as well as can be due to non-random associations of alleles – those potentially located on different chromosomes. The potential for masking the true causal allele was considered to be high.

Non-genetic factors associated with plasma fibrinogen levels

Increased plasma levels are associated with age, female gender[107] and ethnicity[128]. Common cardiovascular risk factors have been positively correlated with elevated plasma fibrinogen levels, namely smoking (including passive smoking[216]), obesity, waist-hip ratio, diabetes, hypertension and elevated LDL-cholesterol levels[107]. Specifically, a number of components of metabolic syndrome, such as decreased HDL-cholesterol, glucose ≥ 5.5 mmol/L., diastolic BP ≥ 90 mm Hg, have been positively associated with increased plasma fibrinogen level, independently of major confounders[217].

In terms of hormonal status, data provided by both cross-sectional and longitudinal evidence have not been consistent. The use of oral contraception[218], menopause and hormone replacement therapy[219] have been associated with elevated plasma fibrinogen levels. However, studies also suggest an inverse relationship between the latter two factors and increased fibrinogen concentration[107]. Interestingly, positive interaction has been observed between the use of oral contraceptive and smoking[220]; conversely, within approximately 3 months of discontinuation of oral contraceptives[221], relatively rapid reduction of plasma fibrinogen levels have been detected after cessation of smoking; although the duration of an average of 10 years is required to return to the state of healthy never-smokers[109]. In terms of alcohol consumption, a U-shaped relationship has been observed between alcohol units and plasma fibrinogen levels amongst men[222] and independent of gender[223]. In a large epidemiological study; higher concentrations of the plasma fibrinogen were detected in non-drinkers or >60g/day of alcohol. The association was stronger in men again; a possible explanation might be linked to the type of alcohol but the precise mechanism is yet to be determined[224].

Psychosocial factors have also been strongly associated with plasma fibrinogen level with stress being one of the strongest candidates[225]. Childhood environment, father's social class and levels of education have also been inversely associated with elevated fibrinogen concentration[226], postulating plasma fibrinogen levels as a possible explanation of an inverse relationship between socio-economic status and coronary heart disease[227]. In large population-based studies (Caerphilly and Speedwell Studies), plasma fibrinogen levels were found as a strong determinants of the effect

of low socio-economic status on increased risk for CHD[228]. Other factors correlated with plasma fibrinogen level include seasonal effect (with higher levels in winter[229]), low birth weight[230] and air pollution[231].

Chapter 2: Literature review

2.1 Aim

The purpose of this literature review is to systematically identify and critically discuss the available prospective epidemiological evidence on the association of inflammatory and acute-phase proteins with cognitive decline in elderly populations. For this purpose, published articles that investigated the nature and strength of association between the systemic inflammation and late-life cognitive ability were identified using a systematic literature search carried out with adherence to a predefined set of inclusion and exclusion criteria. The rationale for the selection of studies and the details of the final search are outlined at the beginning of the chapter. This is followed by a summary of the identified literature search, also including a brief outline of the excluded studies. A critical appraisal of the eligible evidence focuses on methodological aspects of the included studies. Finally, a summary of the appraised evidence and implication for the original work presented in the remaining section of the thesis is presented.

2.2 Literature Review Methods

Search strategy

The electronic databases Medline, EMBASE and PubMed were used to search for prospective evidence on the inflammation-cognition relationship. Searches were last run on these databases, October, 2013.

Key search words included: "*Cognition.mp/cognit$.ti,ab./cognition disorders/cognit$ impair$.mp/*", exploration of reported inflammatory markers included: "*Fibrinogen/fibrin$.ti,ab./Interleukin-6/IL-6.ti,ab./C-Reactive Protein/C-Reactive Protein/CRP.ti,ab./Tumor Necrosis Factor-alpha/TNF-alpha.ti,ab./biological markers/ or exp inflammation mediators/*". The search was restricted to an ageing population by:*"(elderly or aged or "old age").ti,ab*.", and in terms of methodology the key words were: "*prospective.mp. or Prospective Studies/ or Aged/ or prospective.mp./Cohort Studies/Longitudinal Studies/ or longitudinal.mp*.". Pre-determined inclusion criteria included studies that reported findings from investigations on living human participants and for which a full text paper was published in English language.

Inclusion and Exclusion Criteria

Many of the neuropsychological tests commonly administered in epidemiological studies have been shown to be domain-sensitive[19]. Furthermore, some of the individual cognitive tests are deemed as not sufficiently sensitive for detecting borderline changes, especially if the changes in cognitive profile are associated with

particular alteration in brain anatomy (for example, vascular damage to one particular area only[4]) or in cognitively healthier subjects[232]. This applies specifically to the Mini Mental State Examination, a test that was designed for screening purposes only and therefore is not sufficiently robust to confirm an overall cognitive decline[233]. Therefore, only studies with a comprehensive cognitive battery, consisting of multiple testing, were selected in order to allow for a review of findings that are comparable to the presented ET2DS results. To ensure that findings were comparable to the ET2DS, at least one of the markers of inflammation assessed in the ET2DS must have been present in the reviewed articles.

Cross-sectional data are undoubtedly a valuable source of evidence for cognitive impairment relating to cognitive performance at one given time; nevertheless, unlike longitudinal evidence, this does not allow us to observe changes in cognitive ability and to subsequently assess the potential predictive value of measured markers of inflammation[173]. A longitudinal design is therefore better suited for the investigation of a complex, over-time developing condition. For this reason and also to collate evidence comparable with the prospective design of the ET2DS, only prospective evidence was eligible for the current review. In summary, studies must have satisfied these conditions:

Inclusion

- Original research only, full text available (reviews scanned for additional relevant references)
- Prospective design
- At least one of the circulating inflammatory marker investigated in the ET2DS analysis (i.e. plasma fibrinogen, CRP, IL6 and TNF-alpha)
- The cognitive neuropsychological battery contained at least one assessment of at least one cognitive domain that was compatible with ET2DS cognitive assessment
- Articles reported results gathered from live adult/human, age 60 and above
- Articles published in the English Language

Exclusion criteria

- European ancestry
- Post-mortem findings
- Animal studies
- Baseline sample consisted of either solely or comparison of healthy subjects and subjects with clinical manifestation/diagnosis of any kind of MCI or dementia of any type, including any type of a motor-neuron disease; cerebrovascular disease (any type); cardio-vascular disease (any type); depression; post-surgery cognitive performance; rheumatoid arthritis; dialysis

Data extraction

A range of information was extracted from the studies included. In terms of methods, this included: details of sampling; population characteristics; baseline sample size; sub-sample as reported in this review; age (mean and/or, SD and/or range); percentage of male subject; which of the four inflammatory markers were investigated; what cognitive/neuropsychological tests were administered, and if the pre-morbid intellectual ability was estimated. Methodological details of included studies are summarized in Table 2. Co-variants used in multivariate analysis were recorded along with all relevant results; this is presented in Table 17, Appendix A. The Figure 4 below outlines and sumarises consequent stages of the literature search.

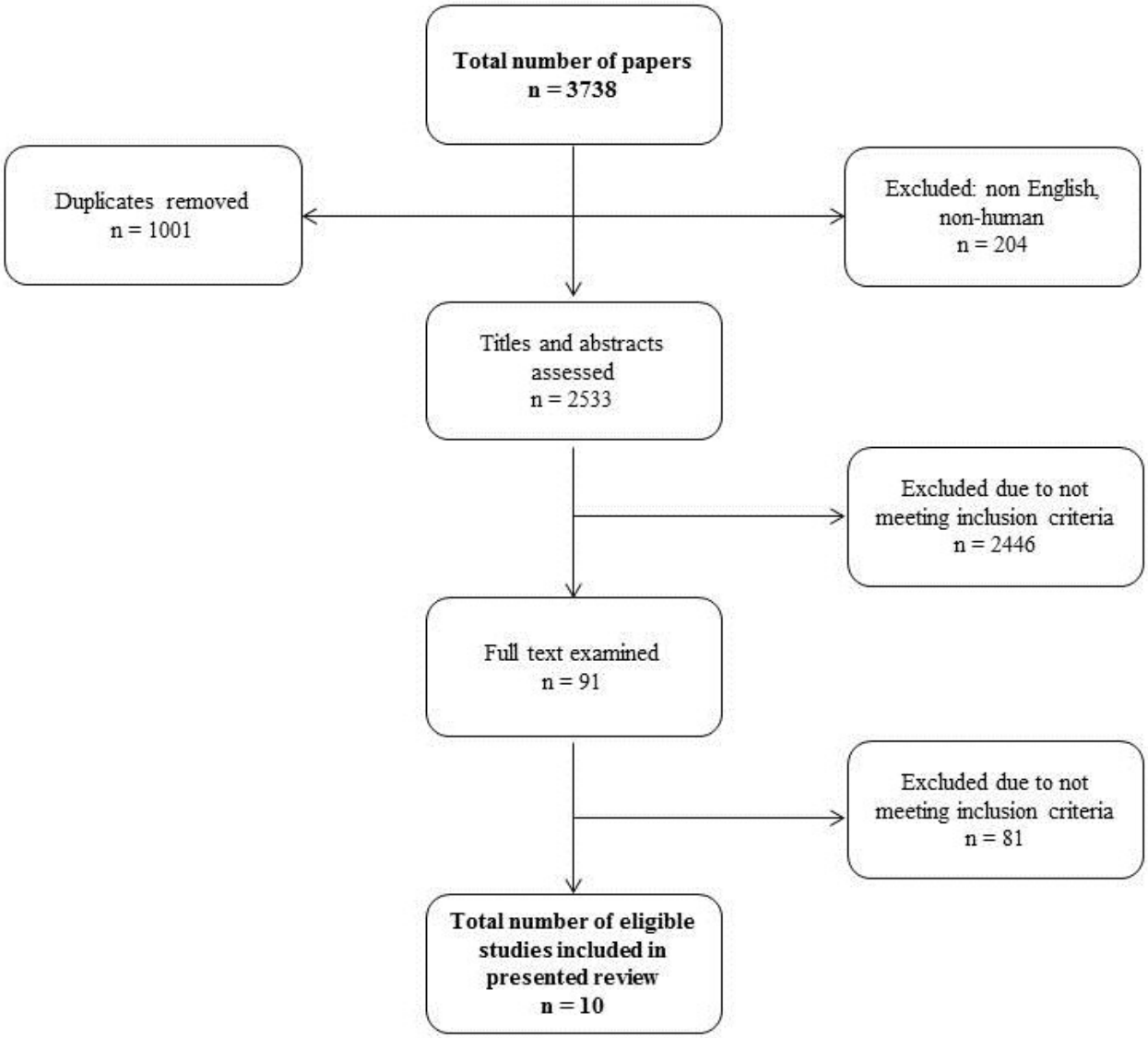


Figure 4: Flow chart of search strategy

Data synthesis

The aim of this review was to critically appraise epidemiological, prospective evidence to ascertain the role of inflammatory markers in the pathogenesis of steeper cognitive decline. Meta-analysis was therefore not intended. In fact, due to a high degree of heterogeneity - in particular in baseline characteristics of selected populations, length of follow-up, selection of cognitive assessment tools, varied combination of the four identified circulating inflammatory markers and selection of co-variants - meta-analysis would not be appropriate. Therefore a narrative, critical approach to the relevant evidence review was adopted.

2.3 Main findings

The primarily aim of the search was to identify original articles reporting single, population-based studies with sufficient selection of inflammatory markers and a sufficient selection of cognitive test battery. It was structured to avoid anything other than prospective/longitudinal studies that focused on the (trajectory of) cognitive decline in relation to levels of inflammation, rather than clinically defined diagnosis (MCI, dementia). Even so, the search also identified four systematic literature reviews; these were examined for additional relevant references if not identified already independently through the search strategy. Additionally, seven conference reports were identified, deemed as relevant to the proposed subject; the original paper was sought, assessed and reviewed according the inclusion criteria stated above. There were also some cross-sectional findings; even when the methods were

clearly stated in a particular article's abstract, the methods and result sections were nevertheless examined to ensure that no prospective data were lost. After careful consideration, one study was accepted, since it presented results that were adjusted for childhood (age 11) IQ levels.

Excluded studies

Of the identified articles, 1001 were removed due to duplicate entry in more than one database and 204 were removed as the paper was either not published in English language, or the study was concerned with non-human or post-mortem data. This left 2533 titles and abstracts to be reviewed. A further 2441 were removed at this stage as the studies were not relevant for the presented review. This left 92 full text detailed review. From the reviewed full text papers, 81 articles were excluded as the studies failed to meet essential criteria for inclusion: primary reasons and the corresponding number of articles excluded are presented below:

Table 1: Distribution of principal reasons for exclusion of studies from review

Reason	**Number of Articles**
No inflammatory marker of interest/IM only as covariates	6
IM-personality with cognitive performance as covariant	1
Restricted Cognitive Battery	4
Non-Caucasian	7
Irrelevant predictors and/or outcome	11
Non-human	1
Post-surgery	1
MCI	2
MCI/AD	3

Reason	Number of Articles
AD (one post-mortem and one AD at baseline and depression as an outcome measure)	13
Parkinson's Disease	1
Post-stroke/TIA	2
CVD condition	6
Subcortical ischemic VD	1
inflammatory status as an outcome measure	1
Depressed (case-control study; healthy controls)	1
Rheumatoid arthritis	1
Asymptomatic Intracranial Atherosclerosis	1
Dialysis	1
Gene-variants-cognitive ability with no cogn results available	3
Duplicate publications	3
Cross-sectional design	8
Conference abstract, no article available	1
Conference abstract, outcome MCI and AD	2
Total	**81**

Included studies

Brief examination of the evidence generated through the initial search revealed a remarkable heterogeneity in terms of methodologies, range of investigated biomarkers and neuropsychological assessment of cognitive ability. Also, there was a great inconsistency in adjustment for relevant co-variants. There were also marked differences between the studies, in the style of reporting results, with the majority of studies failing to report effect size and/or exact p values except for Luciano et al and Marioni et al, both studies based in Scotland).

Nine studies (in 11 publications) were identified which met the full eligibility criteria and were therefore included in the review. Included studies are considered below in chronological order of their publication date. For the MacArthur Study of Successful Ageing, two publications that were eligible for this review were identified and these are considered together. Two publications were also identified for the PROSPER Stud). One study was included despite an apparent cross-sectional design – this study presented analysis in which the cross-sectional association between CRP levels and cognitive ability at age 70 was adjusted for IQ measures in childhood and therefore estimated life-time cognitive change in relation to levels of inflammation was determined[234].

The MacArthur Study of Successful Ageing (MASS)[235] is a population-based cohort study of healthy men and women (aged 70-79 at recruitment in, 1988) based in East Boston, Durham and New Haven, USA. At baseline, relevant demographic data as well as plasma IL-6 and CRP biomarker levels were collected under standard operational procedures. Cognitive functioning was assessed at baseline and again at two subsequent follow-up clinics. After 2.5 years (first cognitive follow-up in 1991), 71 of the initial 851 participants (8% of the original cohort) had died. At the second follow-up assessment that was carried out 7 years after baseline, 273 people had died (32% of the original cohort). Subjects lost to follow-up were more likely to be men, more likely to have higher plasma IL-6 levels and tended to have lower cognitive scores at baseline (by 7 years follow-up, subjects lost to follow-up also had lower baseline physical activity scores and were more likely to be current or ex-smokers). All differences between the groups of participants who did and did not attend follow

up assessments respectively reached conventional levels of statistical significance ($p<0.05$).

In initial analysis from the MASS (Weaver et al., 2006[236]), which reported findings for 779 participants, linear regression analyses were used to investigate the baseline plasma IL6 levels in relation to change in scores for two waves of cognitive assessment. Median IL-6 were on average 4.55 pg/mL (ranged from 0.10 to 3.8 pg/mL) at baseline. For the purpose of non-linear association analyses, tertiles of IL-6 were defined, with values of <2.13 pg/mL in the lowest tertile, followed by any values between 2.13 and 3.8 pg/mL falling into the middle tertile and the highest tertile containing values $\geq$ 3.8 pg/mL of plasma IL6 levels. Results suggested an association between baseline cognitive ability and IL-6, independently of all covariates; although marginal, subjects in the highest tertile of plasma IL6 presented with impaired, baseline cognitive ability (OR for summary cognitive test score below median = 1.46; 95% CI: 0.97, 2.20). During follow-up, tertiles of cognitive decline were defined according to change in a summary cognitive test scores (follow-up score minus baseline score). The first wave of the follow-up testing supported the role for IL-6 in cognitive decline, i.e., both the second and third tertile of IL6 levels were significantly associated with cognitive decline (OR for change in summary test scores falling in bottom tertile of distribution of change scores =2.21; 95% CI: 1.44, 3.42 and OR=2.03; 95% CI: 1.301, 3.19, respectively). However, at the 7 years follow-up assessment, only subjects in the third, (i.e. highest, tertile showed declined cognitive scores (OR=1.90; 95 % CI: 1.14. 3.180).

The second analysis of MASS, in 2008[237], included all 851 study participants. The investigators used growth curve modelling in order to assess change in cognitive ability in relation to baseline levels of IL-6 and CRP levels. As previously, cognitive scores were available from three time points over a period of 7 years. Both biomarker values were subdivided into tertiles, from the highest to the lowest: for IL-6 tertile values in pg/m were <2.15; 2.15 - 3.80 and > 3.80. For CRP tertile values in mg/L were: <1.24; 1.24 - 2.65; >2.64. At baseline, a cross-sectional association between increased biomarker levels and cognitive ability was detected. In terms of follow-up results, an increased risk of cognitive decline was detected forthe top tertile of IL-6 ($p< 0.05$). However, neither the CRP nor the IL-6 levels significantly predicted decline in any cognitive ability before or after adjustment.

In the Longitudinal Ageing Study Amsterdam[238], comprising 998 participants (age range 62 to 85 (male=47.3%), comprehensive cognitive batteries were administered at baseline and at a 3 year follow-up clinic, assessing general cognition (MMSE, no other determination of general cognitive ability), immediate and delayed recall, speed of information processing, and non-verbal reasoning (Dik et al, 2005). Among other biomarkers, baseline measures of CRP and IL6 were obtained. Both markers were log-transformed for regression analysis, although extreme values were not removed. It can be argued that this might have introduced a potentially bias to the analysis; comparison of results with extreme and without extreme values were not presented . No significant association was detected between biomarkers and cognitive decline, measure by subtracting follow-up score from a respective baseline score.

A possible contribution to this null result might be the loss to follow-up testing; whereas the mean age of the total baseline sample was 75.4 years (SD 6.6), the subjects who attended for follow-up were on average 74.6 years old (SD 6.2) at baseline and generally healthier (although, interestingly, there was no significant difference between the baseline and follow-up groups in alcohol consumption and smoking, contrary to previous studies on subjects undergoing repeat cognitive testing[59].

Another study conducted in Holland (Schram et al, 2007[239]), published data collated from two population-based cohorts: the Rotterdam Study and the Leiden 85-plus Study. For the Rotterdam Study, levels of baseline IL-6 and CRP were available for 3874 subjects (mean age 72 years, average follow up 4.6 years). The Leiden 85-plus Study comprised 491 subjects (age range 85 to 90 years, average follow-up 5 years). IL-6 and CRP levels were entered into linear regression modelling to assess change in global cognition, executive functioning and memory performance. Participants from the Rotterdam Study with higher baseline measures of CRP and IL-6 presented with lower baseline cross–sectional global cognitive ability and executive functioning ($p<0.05$); furthermore, accelerated decline in global cognitive ability was observed in relation to elevated IL-6 baseline levels. However, this effect was observed only in APOE ε4 allele carriers ($p<0.05$). Otherwise, no significant associations were observed.

In the Leiden cohort, associations of similar magnitude and direction were observed but power was less, affecting the statistical significance of the findings; higher baseline IL-6 was associated with cognitive impairment in all three domains at

baseline but similar findings for CRP were not statistically significant. In terms of cognitive decline, the only significant association was between higher baseline IL-6 levels and poorer performance in memory function. Steeper decline in global cognitive ability, as predicted by IL-6 levels, was observed only in APOE ε4 allele carriers ($p<0.05$). Therefore results from both cohorts suggested that IL-6 may only significantly influence cognitive performance at advanced age in APOE ε4 allele carriers.

In a Scottish cohort consisting of 1053 community-dwelling participants (the Lothian Birth Cohort 1936, age range 67 to 71 years [234]), the availability of childhood IQ data allowed investigation of life-time estimated change in cognitive ability in relation to late-life measurements of plasma fibrinogen and CRP levels. The results suggested the possibility of a reverse association; i.e., suggest a strong possibility that childhood IQ levels predict elevated levels of these two inflammatory markers at the age of 70 ($p<0.001$). However, the results also indicated that levels of plasma fibrinogen level were a significant predictive risk for late-life decline in general cognitive ability and speed of information processing.

A further Scottish study (Marioni, et al., 2009[240]) reported finding from the Aspirin for Asymptomatic Atherosclerosis Trial, a study that consisted of a baseline sample of 2321 cognitively tested community-based adults (age range 50 to 80 years) of whom 504 underwent repeat cognitive testing at a 5-years follow up clinic. For all participants, pre-morbid peak cognitive ability scores were also available from the Mill Hill Vocabulary Test (MHVT). Therefore, two sets of results from this study are of interest: the life-time estimated change in cognition (2321 subjects) and five-year

actual change in cognitive ability (504 subjects). Baseline plasma fibrinogen and CRP levels were significantly associated with decline in general ability ($p<0.05$), non-verbal reasoning ($p<0.05$), with information processing speed ($p<0.01$), attention and mental flexibility ($p<0.01$). Adjustment for common cardiovascular risk factors attenuated these associations only partially; the CRP-general cognitive ability relationship became non-significant as did the relationship between both plasma biomarkers and non-verbal reasoning. Plasma fibrinogen, adjusted for plasma viscosity, was significantly associated with mental flexibility performance, independently of the CV risk factors. With respect to 5-year cognitive decline, plasma fibrinogen levels significantly predicted decline in executive functioning ($p=0.046$) and CRP levels predicted decline in non-verbal reasoning ($p=0.037$), also independently of the common CV risk factors. The authors also observed an interesting finding in so far as higher baseline CRP levels significantly predicted improvement in verbal memory test score ($p=0.029$).

A third Scottish cohort study (Rafnsson et al., 2010[241]) was based on the Edinburgh Artery Study, a population-based cohort of 1592 subjects. Blood sampling was carried out in 1987-88 with two subsequent cognitive assessments; in 1998/99 with 717 subjects (mean age 73.1 years; SD 5.0) and four years later when 452 participants agreed to return for a follow-up assessment. For these people, mean value of plasma fibrinogen was 2.53 g/L; SD = 0.61 and median value of CRP was 2.0 mg/L (range 0.92-4.39). In terms of the 5-year cognitive change, plasma fibrinogen predicted decline in executive functioning only, also independently of CV risk factors and events (both $p<0.05$). Plasma CRP levels were significantly

associated with a 5-year decline in non-verbal reasoning only ($p<0.05$). However, there was a much more consistent association of biomarkers with cognitive decline measured in terms of estimated lifetime cognitive change (calculated using results of the National Adult Reading Test, NART). Plasma fibrinogen levels were significantly associated with lifetime decline in general cognitive ability ($p<0.01$), non-verbal reasoning (0.05), speed of information processing ($p<0.001$) and attention and mental flexibility ($p<0.001$). A very similar pattern of findings was observed in for CPR levels, which were associated with a decline in general cognition ($p=0.05$) and in the majority of individual cognitive domains: non-verbal reasoning ($p<0.05$) speed of information processing ($p<0.001$), attention and mental flexibility ($p<0.01$) and general cognitive ability ($p<0.05$).

In the Framingham Study (Jefferson, et al., 2011[242]), circulating levels of CRP, IL-6 and TNF-alpha were modelled to investigate their association with cognitive decline on a comprehensive test battery. The follow up cognitive assessment was conducted with 1352 subjects (mean age 60 years, SD 9, range 35 to 85), all of whom were community-dwelling citizens in Boston, USA. IQR values for plasma CRP, IL-6 and TNF-alpha were 2.0 (0.9-4.9), for IL-6 2.6 (1.8-4.2) and for TNF-alpha 1.2 (0.9-1.6), respectively. In linear regression models of cognitive change over 6.3 years, the only significant associations detected were between CRP and naming and lexical retrieval ability ($B=-0.02$; $p=0.044$), TNF-a and attention and mental flexibility ($B=0.04$; $p=0.004$) and TNF-alpha and verbal reasoning and abstraction) ($B=-0.08$; $p=0.046$).

The most contemporary study was the Pravastatin in the Elderly Individuals at Risk of Vascular Disease[243](PROSPER) study. This randomised control trial was

conducted in Scotland, to investigate the efficacy of statins in the reduction of coronary and cerebrovascular morbidity and mortality in an older-aged adults. Prospective collection of cognitive data was also undertaken. Two publications eligible for this review, one assessing the relationship of CRP levels[244] and one assessing the relationship of IL6[245] levels in relation to accelerated cognitive ageing, have been published. At baseline, 5680 participants (mean age 75.3 years, SD 3.4; range 70-82 years) from Scotland, Ireland and the Netherlands were recruited. Cognitive assessment was carried out over an extended period of 36 to 48 months (averaged for 42 months). Higher plasma CRP levels were significantly associated only with a decline in immediate and delayed memory, assessed by a 15-Picture Learning test; this association persisted after adjustment for conventional CVD risk factors (both associations, $p<0.05$). No other significant associations with cognitive decline were detected. With regard to IL-6 levels, this circulating biomarker predicted decline in memory and executive functioning, both at the 0.002 level of significance; as with the CRP levels, here too these associations persisted at analyses adjusted for CVD risk factors. Interestingly, even though concentration of IL-6 was not associated with a significant decline in attention (Stroop test), its polymorphism *IL-6* -174 CC carriers performed significantly worse than their counterparts on this test.

2.3.1 Discussion of main findings

Although overall evidence from prospective studies supports some form of link between systemic inflammation and late-life cognitive skills, it only partially confirms the hypothesis that elevated levels of circulating inflammatory markers have a negative impact on cognitive decline in advanced age. Evidence was inconsistent among the reviewed studies, with some reporting no significant associations, especially after adjustment for conventional cardiovascular risk factors and vascular events. Also, there was evidence of a reverse relationship between concentrations of some biomarker (CRP) and general cognitive ability and in one study improvement on memory test was detected (the AAA trial).

There may be a number of reasons for this lack of consistency between studies. Most notably, there was a great degree of between-study variability in the selection of cognitive tests, in the length of follow up, in population characteristics and the choice of co-variants.

With regard to the cognitive assessment, the inclusion criteria for this review excluded studies with too narrow an assessment of cognitive performance. For example, global cognitive ability, or 'g', is generally understood as a factor derived from suitable, comprehensive cognitive batteries that accounts for a certain level of variability[246]. Whereas some studies conducted appropriate statistical analyses to determine 'g' (such as a principal component analysis), some researchers claimed that MMSE was a sufficient tool for determining general cognitive ability. Given the

possible lack of sensitivity to mild cognitive impairment (MCI)[247], MMSE has failed to detect differences between the MCI and dementia and MCI and healthy adults[248]. Furthermore, it may fail to detect true cognitive impairment in well educated, mentally and physically active people and there can be an increased rate of false positives among people with lower educational attainment[249].

Included studies reported all reported administration of, to a greater or lesser degree, a comprehensive neuropsychological battery that was comparable to that used in the Edinburgh Type 2 Diabetes Study (ET2DS), thus enabling direct comparison of their findings with my own. All reviewed studies assessed memory function. Almost always, verbal and non-verbal, prospective and/or semantic memory was assessed; one study assessed immediate and delayed memory via Picture Learning Test; this differs from the ET2DS where verbal memory test and faces recognition test was administered. Most studies used some test assessing ability to process information. Executive functioning was also frequently examined. Even where neuropsychological tools differed considerably from those used in the ET2DS, the reliability and robustness of all tests was always clearly considered.

In terms of cognitive change or decline over time, it was not always reported whether cognitive decline was examined via 'absolute change' (calculated by subtracting baseline from follow up respective cognitive scores) or adjustment for respective baseline cognitive test score. Both approaches have their own advantages and limitations [250], which will be considered in the interpretation of my own ET2DS study findings. When discussing aspects of cognitive testing, it is also important to note the length of follow-up, which ranged from 2.5 to 7 years (coincidentally both

'extremes' of follow-up are follow up time points for cognitive assessment in the MASS investigation). The average follow up period at all included studies was 3.5 years which is relatively short to detect change in cognitive performance that might be associated with bio-pathological degeneration of the brain. In fact, Dik et al noted that their negative results might be due to an insufficient follow up period, commonly associated with an observation of a practice effect[238]. The effect of practice was also possible in the MASS investigation and both authors admit that two sets of follow-up cognitive assessment with a space of just over three years in between is too short a time period to detect noticeable change in cognitive performance. Likewise, the PROSPER investigation allowed just 39 months in between baseline and subsequent testing.

Another issue which limits the interpretation of findings from the included studies is that approximately half of the studies lacked data on baseline characteristics and did not compare these in subjects who were lost to the follow-up assessment. Only the MASS[235], the Longitudinal Aging Study Amsterdam[238], Edinburgh Artery Study[241] and the Aspirin for Asymptomatic Atherosclerosis Trial[251] provided such data. Generally, subjects lost to follow up assessment tend to be more frail and cognitively less able; this might contribute to health-related choices, leading to generally worse physical health and also higher prevalence of risk factors and/or disease associated with an accelerated cognitive decline[252]. This may produce potentially serious bias in results and needs to be taken into account during interpretation of findings.

In terms of selection of inflammatory circulating markers, the majority of studies investigated elevated levels of IL6 and CRP, few studies assessed the role of plasma

fibrinogen levels, and only one publication reported the role of TNF-alpha. Furthermore, sample size varied from several hundred to several thousand with the majority of studies being of small to average size.

There was also considerable overlap in the selection of covariates, based on a rationale/evidence of the relationship between these confounding and mediating variables either with elevated levels of circulating inflammatory markers and/or cognitive ability at advanced age. In general, adjustment was made for the following covariates: age and sex, education attainment (years spent in formal education or highest education attainment) and socio-economic status. An adjustment for conventional cardiovascular risk factors and/or events was common to all studies with slight variation between each (i.e., myocardial infarction, angina, stroke, TIA, hypertension, type 2 diabetes, HbA1c levels, cholesterol levels, smoking status and alcohol consumption). Three studies also considered mental health (depression as defined by a standardised assessment) as a potential confounder of the relationship between inflammatory markers and cognitive performance[238,241,253]. Additionally, the MacArthur Study of Successful Aging analyses were also adjusted for marital status and, given the variability of sample population, also ethnicity[236,237]. A couple of studies carried out an additional assessment to investigate whether adjustment for the presence of APOE, ε4 allele[238,239] or the presence of APOE, ε4 allele and the country of origin (Scotland, Ireland or the Netherlands) [244] attenuated the strength of the inflammation-cognition relationship. Additionally, adjustment was made for various other medical conditions, such as tumor, thyroid disturbance, allergy, asthma, rheumatic diseases, fracture/osteoporosis[253] and for hip and/or bone fracture[236].

Only the Scotland-based cohort studies reported adjustment for prior peak cognitive ability defined by a standardized test, such as the National Adult Reading Test[241] or the Mill Hill Vocabulary Scale[240] and one article provided results from analyses adjusted for childhood IQ levels[234]. All the reviewed studies reported adjustment for variables that were comparable with those undertaken in the ET2DS, which helped subsequent comparisons and ultimate interpretation of findings from my own study.

Table 2: Characteristics of studies investiation relationship between markers of inflammation and cognitive change

Author/ year	**Population[1]; Baseline Year Baseline Sample**	**Study Design[2], Year[2]; Years of FU; Primary Aim [2,4]**	**Number[2] Sex (*male,%)***	**Age[2] (Mean/SD or range)**	**Biomarker(s), mean levels[3]**	**Cognitive Domains/ Cognitive Measures (tests)**
Weaver, JD, et al., 2002[236] Alley, DE., et al., 2008[237]	MacArthur Study of Successful Aging (1988) East Boston; 1189 subjects	Prospective; 2.5 years and 7 years; Healthy Adults	779 subjects 851subjects	74.5 (70-79)	IL 6 (880 participants) median; 4.35pg/ml; split into tertiles IL6 (median): 2.77 pg/mL (1.86-4.64) CRP (median): 1.79 (0.95-3.13)	Screening (Mental Status Questionnaire) Immediate and delayed memory (Boston Naming Test[1]) Language comprehension (Boston Naming Test[2]) Spatial memory (delayed recognition span test) Abstraction: Similarities (WAIS[R]) Spatial ability (copying geometric figures) *All values add up (range 0-89)*
Dik, V., et al, 2005[238]	The Longitudinal Aging Study Amsterdam, 1284 subjects	Prospective, 3 years	998 (47.3%)	74.6 (6.2)	CRP: 3.2 (5.0) IL6: 1.9 (2.1)	General cognition (MMSE) Immediate and delayed recall (AVLT) Information processing (Alphabet Coding Task)
Schram MT., et al., 2007[239]	Rotterdam Study (1) & Leiden Study (2)	prospective 1: 4.6 years 2: Max 5 Y.	1: 3874 2: 491	1: 72 2: >85	1.CRP:2.4 (1.2-4.6) IL6: 2.11 (1.44-3.32) 2.CRP: 3 (1-7) IL6: 10 (0-57)	Global functioning Executive function Memory

Author/ year	Population[1]; Baseline Year Baseline Sample	Study Design[2], Year[2]; Years of FU; Primary Aim [2,4]	Number[2] Sex (*male,%)*	Age[2] (Mean/SD or range)	Biomarker(s), mean levels[3]	Cognitive Domains/ Cognitive Measures (tests)
Lucciano, M., et al., 2009[234]	Lothian Birth Cohort 1935	Birth cohort; MHT at age at 11 years	1053 (50.2%)	69.6 (0.83) 67-71	CRP: 0-3mg/L: 535 4-10 mg/L: 398 >10mg/L:120 FIB: 3.28 (0.64)	MHT – pre-morbid IQ Verbal Memory (LM) Spatial Span (Verbal Paired Associates) Non-verbal memory (Ravens' Matrices) Executive Function (Verbal Fluency) Working Memory (LNS) Information Processing (DST) Spatial ability (Block Design) Executive Function (Symbol Search) Speed processing (Simple Reaction Time; Choice Reaction Time)
Marioni, RE., et al., 2009[251]	The Aspirin for Asymptomatic Atherosclerosis Trial, 3350	Prospective cognitive assessment 5 years follow up + pre-morbid test	504 (27%)	63.0 (6.85)	Fibrinogen: 3.36 (0.72) CRP: 1.98 (0.89,4.18) log transformed	Memory (Logical Memory) Non-verbal reasoning (Raven's Matrices) Info speed process (DST) Executive Function (VFT) Mental flexibility (TMT-B)
Rafnsoon, S., et al., 2010[241]	EAS; 1987-88	Prospective; cognitive testing on 98-99 and 4 years later + pre-morbid vocabulary test	441 subjects with Fibrinogen data	Baseline: 73.1 (5.0) Follow up:	Fibrinogen (BL) = 2.53 (.061)	Screening (MMSE) Verbal declarative memory (Logical Memory) Non-verbal memory (Faces) Executive Function (BVFT) Information Processing (DST) General cognitive ability factor 'g'

Author/ year	Population[1]; Baseline Year Baseline Sample	Study Design[2], Year[2]; Years of FU; Primary Aim [2,4]	Number[2] Sex (*male,%)*	Age[2] (Mean/SD or range)	Biomarker(s), mean levels[3]	Cognitive Domains/ Cognitive Measures (tests)
Jefferson, AL., et al, 2011[254]	Framingham Offspring Study; 5124 subjects	Prospective design, 1971; 1878 subjects; 6.3 (SD+1) years	BL: 1878; 46% FU: 1352; 45%	60 (SD=9)	CRP: 2.0 (0.9-4.9) IL-6: 2.6 (1.8-4.2) TNF: 1.2 (0.9-1.6)	Memory verbal (Logical Memory) Memory-visual spatial (Visual Reproductions Delayed Recall) Executive Functioning (TMT-B) Language (Boston Naming Task – 30 item) Visual perceptual ability (Hoope visual organisation test) Verbal reasoning (Similarities Subtest) Reading (WRAT-3 reading subtest)
Mooijaart, SP., et al., 2011[244]	PROSPER (The Netherland, Scotland, Ireland) 5804 subjects	Prospective design; 3.2 years	5680; 48%	75.3 (70-82)	CRP: 3.1 (4.9) mg/L	Screening Test (MMSE) Executive Function (Stroop test) Executive Function/working memory: (Letter-Digit Coding test) Memory (Picture-Word Recall: immediate/delayed)
Mooijaart, SP., et al., 2013[245]			Total: 5653	N; male %	Age (SD)	Il 6pg mL^{-1}	Screening test - MMSE
			1st Tertile	1884; 40.8%	75 (3.3)	.21-1.94	Executive Function – calculated fro as a z-score from *Attention* (Stroop test)
			2nd Teritle	1885; 47.9%	75.5 (3.4)	1.94-3.19	*Processing Speed* (Letter Digit Coding) Memory Immediate (picture-learning)
			3rd Tertile	1884; 56%	75.5 (3.4)	3.19-26.81	Memory Delayed (picture-learning)

[1] entire study population, baseline

[2] samples investigated in the presented article, in prospective studies referring to FU variables

[3] only biomarkers of interest reported here (Fibrinogen, CRP, IL6, TNF-alpha), mean levels (SD) provided where available

[4] if other than are related cognitive ability

2.4 Summary

Even though the strength of detected associations between inflammatory biomarkers and cognitive decline differed considerably between studies and the overall evidence was not conclusive, the available prospective evidence does seem to indicate that elevated inflammatory markers are associated with accelerated late-life cognitive decline, occurring after measurement of the circulating biomarkers. Inconsistency in methodologies, particularly in the selection of neuropsychological tests, variation in cohort size and characteristics, and selection of potentially mediating variables that require consideration, most likely account for the current mixed evidence. Furthermore, insufficient length of the follow-up period might not allow for detecting significant brain-related pathological changes that would be subsequently manifested in declined cognitive scores and therefore indicate whether inflammation is, indeed, potentially influencing late-life cognitive ability.

A great deal of evidence suggests that the association of inflammation with late-life cognitive decline occurs independently of common cardiovascular risk factors and conditions, pointing towards the possibility of a direct effect on the brain, most likely in the form of neuro-inflammation. However, this does not provide a sufficient explanation of whether a cardiovascular condition confounds the investigated relationship or mediates the effect of elevated inflammatory markers in these processes.

On the other hand, the magnitude of some results suggests that the pathway by which inflammatory markers might affect cognitive decline is worth investigating further.

Certainly there is a need for larger, prospective studies with a long-term follow up, ideally with repeated, multi-wave measures using the same, comprehensive neuropsychological battery. This will add to evidence on the pattern and speed of changes in cognitive performance, highlighting the main factors in the trajectory of cognitive decline.

The complexity of the gradual, progressive change in cognitive performance, influenced by numerous factors that interact with each other, points towards multiple bio-pathological processes in which systemic inflammation alters cognitive performance. From the result of even prospective observational studies, it is not possible to determine whether systemic inflammation lies on the causal pathway towards defined cognitive decline (and potentially dementia). One possibility for further research in this respect is investigation of association between cognition and biomarkers' related genes and gene variants (i.e. those genes variants which affected the circulating levels of the biomarkers).

As these are not confounded by the main associated factors, such as CV conditions, association between gene variants and defined cognitive phenotype could help determine whether associations between biomarkers and cognition are causal.

The ET2DS was designed with the first wave (baseline) assessment providing data for cross-sectional analysis[255,256] but with the advantage of subsequent prospective phase scheduled for re-assessment four years after the baseline. The cohort is also of a sufficient size to detect meaningful changes in cognitive performance in relation to altered levels of baseline inflammatory markers. The comprehensive

neuropsychological assessment provides cognitive scores across all main domains of cognitive functioning as well as general cognitive factor (derived using all cognitive scores) and estimated peak, pre-morbid cognitive performance.

The most likely inflammatory candidates, upstream markers (IL-6 and TNF-alpha) and downstream markers (plasma fibrinogen and CRP) were measured at baseline; these scores can be modelled independently or can be used in further analysis as a 'inflammation factor' (derived using scores from all four markers).

As Type 2 Diabetes is one of the strongest risk factors for many conditions associated with more rapid deterioration of the brain – potentially making people with this condition to decline faster than general population – the investigation undertaken in the first part of this thesis (on the association between plasma inflammatory biomarkers and cognitive decline) represents investigation of an accelerated model of the process of degeneration and consequent cognitive decline.

2.5 Study Aims

People with type 2 diabetes are at a greater risk of age-related cognitive decline. In light of the current evidence, the aim of this thesis was to test whether higher plasma levels of circulating biomarkers are related to the four-year change and to the life-time change in performance in general cognitive ability and in specific cognitive domains in the diabetic older population. For this, data from the baseline and four-year follow up phase of the Edinburgh Type 2 Diabetes Study was used (detailed description is presented in the following chapter). The following baseline levels of circulating inflammatory markers were examined: cytokines IL-6 and TNF-alpha and acute-phase proteins fibrinogen and CRP. Additionally, the general inflammation marker, derived using baseline measures of the four markers, was examined. The main outcomes were the follow up scores of seven neuropsychological tests, providing data for cognitive domains of memory, executive functioning and processing of information. The score of general cognitive ability 'g' was derived using follow up scores of the seven neuropsychological tests. Four-year cognitive decline was assessed by using the respective baseline cognitive scores as covariates, and the life-time cognitive change was estimated by adjusting for a score of the Mill Hill Vocabulary Test.

To examine the mechanisms of the underlying, potential causal relationship, genetic association analysis was conducted. Gene variants (SNPs) known to have an effect on circulating plasma fibrinogen levels were selected via literature search and relevant gene-databases and modelled against scores of general cognitive ability 'g'.

For this purpose, data from seven population-based cohorts were collated, providing genetic and cognitive data for 14033 subjects. General cognitive ability factor ‘g’, based on cognitive scores for mutually compatible data of three cognitive domains (memory, executive functioning and information processing) was calculated for each study. Results from individual studies were further used for meta-analysis, determining the relationship between fibrinogen-related SNPs and general cognitive ability.

The following chapters describe in detail the methodology of the ET2DS and the information on the seven collaborating cohorts, statistical analysis and the results generated from analyses as well as their interpretation and implications.

Chapter 3: Methods – The Edinburgh Type 2 Diabetes Study

This chapter describes the design of the Edinburgh Type 2 Diabetes (ET2DS), study investigating the association of circulating inflammatory markers with cognitive impairment and decline in adults with type 2 diabetes. Methods of data collection and analysis are reported, both for the baseline and follow up phases of the study, where these are relevant to the research described in this thesis. Baseline data for the study were already available when I started my PhD (methods published in detail previously[257]); I was responsible for collecting follow-up cognitive data, assisted by other researchers from the ET2DS research team.

3.1 The Edinburgh Type 2 Diabetes Study

The principal aim of the ET2DS was to investigate the role of potentially modifiable risk factors, such as circulating inflammatory markers, vascular disease and other potential risk factors involved in aetiology of cognitive impairment and cognitive decline in elderly people with Type 2 Diabetes. The Edinburgh Type 2 Diabetes study is a prospective, population based study that commenced in 2006-07 and has continued as a longitudinal study with cognitive follow up in 2010-11.

3.2 Study Recruitment, Selection Criteria and Population

Baseline Sampling Frame

ET2DS participants were selected from an elderly population of Lothian, Scotland, with diagnosed Type 2 Diabetes via Lothian Diabetes Register (LDR), a computerized database containing clinical details of approximately 20,000 diabetic inhabitants. Through comparison of the prevalence of diabetes on the LDR with that calculated from other sources data in Scotland, it has been established that the LDR captures almost everyone diagnosed with this condition in Lothian[257].

To ensure the validity of T2DM diagnoses recorded on the LDR, the ET2DS team performed confirmatory checks according to several conventionally accepted diagnostic criteria for T2DM. Patients were accepted as having diabetes if they were

treated with oral anti-diabetic medication or insulin and if those receiving dietary treatment alone had a research clinic HbA1c measurement >6.5%. Clinical records of subjects with HbA1c below 6.5% and those who may have forms of diabetes other that type 2 were reviewed by a consultant diabelogist for validation of a correct diagnosis.

Exclusion Criteria

Exclusion criteria were as follows:

- subject not confirmed diagnosis of T2DM (i.e. T1DM)
- subject not willing to consent to the study or unable to provide a fully informed participation consent form
- subject was not physically fit to complete clinical and cognitive assessment
- subject confirmed to be non-native English speaker
- subject did not have normal (corrected) visual acuity – corrected visual acuity was below 6/36 for distance vision and/or sufficient to read large print

Baseline Study Population

A total of 9.464 men and women were identified from the LDR on the 20th July, 2006, within the study age range of 60 to 74 years as of the 1st August, 2006. Of the 5.561 randomly selected people, 107 people were no longer on the LDR list by the time invitations were ready to send, therefore an invitation for participation was sent to 5,454 people. A total of 3,286 people (60.2%) replied to the invitation letter with 2,034 declining participation. Random selection from the same sex and 5-year age

band was carried out to replace those who declined or did not reply to an invitation. Of those, 1,252 (23%) people who declared an interest in participation, 1,077 (19.7%) ultimately attended the baseline clinic. Failure to meet the essential study inclusion criteria further reduced the number of the final study participation to 1,066 subjects and these formed the ET2DS study population (figure 5).

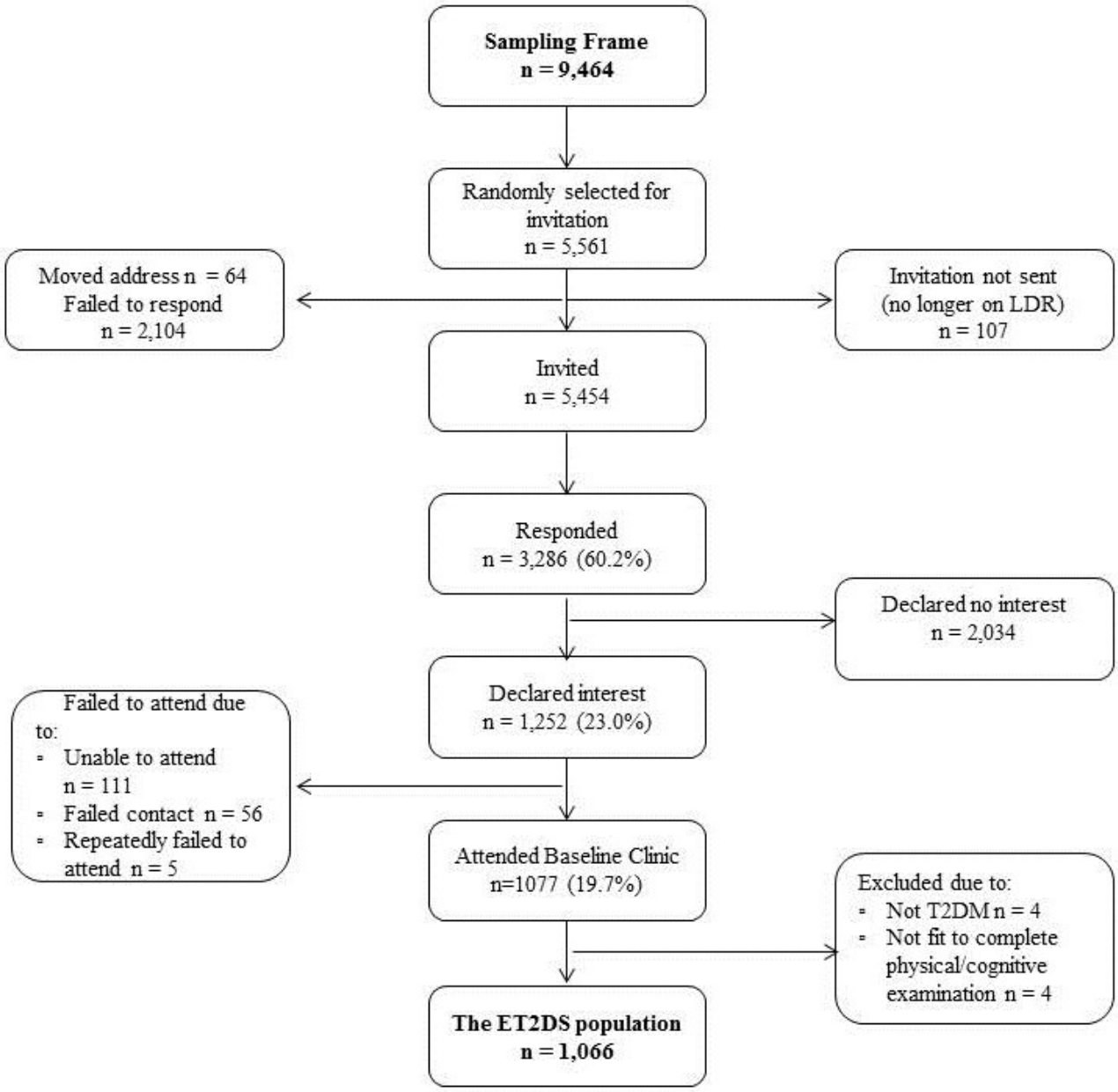


Figure 5: Recruitment for the ET2DS and attendance at baseline clinic

Follow Up Clinic Study Population

All ET2DS participants were considered for follow up clinic assessment in 2010/11. Of the initial 1,066 participants, 88 (8.3%) had died, 26 (2.4%) were deemed unfit for the follow up examination and 9 (0.08%) had declined further clinic participation. Of the remaining 943 (88.5%) subjects invited to the follow up clinic, a further 98 persons (9.2%) declined to participate and 14 (1.3%) could not be contacted. The Follow up Clinic was therefore attended by 831 (77.9%) participants; of these 3 (0.3%) people were deemed unfit or refused to proceed with cognitive testing. Therefore cognitive data were available from 828 (77.6%) of the initial baseline study population (Figure 5).

Of the 235 (22%) people who did not attend the follow-up clinic; 88 (8.3%) had died and of the remaining 147 participants, a small group of 6 (0.6%) had declined any further contact with the research team. A questionnaire investigating health and personal circumstances was sent to all remaining participants (returned by 67 subjects) and/or their General Practitioners (returned for further 42 subjects). The main reasons given for not attending the follow up clinic were poor health and time constraints due to duties as a family carer. In addition to questionnaire data, further information on all study participants was gained from record linkage to hospital discharge and death certificate data and from hospital notes to confirm clinical

diagnoses, including dementia. Over all, information from 32 (3%) participants could have not been obtained.

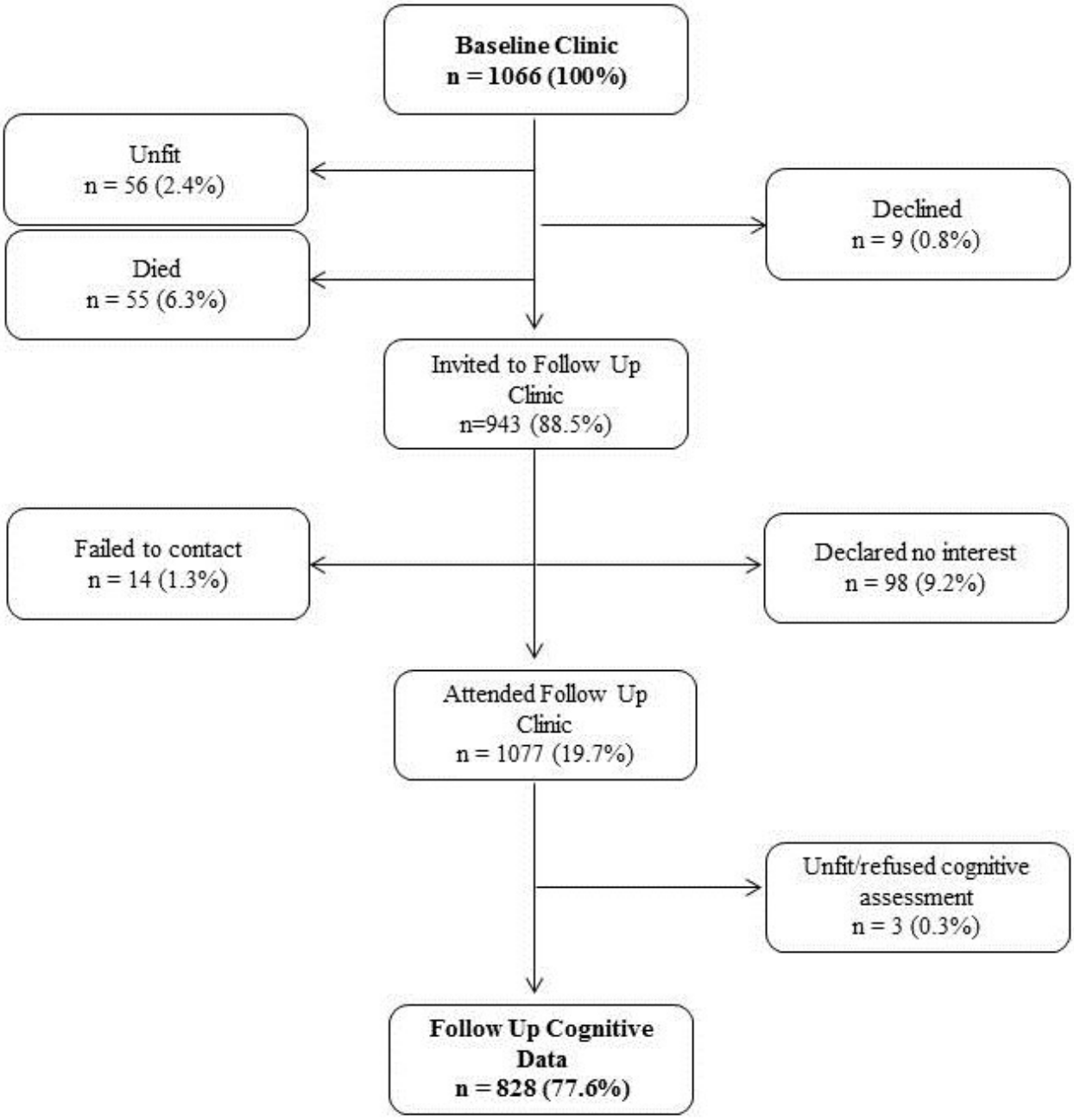


Figure 6: Participation in the ET2DS research clinic at baseline & and at follow up

3.3 Sample Size

When the ET2DS was designed in 2005[257], the targeted sample size of 1,000 was estimated on the basis of the number required to conduct a baseline assessment with sufficient statistical power of 90%; α=.05 at two-sided significance level to detect a standard Pearson correlation of 0.10 and above between the cognitive test scores and the independent variables. Estimated targeted sample of the follow up wave was 800 subjects in order to retain power of 90%; α=0.05 at two-sided significance to detect correlation 0.12 or greater. At the four year follow-up of the study with 828 participants, the regression analyses remained powered over 95% with a=.05, two sided significance, detecting 0.01 increase in multiple correlation coefficient.

3.4 Ethical Approval

Ethical permission was obtained for the ET2DS from the Lothian Medical Research Ethics Committee. Informed consent was obtained from all participating patients attending baseline, follow up clinics and for the record linkage use. Data collection at all stages complied with the ethical rules of Declaration of Helsinki.

3.5 Clinical Assessment at Baseline and Follow up Clinics

The primary aim of the 2010-11 follow up clinic was to repeat the baseline cognitive assessments in a manner that would provide comprehensive and comparable data required for assessment of 4 year change in cognitive function. The project presented in this thesis aimed to determine the relationship between baseline circulating levels of the acute-phase proteins and central pro-inflammatory cytokines with 4 year subsequent change in cognitive performance and estimated cognitive decline. Therefore the following section provides information on baseline measurement of the inflammatory markers and potential confounding and mediating variables considered in subsequent analyses as well as both, baseline and follow up cognitive assessment (including derivation of general intelligence factor *'g'*). It also reports on data management and data analyses.

Importantly, all data collection at both baseline (BL) and follow up (FU), was carried out by trained nurses/researchers, in accordance with standard operating procedures [257]. All forms and questionnaires were created or chosen with an aim of ensuring reliability between and within nurse/researcher assessments. The self-administered questionnaire contained validated questions on a range of demographic, personal and clinical characteristic, including age, sex, marital status, participant and their spouse educational attainment and their employment/occupation status), current socio-economic status (current post code/SIMD smoking history, alcohol consumption,

history and control of diabetes (age of onset, HbA1c levels) and current and previous medical diagnoses of cardiovascular and other relevant comorbidities.

Inflammatory Markers Measurement

A baseline fasting venous blood sample provided measures of a range of biomarkers, including plasma levels of fibrinogen, CRP, IL-6, and TNF- α, as well as high-density lipoprotein cholesterol, total cholesterol, HbA1c and also blood samples for DNA extraction. Blood samples were processed at the research clinic and plasma stored at -40^{0}C. Assays for inflammatory markers were performed in the University Department of Medicine, Glasgow Royal Infirmary. Plasma fibrinogen was determined from stored plasma, anti-coagulated with trisodium citrate, using the automated Clauss assay (MDA-180) coagulometer, Organon Teknika, Durham, NC). Plasma CRP was determined by a high-sensitivity immunonephelometric assay [114]. IL-6 and TNF antigen levels were determined by high-sensitivity ELISA kits from R&D Systems, Oxford, UK [256].

Assessment of Baseline Cardiovascular Disease and Risk Factors

Questionnaire

The self-reported questionnaire contained questions on current and previous medical diagnoses ('doctor-diagnosed') and treatment for myocardial infarction, angina,

stroke and transient ischaemic attack (TIA) and also the presence of cardio-vascular risk factors. Information was gathered about the year of diagnosis/event and the diagnosing general practitioner/hospital. This facilitated confirmation of a clinical diagnosis using scrutiny of medical records and/or record linkage to Scottish hospital discharge data (SMR01 scheme) obtained from Information Services Division (ISD) of NHS Scotland. The WHO Chest Pain Questionnaire [258] was also completed.

Data on smoking and alcohol consumption were collected using standard questionnaire as used in previous epidemiological studies[259]. The smoking variable used in subsequent analyses was the estimated pack per year of smoking. This variable was derived by multiplying the number 20 (estimated cigarettes smoked per day) by the number of years as a smoker. For non-smokers, zero value was entered. Alcohol intake was estimated on self-reported alcohol units consumed in a typical week in last 12 months before the clinic visit.

Physical Examination

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

A standard 12-lead ECG was taken to assess evidence for myocardial ischaemia; this was coded as evidence of coronary heart disease if Minnesota codes were 1.1. to 1.3; 4.1 to 4.2; 5.1 to 5.3; 7.1 and as indicating definite MI, if Minnesota codes were 1.1.1 to 1.2.5; 1.2.7; or 9.2 plus 5.1 or 5.2[260] .

Definition of Cardiovascular Disease

Cardiovascular disease was defined according to pre-specified criteria, using evidence collected from the questionnaire (including the WHO chest pain questionnaire), the ECG, hospital discharge codes from record linkage and, if necessary, review of medical notes.

In terms of *myocardial infarction (MI),* this was recorded if two out of the first three of the following criteria were met, or if the first and last criteria were met:

i. Self-reported history of heart attack (subject recall of doctor's diagnosis)
ii. MI indicated by WHO chest pain questionnaire for MI
iii. ECG evidence of ischaemia
iv. Prior hospital discharge ICD10 code for MI (I21-I23; I252)

In terms of angina this was recorded if two out of the first three of the following criteria were met, or if the first and last criteria were met:

i. Self-reported history of angina (subject recall of doctor's diagnosis)
ii. Angina indicated by WHO chest pain questionnaire for angina
iii. Ischaemic ECG codes

iv. Prior hospital discharge ICD10 code for ischaemic heart disease (I20- I25)

For *stroke*, two out of the first three of the following criteria had to be present:

i. Self-reported history of stroke (subject recall of doctor's diagnosis)
ii. Prior hospital discharge ICD10 code for stroke (I61; I63-66, I679, I694)
iii. Review of clinical notes confirmed history of stroke as opposed to TIA

For *transient ischaemic attack*, two out of the first three of the following criteria had to be present:

i. Self-reported history of TIA (subject recall of doctor's diagnosis)
ii. Prior hospital discharge ICD10 code for TIA (G45, G659)
iii. Review of clinical notes confirmed history of TIA

3.6 Cognitive Assessment at Baseline and Follow Up

Assessment of cognitive function was carried out at baseline (2006-07) and the same battery of seven standardised, psychometric neuro-psychological tests was administered at the follow up clinic (2010-11). This provided a comprehensive and validated assessment of cognitive functioning allowing for comparison and thus assessment of a possible 4-year change across a range of cognitive domains as well as general cognitive ability. At both waves of the study, the following steps were taken:

- All nurses/researchers followed a standard operating procedure.
- The ET2DS investigator with a high level of expertise in psychological testing completed observation for validation
- Prior to commencing with cognitive testing, mood quality was assessed (assessment of anxiety and depression, using hospital anxiety and depression scale to identify potentially high score on anxiety and depression scale (HADS)[261]
- Prior to commencing with cognitive testing, capillary blood glucose was measured to confirm that subjects were not hypoglycaemic. Any subjects with glucose < 4.0 mmol/l would be asked to return on a different occasion for testing.

At baseline, cognitive testing was conducted by trained research nurses during one clinical session, approximately for one hour, after collection of blood and urine samples and a short break for breakfast. Due to resources available at the follow up clinic participants completed their clinical and cognitive assessments at different times during a total visit of approximately 4.5 hours. Sample of the cognitive test pack is provided as an Appendix B.

Mill Hill Vocabulary Test

Unlike the remaining part of the cognitive tests battery measuring current cognitive performance, the Mill Hill Vocabulary Scale (MHVS) test is designed to provide an estimate of prior, pre-morbid, peak cognitive ability. It is based on the notion that

'crystallised' intelligence, assessed via vocabulary test, is known to decline very little with age thus provide a valid pre-morbid, pre-clinical cognitive functioning [17,232]. In this study the combined Junior and Senior Form A synonyms MHVS[262]. It is a 44-items, self-administered test where subjects are presented with a key word and are required to select the closes synonym from six given words; overall score ranged 0-44. Vocabulary assessment vary very little across the life-span; as such the major advantage of this test is that it allows a direct comparison between population as well estimated premorbid assessment thus life time cognitive change, it provides a crude measure to those lost in follow up as it correlates with performance in other cognitive domains[17].

Mini-Mental State Examination

Mini-Mental State Examination (MMSE) is a screening 30 item scale designed to detect severity of cognitive difficulties, particularly in elderly population; it comprises of 20 questions and task assessing functioning in those domains: orientation time and place, memory (immediate and recall), attention and calculation, praxis-ideational, praxis-copying and drawing and spontaneous praxis-writing. A single score is given for each correctly completed item, ranging 0-30. The cut off point for dementia is <24, which leaves 6 points for detection of MCI from healthy cognition. Its major advantage is its practicality, only taking approximately 10 minutes to complete[4] and consistency between MMSE scores and outcomes of other standardised tests was documented (Royall et al. (2007) However, as a screening

test, it had lacked sensitivity to detect subtle changes in specific cognitive domains and to differentiate between people whose cognition deteriorated over 4 years and those whose cognition remained stable[233] and also differences between the MCI and dementia and MCI and healthy adults[263]. Furthermore, cognitive impairment might not be detected in subjects with high educational attainment and thus increased rate of false positive among people with lower educational level[264] .

Logical Memory Test (Immediate & Delayed Recall)

Verbal declarative memory, measured by *Logical Memory Test* (LM), was assessed by immediate (subtest 1) and approximately 30 minutes delayed (subtest 2) recall of a short story of a 3rd Edition (WMS-III, UK)[265]. A brief story of 25 elements is read aloud by to the participants. After reading, subject is encouraged to recall as much as they can (part I). Delayed recall is tested and recorded in identical way; participants are asked to recall as much as possible without hearing the story again. Each correctly recalled element is scored; the two sub-scores are summed and combined, providing a scoring range 0 – 50. Due to a high inter-ratter reliability, this test has been deemed as a valid sensitive measure of memory functioning[266].

Faces (Immediate & Delayed Recall)

In a very similar manner, non-verbal, visual, memory was assessed by two subtests of *Faces test*, [ref: Edition (WMS-III, UK]. Subjects are presented with 24 photos of

faces, each on display for approximately 2 seconds and with each photo subjects are reminded to remember the image. The 'recall test' collection contains 48 photos; the initial 24 photos (target) and 24 distractor photos. Subjects are required to indicate whether photo was already seen by yes/no answer at two time points, immediately (subtest 1) and approximately 30 minutes delayed recall (subtest 2).These are scored with 1 point for each correct answer; sub-scores are summed and combined, providing a range of 0-98.

Letter-Number Sequencing

Working memory along with attention, active maintenance and information organisation was evaluated by the *Letter-Number-Sequencing* subtest (LNS), another test utilised from the Wechsler Adult Intelligence Scale, 3rd Edition (WAIS-III, UK). Participant is verbally presented with a string of numbers and letter in randomly alternating order and is required to re-schedule these according to certain criteria (numbers first is raising order followed by letters in alphabetical order). Strings commence with three items and at successful completion of three strings per block, subject move toward longer, more complex collections up to the point of 8 items per string. The test is discontinued if participants fail to provide three consequent correct answers or at successful completion of all 21 strings. Participants score one point for each correct response, providing a range of 0-21.

Digit-Symbol Coding Test

Digit Symbol Test (DST), WAIS-III, UK test was administered to assess speed of information processing of adults in age range 16-89[267]. It is another pen-paper task where participants are presented with a template of numbers 1-9, each corresponding with a specific unique symbol. This template remains present for the duration of the test. Participants are required to match symbols with numbers using a pen and predesign table of randomly allocated digits and empty boxes below for relevant symbols, as many as possible within allocated 120 seconds. Boxes must be filled in linear order, i.e. alternating across the sheet is not allowed. Prior the actual tests, a trial of 7 boxes is provided; this allows the examiner to confirm that subject understood the nature of the task. Score ranges between 0-93. DST has demonstrated a high correlation with other cognitive tests, particularly those assessing memory functioning[268].

The Borkowski Verbal Fluency Test

Executive functioning was assessed by a phonemic, *Borkowski Verbal Fluency Test* (BVFT). This consists of 1 minute three sub-task where participant is required to name as many words as they can think of, beginning with three different letters with the exception of proper nouns, numbers and a repetition of words with identical semantics[19]. As such it is orally presented test with the overall score calculated by sum of correctly named words form all three trials. Particular letters have been chosen on the relative frequency in English language words beginning with each

particular letter, i.e. 'C' being relatively common, 'F' less common' and 'L' least common. Despite the test has well established reliability and validity[19,266], however, demographic characteristics such as age, educational attainment and gender might affect overall performance[269]

Matrix Reasoning Test

The *Raven's Matrices* (MR), another subtest selected from the Wechsler Adult Intelligence Scale-III, allows for assessment of a person's non-verbal reasoning, i.e. person's ability to reason by induction, therefore relies heavily on problem solving skills[19]. It consists of a sequence or group of 26 designs. In each there is a one block of the pattern missing and the participant is required to fill in a missing design from a 6 choices provided below. Items/patterns are becoming increasingly difficult and complex, requiring higher ability to understand and apply the rules of the missing point of the matrix. Maximum score is 26. Both reliability and validity[270], including between culture, has been assessed and supported[271].

Trial Making Test – B

The *Trial-Making Test,* part B (TMT-B), selected from the Halstead Retain Battery has been administered to assess mental flexibility predominantly[272]; further, it shows to reliably assess other cognitive domains, such as executive function[273], sustained attention and visual scanning and attention[4]. Subjects are presented with a list of

circles, containing numbers (1-13) and letters (A-M) and required to connect these circles in numeric and alphabetical order, evenly alternating digits and letters in an upward order and it is measured in seconds. Throughout the completions, subjects are required to keep pen on the paper. Time to complete this test provides a final score, therefore the higher the score, the poorer performance on this task. Errors are not counted (unlike in part A of this test). TMT-B has demonstrated well above average reliability estimate in normal or neurologically stable adults[274].

Hospital Anxiety and Depression Scale

Also, to assess patients' mood, every participant completed a self-reported *Hospital Anxiety and Depression Scale* (HADS) questionnaire[275] [52]

There are 14 questions in total, seven assessing anxiety and seven assessing depression. Each item is presented with a 4-multiple choice, allowing the person to indicate severity of the item with regards to last 3 days mood. Scores range 0-3, as follows:

- Most of the time (3)
- A lot if the time (2)
- From time to time, occasionally (1)
- Not at all (1)

Therefore the maximum possible score on each scale is 21; evidence suggest depression and/or anxiety approximately at the score 11 and above. However, it shall

not be understood as a definite diagnostic tool but rather as a dimensional indicator of mood state[276].

3.7 Data Management

A master Microsoft Access database was created for all baseline and follow up, coded data. Results of plasma assays were entered onto the same database, either from paper records (biochemistry and haematology) or from electronic files provided by participating laboratories. At baseline, the majority of data from paper records were double entered and possible discrepancies were resolved by referring to the original, paper format record. Remaining data were randomly sampled and double checked. In terms of the follow up cognitive data, an identical approach was applied; data were manually entered on a daily basis. On completion of all cognitive test, randomly selected 20% of records were double entered by any member of the research team except the member providing the initial scores. As at baseline data management, all discrepancies were resolved by referring to original paper records. At both time points, all data were handled only by the ET2DS research team and all sets of data, including the master database, were securely stored (password protected) on dedicated university computers and backed up securely on a dedicated university server. All paper records were securely stored and with only authorized access possible.

3.8 Data Analyses

Prior to statistical analysis, data sets were examined for outliers and missing data. Specifically for outlier values in a cognitive dataset, the threshold was set at '>1.5 interquartile range of distribution'; all values out-with this threshold were checked against a paper record and corrected where necessary. The intention was to preserve as much information as possible to satisfy the required sample size and retain statistical power. Therefore only the true outliers were removed from any subsequent analyses.

All categorical variables were described according to their frequency (percentage/number). All continuous data were first explored for normality of distribution, assessing QQ plots along with descriptive statistics; majority of data followed a normal distribution except for positively skewed IL6, CRP and TNF- α, smoking-pack-per-year and cognitive test TMT-B (both baseline and follow up scores). These values were transformed to their natural logarithm values; for these variables, median and inter-quartile range is reported for the raw values of all transformed variables; further analyses were carried with the transformed values.

Prior to performing linear regressions a number of preliminary calculations and analyses were carried out, relevant to cognitive data. Two cognitive tests, Logical Memory and Faces, consisted of 'immediate' and delayed' tasks; Pearson correlation between respective scores in each test showed a moderate to high coefficients, therefore immediate and delayed scores were combined into a single value, i.e. 'total Logical Memory' value and 'total Faces. This approach was taken in both baseline

and follow up data sets. Further, standardised residual scatter plots were inspected visually for possible violations of homoscedasticity. Set of correlation analyses was run to assess potential issues of collinearity; this concerned with relationship between predictive variables and between cognitive tests.

3.8.1.1 General intelligence factor –Principal Component Analysis

One of the most common methods used in the area of epidemiological studies of cognitive ageing is Principal Component Analysis (PCA). It is reasoned that people perform consistently across a different cognitive domains[277]. PCA is a data reduction technique that condenses a multidimensional dataset by creating components (factors) that account for the correlated variance amongst the cognitive scores[278]. In studies concerned with cognitive performances, the largest component that explains the largest single proportion of variability, i.e. the principal component factor, is commonly known as the general intelligence factor, or '*g*'[277]. Derivation of this single dimension cognitive competence enables an interpretation of results from a variety of neuropsychological tests in a meaningful way that allows comparison of cognitive scores across varied cohorts.

Prior to performing the PCA, set of Pearson correlations was run to assess associations between seven cognitive test scores at each time point; moderate to high correlation coefficient were observed, therefore it was possible to determine the baseline and follow up 'g' as well as a follow-up 'g' that was adjusted for its respective baseline score.

In terms of calculation of the actual '*g*' factor, firstly two single scores of '*g*' were determined: baseline and follow up '*g*', each combining respective seven cognitive scores (LM, Faces, MR, DST, LNS, BVFT and TMT-B). In terms of the baseline '*g*', the principal component accounted for 44, 0% of the variance with each test loading strongly on the *'g'* (range .0454 - (-.0794)). All loading was of a positive value except for the TMT-B (measured in seconds), suggesting a higher scores and thus lower level of performance on this test. The follow up '*g*', principal components accounted for 46.3% of the variance, each test again loading strongly on the *'g'* (range .481 – (-.807)). As above, TMT-B negative value reflected higher time need to complete the task and thus a lower cognitive performance. Table 3 provides each test contribution to principal component *'g'* (baseline and follow up data), that demonstrates the strength of loading onto the first component und thus the occurrence of the general intelligence factor. The consistency between all values, the pattern detected within each time point scores as well as the steadiness between those two time points, is in line with relevant evidence suggesting that performance on individual cognitive test remains of a same level across other cognitive tests [279].

Table 3: Principal Component Analysis: baseline and follow up cognitive scores

	Factor Loading	Extraction (%)	Component	Variance (%)	Eigenvalue
LM_BL	0.530	28.1	1	44.0	3.08
Faces_BL	0.456	20.8	2	13.0	0.91
VFT_BL	0.678	45.9	3	11.2	0.78
MR_BL	0.663	44.0	4	9.8	0.69
DST_BL	0.753	56.6	5	9.3	0.65
LNS_BL	0.710	50.4	6	7.8	0.54
- ln_TMT-B_BL	0.793	62.8	7	4.9	0.34
LM_FU	0.182	36.6	1	47.4	3.32
Faces_FU	0.154	26.2	2	11.7	0.82
VFT_FU	0.188	38.8	3	10.5	0.74
MR_FU	0.207	47.4	4	10.2	0.71
DST_FU	0.237	61.8	5	8.4	0.59
LNS_FU	0.224	5.55	6	6.9	0.49
- ln_TMT-B_FU	0.244	65.8	7	4.7	0.33

BL - Baseline Scores; **FU -** Follow Up Scores; **DST** – Digit Symbol Test; **LM** – Logical Memory; **LNS** – Letter Number Sequencing; **MR** – Ravens' Matrices; **TMT – B** – Trial Making Test- B; **VFT** – Verbal Fluency Test

Final step was to determine a follow up *'g'* that was adjusted for a respective baseline *'g'* scores. A simple comparison of the individual (i.e. baseline vs. follow up) mean standardised scores would be uninformative because the means and standard deviation at both waves are approximately 0 and -1, respectively[59]. Therefore to ensure that the comparison of the mean scores across time is meaningful, PCA was carried out on the age-adjusted residuals of each cognitive test in the following steps:

- all data (baseline and follow-up) were combined into a single column;
- standardised residuals from PCA were saved as composite ability score;
- this was split into 2 variables according to time of data collection;

- the follow-up composite residual was entered as an independent variable into regression analysis, with adjustment for baseline composite score.
- residual from this analysis provided a variable indicating a cognitive decline over 4 years.

This variable was further entered into all models that were set to establish the strength of association between baseline levels inflammatory markers and a change in general cognitive ability.

3.8.1.2 Inflammation Factor – Principal Component Analysis

Pearson correlation between baseline levels of biomarker values, carried out on results from the entire baseline sample, revealed a significant inter-correlation with a moderate effect size. Reduction to the attenders weakened these coefficients slightly; however, all values reached a significant level of $p< 0.001$. Also, the same pattern remained the same across the two time points, i.e. the strongest associations were observed between plasma fibrinogen and CRP levels ($r=0.521$ and $r=0.497$). Slightly weaker associations were observed between IL-6 and CRP ($r= 0.433$ and $r=0.415$) and between IL-6 and plasma fibrinogen ($r=0.339$ and $r=0.344$). Overall, TNF - α showed the lowest magnitude of correlation values with the weakest relationship observed between CRP and TNF - α ($r = 0.133$; and $r=0.126$) (Table 4 and 5). These results supported previously identified biological (cellular and molecular) multi-factorial relationship between these four specific biomarkers that mutually affect production and regulation of other cytokines and acute phase reactants[86,87].

As mentioned above in a sub-section 5.1.7, principal component analysis reduces multi-variable measures into one principal factor that encompasses the correlated variance between individual variables. Provided the biological link and significant correlation coefficients between individual biomarkers, it was rationalised to conduct a PCA on values of baseline biomarkers values.

PCA revealed that all loadings were of a positive value; Table 6 provides each biomarker's contribution (percentage and factor loading value, baseline measures), that demonstrates the strength of loading onto the first component und thus the occurrence of common component. This principal component accounted for 48.6% of the variance, each biomarker strongly loading on it (range 0.789 to 0.435). This unrotated, saved component, referred here as an 'inflammatory factor', was later subjected to same multiple regression analysis as each inflammatory marker individually.

Table 4: Pearson correlation of baseline inflammatory markers (whole study population)

	Fibrinogen	**CRP**	**IL6**	**TNF – α**
Fibrinogen	1	0.521**	0.339**	0.146**
CRP		1	0.433**	0.133**
IL6			1	0.324**
TNF – α				1

** p<0.001

Table 5: Pearson correlation of baseline inflammatory markers (attenders sub-sample)

	Fibrinogen	CRP	IL - 6	TNF – α
Fibrinogen	1	0.497**	0.344**	0.133**
CRP		1	0.415**	0.126**
IL - 6			1	0.296**
TNF – α				1

** p<0.001

Table 6: Principal Component Analysis: baseline inflammatory markers

	Component	Variance (%)	Eigenvalue
Fibrinogen	1	48.62	1.95
CRP	2	24.18	0.97
Interleukin - 6	3	15.05	0.60
TNF-α	4	12.14	0.49

3.8.1.3 Relationship between T2DM and baseline levels of inflammation, association with follow up cognitive scores

Interaction between Type 2 diabetes and elevated systemic inflammation that is either mediated or confounded by vascular risk factors has been documented[94,280]. Provided the rationale and research question of this study, i.e. investigation into relationship between elevated levels of inflammatory markers and accelerated cognitive decline in solely diabetic population, a series of regression analyses were run to explore a potential interaction between type 2 diabetes duration (defined by

the baseline data) and the baseline HbA1c levels and all above listed inflammatory markers to confirm such associations in this particular cohort.

Results, presented in Table 7 show a significant relationship between T2DM, as defined in the paragraph above, and inflammation status of all measured markers, including the inflammatory factor. In case of plasma fibrinogen and TNF - α, here significant associations vanished in analyses adjusted for common cardiovascular factors. The remaining biomarkers showed significant relationship with T2DM, independently of cardiovascular factors and events.

Table 7: Associations between baseline measures of inflammatory markers & HbA1c

	Fibrinogen	**CRP**	**IL – 6**	**TNF - α**	**IF**
HbA1C, Age, Sex,	.067 (.036)**	.146 (.036)***	.113 (.035)**	.092 (.035)**	.168 (.035)***
HbA1C, Fully adjusted[$]	.037 (.043)	.155 (.045)***	.091 (.045)*	.049 (.045)	.153 (.045)***

[$] Age+ Sex + BP (S) + Total cholesterol + smoking + alcohol +HbA1c + MI + angina + stroke + TIA + duration of T2DM

IF – Inflammatory Factor; **BP(S) –** Blood pressure (systolic); **TIA –** (transient ischaemic attack)

3.8.1.4 Assessment of missing data, imputation of cognitive scores

Missing data were present at both time points, in independent as well as dependent variables. For the purpose of this analysis, cognitive data were imputed for baseline and follow up. Specifically, adjusting for age and sex, missing cognitive score were imputed for any person where data were missing on one, two or three out of the seven tests (LM, Faces, LNS, MR, DSC, TMT-B, BVFT). This procedure was

performed separately for cognitive test scores at baseline and at year 4 follow-up. For participants with missing data on Faces or Logical Memory, the sum scores on the respective test were imputed. A potential for an underestimation of error following imputation may be problematic. In the present analyses, imputation of missing data outweighed by the increased statistical power, in particular in the analysis of the global ability factor *g*. The calculation of the factor, which is further described below, requires that data is available on each contributing test, so that any missing data on individual tests would severely restrict the number with participants with a value of g. The number of cases with data was imputed is shown separately for each cognitive test in Table 8, below.

Table 8: Imputation of cognitive data collected at baseline and 4-year follow up

	Baseline Cognitive Data (n=1066)		**Follow-up Cognitive Data (n=828)**	
Cognitive Test	Number of imputed cases	Data missing after imputation (%)	Number of imputed cases	Data missing after imputation (%)
Logical Memory	12	0.38	5	0.72
Faces	2	0.47	3	0.72
BVFT	2	0.38	4	0.60
MR	9	0.28	1	0.72
DST	4	0.47	21	0.96
LNS	13	0.47	32	0.84
TMT-B	9	0.47	21	0.96

In terms of predictors and demographic variables, complete data were available for age, sex, incidence of cardiovascular events (MI, angina, stroke, TIA), duration of

T2DM, SIMD, highest education attainment. Small amount of data were missing in the remaining variable sets (table 9) but except for the percentage representation, no other formal analyses of missing data was conducted on this data.

Table 9: Percentage representation of missing data (predictors & covariates, baseline measures)

	Number	Missing %		Number	Missing %
Age	0	0%	**BP (S)**	2	0.2%
Sex	0	0%	**Cholesterol**	9	0.8%
Fibrinogen	3	0.3%	**MI**	0	0%
CRP	30	2.8%	**Angina**	0	0%
IL-6	14	1.3%	**Stroke**	0	0%
TNF	61	0.8%	**TIA**	0	0%
HbA1c	38	3.6%	**Smoking**	47	4.2%
Dur_T2DM	0	0%	**Alcohol**	49	4.6%
Education	0	0%	**SIMD**	0	0
HADS_A	1	0.1%	**HADS_D**	1	0.1%

3.8.1.5 Multifactorial analyses

To avoid possible violation and biased results, prior regression analyses a number of preliminary calculations were taken to assure that assumptions necessary for this type of analyses were met. Specifically, this concerned with data distribution and extreme values, linearity and homoscedasticity.

The modelling was three-tiered process. Firstly, Pearson's correlation, adjusted for age and sex was calculated to assess a cross-sectional association between individual

biomarker and cognitive scores. Next two cumulative steps, association between the baseline level of each of the four circulating biomarkers/cluster of biomarkers and cognitive function was assessed. At step one, the follow up cognitive scores were adjusted for respective baseline scores, determining a 4 year change in cognitive functioning. Alternative to this approach would be modelling that would explore an effect of inflammatory markers on an 'absolute' change in cognitive performance (i.e. subtracting follow up score from the baseline, results being entered as dependent variable). An adjustment for baseline score ameliorates a possible bias caused by baseline scores exceeding the follow up measures, especially in case if the reliability of dependent variable measure is uncertain[250]. Though the neuropsychological battery of standardised tests used in this study has been specifically selected for its robustness, validity and reliability, in the case of subtest assessing a verbal (Logical Memory) and non-verbal (Faces) memory, follow up scores improved significantly. Therefore in regression adjustments, respective baseline scores were entered as covariates.

Next, all potential confounding variables, measured at baseline, were introduced into the models; those included: age and sex, vascular risk factors (systolic blood pressure, cholesterol levels, alcohol intake and smoking), the presence of cardiovascular disease (incidence of MI, angina, stroke and TIA), and diabetes relevant variables (duration of diabetes and HbA1c levels). At the step two, all follow up cognitive scores were adjusted for the pre-morbid, estimated peak cognitive ability to determine a life-time cognitive ability (MHVS scores were entered as measure of peak prior cognitive ability). Lastly, adjustments were made

for all variables in exactly the same manner as in the previous multifactorial analysis. This enabled a direct comparison of the predictive value of inflammatory markers on 4 years and life time change in cognitive functioning of elderly diabetic population.

Associations between socio-economic factors and performance on cognitive testing at advanced age has been well documented[59,281]. Therefore many studies assessing the strength of relationship between potential risk factor and cognitive performance include various forms of valuation of socio-economic status as a covariate in adjusted analysis[61,282,283]. In the ET2DS, socio-economic status was defined by SIMD value. SIMD, *the Scottish Index of Multiple Deprivation* is used to identify deprivation based domains, such as current income, employment, health, education skills and training, geographic access to services, and housing and crime[284]. Providing the significant relationship between SIMD and trajectory of cognitive ageing[15], it would seem reasonable to include this variable in fully adjusted models, assessing change in cognitive performance in the ET2DS. However, the primary aim of this analysis was to assess the biological link between circulation inflammatory markers and cognitive scores in the diabetic population were a role conventional cardiovascular risk factor and event were assessed in fully adjusted models. Previous research indicates that cardiovascular risk factors and evens largely correlate with the socio-economic status[285]. Therefore it was reasoned that including SIMD values in the presented regression analyses would weaken the statistical power without adding any apparent benefits to the results and consequent interpretation.

Lastly, consideration was made whether to conduct analysis determining a potential associations between the MMSE test score with four inflammatory biomarkers. In

this study, scores on this test were obtained for screening purposes only. Furthermore, scores on MMSE are known to be influenced by intra and inter-tester variability; a previous study found that MMSE scores for identical subjects were dependent on testers[286] and very sensitive to a ceiling-floor effect[287]. In light of the methodological issues, it was therefore not included in the selection of cognitive tests that comprised the general intelligence factor.

Chapter 4: Methods – Collaboration of Seven Population Based Cohorts

The second aim of the work presented in this thesis was to explore the relationship between fibrinogen - related single nucleotide polymorphisms (i.e. SNPs associated with altered plasma fibrinogen levels in previous studies) and general cognitive ability. The rationale was that if fibrinogen levels are casually associated with cognitive impairment in older age, then it might be possible to detect direct association between '*g*' and SNPs affecting plasma fibrinogen levels. In order to explore this, it was recognized that a larger sample size than was available from the ET2DS alone would be required, and so collaboration between seven population-based cohorts which were known to have cognitive data and at least some fibrinogen-related SNPS was established. Plasma fibrinogen measures were also available in the collaborative cohorts. The procedures which I went through to obtain and collate data from these cohort studies and the characteristics of each of the studies are described in this section. Particular focus has been paid to studies description, collection of genotypic and fibrinogen data and cognitive test battery administered in each cohort. Furthermore, method of analyses, including calculation of general intelligence factor '*g*', details of analyses conducted in each cohort as we as well as the method of meta-analysis is presented here.

The last part of this chapter described methods of identification and selection of fibrinogen related Single Nucleotide Polymorphisms (SNPs). This section outlines methods of systematic literature search and search conducted in two databases, the

International Human Genome Project (HapMap) and the National Human Genome Research Institute (NHGRI). Process of deriving to the final selection of fibrinogen related SNPs, subsequently used in analysis determining strength of relationship between these gene variances and cognitive functioning is outlined at the end of this chapter.

4.1 Data collection from participating cohorts

Data request

Cohorts were identified as suitable for inclusion in this work if they contained the variables necessary for the proposed analysis, i.e. to explore associations between fibrinogen-related SNPs and cognitive functioning in older population. Studies were selected on the basis of comparable populations, availability of genotype and cognitive data and, provided the time scope available to complete the analysis, judged to be reasonably easy to access. This included studies in Edinburgh and two large studies based in London with which investigators of the Edinburgh studies had previously collaborated:

- The Aspirin for Asymptomatic Atherosclerosis Study (AAA)*, $
- Edinburgh Artery Study (EAS)*, $
- English Longitudinal Study of Ageing (ELSA)*
- The Edinburgh Type 2 Diabetes Study (ET2DS)*, $
- Lothian Birth Cohort 1921 (LBC 1921)$

- Lothian Birth Cohort 1936 (LBC 1936)[$]
- Whitehall II (WII)*

*studies with previous history of collaboration and now part of UCLEB - Consortium of UCL (London) – Edinburgh – Bristol population based prospective studies[288]; [$] Scottish population - based studies

Firstly, all identified collaborative centers were contacted to discuss potential collaboration and conditions for data sharing. Subsequently, an appropriate *Data Request Form* was forwarded to each external center (ELSA, LBC 21, LBC36, ELSA and Whitehall II; Appendices C). Data from AAA, EAS and ET2DS were requested internally, no official form was required. Study objectives and aims were outlined and appropriate datasets necessary for analyses were requested as follows:

- any person with genotype data including 1 or more fibrinogen-related SNPs (list of eligible SNPs described in section 5.3, Appendix D)
- any person with cognitive test scores, regardless of missing values
- plasma fibrinogen measures if available

4.2 Studies description, Genotypic and Fibrinogen Data, Cognitive Test Battery and calculation of general intelligence factor *'g'*

This section describes the key characteristics of each collaborating cohort, relevant to the presented study. All 7 studies are from the UK, population based with a prospective cohort design. Two studies are birth cohorts (LBC 1921 and LBC 1936). The AAA trial was initially set up as a RCT; however, prospective data collection was conducted, hence this trial could have been identified and included as a cohort study. Each study has clearly defined sampling methods, inclusion/exclusion criteria, method and procedure of genotypic, biological and cognitive data collection that were necessary for the presented investigation. All studies have reasonable even gender representation (except for the AAA trial were approximately 2/3 were female and, reversely, Whitehall II with approximate 2/3 of male subjects), with all subjects falling into 7-8th decade of life, mean age ranged from 60.8 (Whitehall II) to 79.1 (LBC 1921). This is in line with recruitment criteria within those cohorts. Each study details, relevant to the presented analysis, are described below.

Collating and sorting data according to the above stated criteria resulted in reduction in the number of subjects from each study that was included in the final analysis There was a substantial overlap between cognitive tests used in individual cohorts for the neuropsychological assessment; majority of those have been described in a sub-section 5.1.5, *Cognitive Assessment at Baseline and Follow up (ET2DS).* Where further test was administered, description was outlined in a relevant cohort section.

Overview of all tests for which results were available in each collaborating study is provided in table 10. Calculation of general cognitive factor was conducted in each study independently.

Collaborating centres were requested to share all available genotype data (all available according to the final fibrinogen related SNPs selection), cognitive data (all available scores that were obtained through standardized cognitive assessment) and, if available, plasma fibrinogen measures. For each variable, year/date of data collection was requested to assure consistency between cohorts in terms of time difference between each type of data collection. This resulted in receiving partial subsets of the original data from the each of the original cohort population. For this reason, data were not always available for analyses assessing differences in sample characteristics between included and excluded subjects; therefore potential selection bias could have not been assessed. Furthermore, this inconsistency meant that adjustment for factors potentially mediating association between fibrinogen related genetic markers and cognitive outcome. From those, age and sex were consistently reported across studies and thus entered as covariates. However, demographic and laboratory data were available for the received datasets; description of each study characteristics is provided in a table 16, descriptive statistics was performed on a sub-sample analyses in this study.

The Aspirin for Asymptomatic Atherosclerosis Study

The Aspirin for Asymptomatic Atherosclerosis (AAA) commenced in 1998 as a placebo, double blind controlled, randomized trial. The primary aim was to investigate the effect of low-dose aspirin on cardiovascular events and cognitive ability in people with asymptomatic atherosclerosis, i.e. healthy subjects with relative risk of cardiovascular disease. Subsequently, the study has been analyses as a prospective, observational cohort to assess the relationship between inflammatory biomarker and cognitive performance at an advanced age, including cognitive decline and estimated life-time cognitive change. The trial initially recruited 3350 men and women (n= 2396), age range 50-75 years, all residents in central Scotland (Edinburgh, Glasgow and Lanarkshire), of whom 2312 underwent cognitive assessment (mean age of cognitively tested sample 61.6; SD=6.54, male = 27.4%). Detailed description of sample recruitment, baseline clinical and cognitive examination has been published previously[289-291]. Ethical committees of Lanarkshire, Edinburgh and Glasgow granted the ethical approval for this study. Further, all subjects provided informed, written consent prior the start of their participation.

Blood samples used for genotyping were collected at a clinic visit three months after recruitment (baseline). Genomic DNA was isolated by standard procedure at the Welcome Trust Clinical Research Facility Genetic Core, Western General Hospital, Edinburgh. Genotyping was carried out by KBioscience (Herts, UK) using their in-house chemistry of Competitive Allele Specific PCR (KASPar). Assays for plasma fibrinogen levels were performed in the University Department of Medicine,

Glasgow Royal Infirmary; this was carried out in the same manner as in the ET2DS, described above.

Of the initial 3,350 subjects in the AAA Trial, 2312 undertook cognitive assessment at a 5-year follow up clinic, administered by a trained nurse in accordance with standard operating procedures. For the purpose of the current analyses, the sample of 2,061 subjects who completed the entire cognitive battery was used. The battery of neuropsychological assessment included tests of immediate and delayed memory (Auditory Verbal Learning Test; AVLT, score ranged 0-75). In this test participants receive five presentations with recall of a list of 15 words, one presentation of an alternative list of 15 words and then they are presented with the sixth recall trial. Delayed recall is assessed approximately 30 minutes after the initial presentation. Furthermore, subjects in the AAA trial undertook a test of executive functioning (Verbal Fluency Test; VFT), processing speed (Digit Symbol Test; DST), non-verbal reasoning (Raven's Standard Progressive Matrices; MR) and mental flexibility and attention (Trial Making Test, part B; TMT-B). Scores from all five tests showed moderate to high correlation ($r = -646$ to $r = 0.343$; $p<0.01$) and were entered and used to conduct a principal component analysis to derive 'g'. The principal component accounted for 55.83 % of the variance with each test loading strongly on the *'g'*, ranged 0.640 – 0.821.

Edinburgh Artery Study

The Edinburgh Artery Study (EAS) commenced in 1988 with the primary aim to investigate the epidemiology of peripheral arterial disease – assessment of cognitive function was subsequently added. Initially, the cohort consisted of 1592 men and women, age range 55-74, randomly selected from age-sex register of eleven general practices in Edinburgh. Several follow up assessments clinics were organized with blood being sampled for genetic testing at a five-year follow up clinic (1992) and cognitive testing commencing in 1998; at this time point, 1209 subjects undertook a cognitive battery of 4 neuropsychological tests; for those, 717 full cognitive dataset was available. Out of this sub-sample, genotype and cognitive data were available for 534 of subjects (male 50.7%), mean age 73.9; SD = 5.32. A full description of study recruitment, clinical and cognitive assessment of this cohort has been published previously[114,292]. This study gained ethical approval from the Lothian Health Board Ethics of Medical Research Sub-committee for Medicine and Clinical Oncology. Written informed consent was provided by each person prior their participation.

Blood assays were carried out according to relevant standards in accredited laboratory. Genotyping was carried out from five year examination blood samples, using a 5'-nuclease assay (TaqMan) by Helen Ireland in the laboratories of Professor Steve Humphries. Plasma fibrinogen levels were determined at baseline from a fasting blood samples, measured by a thrombin-clotting turbidometric methods[134].

A cognitive assessment consisting of battery of 5 neuropsychological tests was administered to all willing participants by trained researcher, according to

standardized operating procedures. It assessed immediate and delayed memory (Logical Memory; LM), executive function (Verbal Fluency Test; VFT), non-verbal reasoning (Raven's Standard Progressive Matrices; MR), mental flexibility and attention (Trial Making Test, part B; TMT-B) and processing speed (Digit Symbol Test; DST). There was a slight variation in the administration and scoring the Logical Memory test compared with other studies; in EAS, two stories were read and scored for immediate and delayed recall, therefore scores ranged from 0-100. Correlation between individual cognitive tests ranged from r= 0.186 to r= 0.400; $p<0.01$); Principal component factor '*g*' accounted for 49.61 % of the variance between tests with each loading strongly on the factor '*g*', ranged 0.635 – 0.768.

The English Longitudinal Study of Ageing

The English Longitudinal Study of Ageing (ELSA) is a longitudinal, population study. It commenced in 2002 and was conducted by the Department of Epidemiology and Public Health, University College of London and the National Centre for Social Research. The primary aim of this study was to explore and determinate a wide range of factors associated with an advanced age; cognitive functioning of older people being one of the outcome measures. Detailed description of study aim, recruitment and clinical assessment has been published[293]. Initial 12,000 participants formed a nationally representative sample of the English population, aged 50 years and above. For the purpose of this analyses, data from wave 4 (2008-09), available for 5606 subjects of mean age 66.1 (SD=9.63) of which were used. At this time

point, the largest number of subjects with both genotyped and cognitive data was available; also, as this was the most recent data provided by this collaborator, mean age of participants matched the closest with mean age of remaining cohorts. The ELSA has received an ethical permission from the London Multi-Centre Research Committee. All subjects provided a signed informed consent prior their participation.

Analyses of blood sample for plasma fibrinogen concentration was conducted in the Royal Victoria Infirmary (Newcastle-Upon-Tyne). Detailed information of internal and external quality assessment for the laboratory was published elsewhere[294] .

Cognitive data for the presented analyses were available for 5,600 subjects; however, calculation of general intelligence factor reduced this sample to 5,458 subjects. The ELSA cognitive battery consisted of four neuropsychological tests: immediate and delayed word recall of 10-words recall list (words recall, scores 0.10; subjects presented with a random one to two syllables words and are required to recall in writing as many as possible), semantic fluency was assessed by a number of animal names that the subject can freely name within a 1 minute time limit (number of animals); ability to write initials after 20 minutes delay, i.e. when instructions was given (prospective memory, PM, range 0-5); lastly, unlike in other cohorts, literacy skill was assessed to determine subjects ability to memories and comprehend context relevant scenario (literacy, range 0-3, subjects were provided instructions with regards to medication administration and consequently tested on comprehension of it). Detailed description of neuropsychological battery is presented on www.ifs.org.uk/elsa/ Person correlations revealed moderate values, ranged between $r= 0.194$ to $r= 0.434$; $p<0.01$. PCA was conducted and as with other studies, it yield a

presence of principal component that accounted for 45.7% of variance between tests with each score loading sufficiently, ranged 0.528 – 0.768.

The Edinburgh Type 2 Diabetes

As detailed above, the ET2DS is a population based, prospective study of 1066 randomly selected subjects with diagnosed T2DM, aged 60-74. For the presented analysis, the baseline data set of 1,066 subjects was reduced to 1045 of participants with both genotype and cognitive dataset available; mean age 67.9 (SD=4.2), of those 51.2% were male.

Blood samples collected at the ET2DS baseline clinic were used for DNA extraction and subsequent genotyping. Genomic DNA was isolated from whole blood by standard procedure at the Welcome Trust Clinical Research Facility Genetics Core, Western General Hospital, Edinburgh. Genotyping was undertaken at KBioscineces, using their in house chemistry of Competitive Allele Specific PCR (KASPar).

Fibrinogen assays were performed in the University Department of Medicine, Glasgow Royal Infirmary, using stored plasma anti-coagulated with trisodium citrate and automated Clauss assay (MDA-180 coagulometer, Organon Teknika[114].

Detailed description of collection of data cognitive scores at baseline was described above in a section 5.1.5. PCA for the current analyses was calculated on raw (i.e. non-imputed data) in order remain consistent across cohort studies. All scores showed moderate to high correlation coefficient ($r = -0.630$ to $r = 0.471$; $p<0.01$).

Calculation of general intelligence factor revealed a principal component accounting for 44.09% of variance with each cognitive score loading strongly, ranged 0.530 – 0.793.

Lothian Birth Cohort 1921 and Lothian Birth Cohort 1936

Lothian Birth Cohort 1921 (LBC1921) and Lothian Birth Cohort 1936 (LBC1936) are a unique epidemiological studies of cognitive ageing, both comprising of alive participants of the initial 1932 and 1947 Mental Health survey, specifically The Moray House Test, Number 12, a validated test of general cognitive ability[295,296]. Detailed description of background information has been published previously[61,296].

The LBC1921 study had commenced in 2000 with the primary aim to establish individual differences in genetic determinants in cognitive ageing. This cohort consisted of all living people who undertook the Scottish Mental Health Survey at 1932. Then, it was completed by 87,498 children of both genders. Of those, sample of 550 relatively healthy man and women was undertook clinical and cognitive assessment at 2000,. For the current analyses, the required genotype and cognitive data were available for 517 participants, mean age 79.1 (SD=0.57) years, male representing 41.6%.

The LBC 1936 study began at 2004 with the goal to investigate and establish a range of contributors associated with brain ageing and assess multidirectional relationship between them. This cohort comprised of 1,091 survivals of the original 70.805

participants who completed the Scottish Mental Health Survey at the mean age of 11, i.e. in 1947. At the time of the first wave,); data required for this analyses were available for 1005 subjects, mean age was 69.6 (SD=0.83), male representing male 50.6%.

Ethical permission for the LBC 1921 and LBC 1936 was provided by the Multi-Centre Research Committee for Scotland and the Lothian Research Ethics Committee. As above, prior the study all subjects provided a written informed consent indicating voluntary participation.

In case of both cohorts, blood sample were used for genomic DNA and plasma fibrinogen extraction. Genomic DNA was isolated from whole blood by standard procedure at the Welcome Trust Clinical Research Facility Genetics Core, Western General Hospital, Edinburgh. Genotyping was carried out by KBioscience (Herts, UK), using the in-house chemistry of Competitive Allele Specific PCR (KASPar)[297]. Fibrinogen was measured using an automated Clauss assay (TOPS coagulometer, Instrumentation Laboratory[298].

At the follow up wave of the LBC 1921 study at 2000, participants completed assessment of immediate and delayed memory (Logical Memory; LM), executive function (Verbal Fluency Test; VFT) and non-verbal reasoning (Raven's Standard Progressive Matrices; MR). Correlation coefficient between those three tests showed lower to medium values ($r= 0.175$ to $r= 0.386$; $p<0.01$). Principal component analysis was conducted and principal factor 'g' was saved; it accounted for 52.14% of overall variance with all test loading strongly of the factor '*g*' (ranged 0.619 – 0.723)

The cognitive battery administered in the LBC 1936 provided scores for the following: immediate and delayed Memory (Logical Memory; LM); executive function (Verbal Fluency Test; VFT), non-verbal reasoning (Raven's Standard Progressive Matrices; MR), mental flexibility and attention (Trial Making Test, part B; TMT-B) and processing speed (Digit Symbol Test; DST). Individual scores were correlated at a levels similar to the LBC 1921 ($r= 0.181$ to $r= 0.400$; $p<0.01$). A general intelligence factor '*g*' was calculated; results indicated a principal component which accounted for 48.4% of variance, with individual tests loading at a high level (0.645 – 0.775).

Whitehall II

The Whitehall II study is a prospective cohort study of British civil servants. It commenced in 1985 with initial sample number of 10,308 men and women (men 70%), aged 35-55. In comparison to remaining six cohorts, the population of Whitehall II was slightly younger (mean age 60.7, SD = 5.9); also, unlike other cohorts, here the criteria of recruitment included occupational status (civil servants), ethnic background (British) and residency (London). Furthermore, all enrolled participants were 'white collar' worker, despite some hierarchy of salary scale has been observed. The primary focus of clinical examination is cardiovascular and metabolic risk factors and disease and provides a longitudinal evidence of inter-relationship between wide range of these factors as well as their effect on cognitive ageing[299]. As requested, subjects with genotype and cognitive data were provided, in

total 3,431. Of those, all subjects had SNP data available, however, sufficient data set to calculate general intelligence factor 'g' reduced this sample slightly to 3,340 valid cases, mean age 60.7 (SD=5.94), of those 75.6% being male participants.

Genotype data were drawn from a blood samples collected in 2004 (approx. 6000 subjects).

Blood samples were used for an extraction of DNA, using magnetic bead technology (Medical Solutions, Nottingham). Medical Solutions used SNPLex (Applied Bioscinece) to determine relevant genetic variances[300]. For assessment of plasma fibrinogen concentration, blood samples were frozen immediately after venipuncture and stored until assays. Plasma fibrinogen was determined by an automated modification of the Clauss methods. Technical error was estimated by assaying blinded duplicate samples for 5% of subjects[301].

Though the study was established in 1985, collection of cognitive data was not conducted until the phase 5 (1997-99). Comparatively to other collaborative cohorts, the mean age of Whitehall II population was slightly lower. Therefore, to match as close as possible the mean age across all cohorts, data from wave 7 (2002-04) were selected. The battery of neuropsychological tests assessed immediate, short term memory (word list immediate, score range 0-20, subjects are required to freely recall in writing as many words as they can from a previously presented list of one or two syllable words); verbal and mathematical reasoning and test of ability to infer patterns, principles and rules (the Alice Heim 4-1; AH4-1, score range 0-65). The AH4_1 consists of 65 items, 32 verbal and 33 mathematical; complexity of items

progresses with the test. Furthermore, phonemic verbal fluency (freely recalled words beginning with S, test identical to previously described Verbal Fluency Test with e exception of reduced presentation of three letter to single letter, in this case letter 'S') and semantic fluency (freely recalled names of animal; same principle applies as in a phonemic verbal fluency test), each allowed 1 minutes recall time. Detailed description of cognitive assessment procedure has been published previously[283,299] and is also available at www.ucl.ac.uk/whitehallII/. Person correlation showed moderate relationship between individual test scores (r= 0.328 to r= 0.585; p<0.01). The general intelligence factor was derived from 4 cognitive tests with a strong principal component accounting for 59.7 % of the variance and each test loading strongly on the *'g'*, ranged 0.603 – 0.838.

4.3 "Summary of data available for all collaborating cohorts

Table10 below provides a breakdown of numbers of subjects in each collaborating cohort for which genetic, cognitive and/or plasma fibrinogen data were available. The aim of the analyses was to assess relationship between fibrinogen related SNPs and cognitive performance, therefore the final number subjects of each cohort was determined by availability of this data. The total number of eligible subject was 14 033.

Table 10: Final number of subjects per individual cohort

	AAA	**EAS**	**ELSA**	**ET2DS**	**LBC 21**	**LBC 36**	**W II**
Initial cohort (*n*)	3350	1592	12000	1066	550	1091	10308
Received (*n*)[1]	2312	852	5606	1066	517	1005	3413
SNP data (*n*)[2]	2091*	852	5606	1060*	517	1005	3413
Fibrinogen & SNPs data (*n*)	1918	523	5532	1034	487	997	3187
'*g*' & SNP data (*n*)[3]	**2061**	**534**	**5458**	**1045**	**517**	**1005**	**3413**
Total Number				***14033***			

1. Number of cases received from collaborative centers, based on availability of fibrinogen related SNPs (1 or more) from the requested list of pre-identified fibrinogen related SNPs and cognitive test scores
2. Final number of subject with SNPs data after quality control; *additional cases above the requested dataset as according to selection criteria for all studies
3. Final number of subjects with valid 'g' score and SNP data
4. Final number of subjects with valid plasma fibrinogen measures and SNP data

4.4 Data Management

Data Storage

Collaborative centers for ELSA and Whitehall II provided data in a password protected format; this was valid for a limited period of time. Data retrieved from the mail box were saved in a password protected University server with an appropriate, password protected backup. Data from LBC cohorts were received via email exchange; upon password protected saving, these email were deleted from the mailbox, therefore no unauthorized access could have been gained. Internally obtained data for the AAA, EAS and ET2DS are securely stored at the University server. At all stages of the study, all datasets were password protected, back up was on a secure university server, therefore only authorized access was possible.

Cleaning and Preparation of individual data set

The data files received from the collaborating studies differed considerably in format, layout and structure, therefore a direct synthetizing was not possible. To ensure compatibility of all datasets required for individual analyses and the meta-analyses, studies were unified on the following variables:

- ID — unique subject identifier as defined by individual study
- rs number — SNPs
- g — general intelligence factor, derived via PCA
- fib — plasma fibrinogen levels, all available in g/L
- sex — male/female (categorical variable)
- age-years — age in years format at the time of cognitive testing

Exclusion criteria

Subjects were excluded if the following four conditions were not satisfied.

- SNPs (i.e. at least one SNP from the required selection must be present)
- SNPs satisfied a quality control check (details presented below)
- European subjects only
- Cognitive scores available for more than half of the possible cognitive results derived from a cognitive test battery specific to each particular cohort

Genotype Data Cleaning

The genotyping was carried out independently by each research team independently. The format of the received genotype data differed between cohorts. Specifically, ELSA genetic data were coded as 'A'=major allele and 'B'= minor allele, whereas the remaining files were received in the conventional format. Therefore the ELSA cohort genetic variables were recoded first to match the remaining cohort. In Whitehall II, LBC 1921 and LBC 1936 datasets, each SNP was presented in two separate columns, one for each allele; in such case, these were combined into one column. Lastly, the major allele frequency was calculated for each gene variable and coded as a 'scale' variable according to the major allele frequency, i.e. with the highest frequency assigned to highest number (2-1-0). This ensured consistency across all genetic data and thus allowed merging cohorts as well as conducting and interpreting results from subsequent meta-analysis.

A sample call rate threshold > 0.95 was applied in AAA, EAS, ET2DS, ELSA and Whitehall II in order to identify samples with a low DNA quality or problematic arrays. SNPs that were outside the Hardy Weinberg equilibrium threshold (HWE $p<0.05$) and duplicate SNPs were removed. The filter for Minor Allele frequency was set at MAF ≥ 0.5%, excluding rare SNPs. In terms of varied ethnic background, Principal Component Analyses was conducted to identify non-Caucasian subjects; these were subsequently excluded from further analyses to address potential population stratification[288].

In LBC 1921 and LBC 1936, quality control was as follows: individuals were excluded from this study based on unresolved gender discrepancy, relatedness, call rate (less than or equal to 0.95) and evidence of non-Caucasian descent. SNPs were included in the analyses if they met the following conditions: call rate greater than or equal to 0.98, minor allele frequency greater than or equal to 0.01 and Hardy–Weinberg equilibrium test with P greater than or equal to 0.001[297].

Cognitive data

Table 11 provides an overview of cognitive test batteries available used in each study. There was a substantial overlap between the Scotland based cohorts (AAA, EAS, ET2DS, LBC 21 and LBC 36); the two cohort of populations based in England differed. However, all tests were standardised for the population and purposes they were used for; also the three main cognitive domains were tested in each cohort. Pearson correlation showed a moderate to high correlation between test score in each study separately. Therefore all conditions to conduct principal component analysis

were satisfied. It was conducted in seven steps (one step for one study), this is presented in Chapter 6, Section 6.2.

Table 11: Cognitive tests for which results were available in each collaborating study

	AAA	**EAS**	**ELSA**	**ET2DS**	**LBC 21**	**LBC 36**	**W II**
Memory	AVLT	LM	word list 'PM'	LM Faces LNS	LM	LM LNS	word list
Executive Function	VFT	VFT	semantic fluency	VFT	VFT	VFT	phonemic & semantic fluency
Attention; Mental Flexibility	TMT-B		'literacy'	TMT-B			AH 4-1
Processing speed	DST	DST		DST		DST	
Non-Verbal Reasoning	MR	MR		MR	MR	MR	AH 4-1
Pre-morbid Function	MHVS NART	MHVS	Education level	MHVS	MHT (age 11)	MHT (age 11)	MHVS

AVLT – Auditory Verbal Learning Test; **AH-4** – Alice Heim - 4; **DST** – Digit Symbol Test; **LM** – Logical Memory; **LNS** – Letter Number Sequencing; **MHVS** – Mill Hill Vocabulary Scale; **MHT** – Moray House Test; **MR** – Ravens' Matrices; **NART** – National Adult Reading Test; **TMT – B** – Trial Making Test- B; **VFT** – Verbal Fluency Test

4.5 Data Analyses

The primary aim was to conduct meta-analysis of data collated from the 7 collaborative cohorts in order to test the significance of associations between plasma fibrinogen gene variances (SNPs) and cognitive ability, indexed by general cognitive factor 'g'. Prior to this meta-analysis, a number of consecutive steps were carried out. Firstly, individual associations were modeled in two steps, using a multiple regression analysis:

- Plasma fibrinogen values ~ SNPs (+ age +sex)
- 'g'~ SNPs (+ age + sex)

Age and sex are known to affect frequency of gene variants[183]. A set of linear regressions was therefore conducted before and after the adjustment for age and sex.

Results of these regression analyses, i.e., unstandardized parameter values, standard error and p values for each of the 61 SNP for all collaborating study were further used to conduct a meta-analysis. In the first step, the relationship between plasma fibrinogen and selected SNPs was assessed. Although the nominated SNPs were selected especially for their effect on plasma fibrinogen concentrations, linear regression and meta-analysis were conducted in order to confirm the strength of this relationship. The same approach was taken to investigate the association between 61

selected polymorphisms and the 'g'. All steps of the meta-analyses were conducted using statistical package R 2.1.4.

The meta-analysis technique enables combining results from different studies. Generally, three different approaches to meta-analysis are taken. Systematic reviews quite often depend on data that are extracted from the published trials. This can be rather problematic due to number of factors, such as lack of control over co-ordination of data and analytical processes and bias towards publishing statistically significant results[194]. Alternative approaches are either individual data analysis (IPD) or aggregate patient data (APD)[302]. Both IPD and APD share a scale of strengths and limitations, majority of currently published meta-analyses are conducted on APD, especially in gene-association studies and medical studies. In genetic epidemiology, these approach is particularly efficient, especially when raw dataset for each cohort is available and if the overall number of subjects exceeds 1000[194]. In the data available for the presented meta-analysis, there were number of issues. Most notably, methodological rigor in terms of measurement of the outcome of interest, i.e. choice of neuropsychological tests assessing cognitive ability, lack of control over standardization of data collection and scoring, etc. was not feasible. Additionally, one of the key criterion for selecting the fibrinogen-related SNPs was availability in at least two of the seven collaborating cohort. Many SNPs were present in less than all seven cohorts (appendix E) and this would have introduced a major issue of missing data in the IPD approach. Therefore, the presented meta-analysis was based on aggregate patient data.

Correction for multiple testing

One of the common problems in studies assessing multiple variables is the possibility of 'false positive' when the predictor is claimed to have an effect on the outcome when, if fact this has been a false positive finding (type I error)[278]. In this study, for a 95% confidence level, the maximum probability that we have falsely identified positive association was 0.05. In other words, significant associations between fibrinogen-related SNPs and plasma fibrinogen levels and between fibrinogen-related SNPs and 'g' was set at the nominal level of significance of 0.05, expecting to find 5% false positives.

There are number of ways how the type I error can be controlled, which one of the most common statistical approach is the Bonferroni correction for multiple testing[278]. By dividing the alpha value by the number of associations, the cumulative type I error decreases below the set alpha level. In this study, 61 associations/hypotheses were tested. Bonferroni adjustment, calculated in the formula of 0.05/61 would lower the new critical p value at 0.0008197.

Nevertheless, P-values are incredibly sample-size dependent[278]. Conceptually, if two identical underlying sets of effects are studied by one smaller and one larger study, for the smaller size study the multiple testing correction would eliminate evidence of any effects whereas it but make some impact in the second, larger study, despite the underlying situation is identical. Therefore it can be argued that placing more emphasis on presenting *raw p-values*, then considering the possible impact of multiple testing is more informative. Specifically to the present analysis, genetics

studies often suffer from achieving sufficient power. Unlike any hypothesis-free analysis, such as Genome Wide Association Studies, where the nominal level of p value is set at much lower level, the presented, the presented analysis was hypothesis driven. It used a set of 61 SNPs that have been selected for their documented significant association with the predictor of interest, i.e. plasma fibrinogen levels. Therefore the conducted meta-analysis is a replication stage of a (conceptually) larger process. Thus, the underlying probability of a false positive in this analysis is somewhat reduced in a rather subtle way that isn't really satisfied in standard multi-test methods. Bonferroni adjustment, resulting in p-value modification, has been evaluated and number of issues was pointed out[303]. Specifically, this adjustment is predominantly concerned with rejection of null hypothesis. Artificially modifying probability level can result in misinterpretation of the analysis when true associations are deemed as no significant despite their true association with the outcome of interest (type II error)[303]. Many researchers argue that, especially in genetic-association studies, cconfidence intervals are more informative than p values because they provide a range of values, which is likely to include the true population effect[193]. They also indicate whether a non-significant result is or is not compatible with a true effect that was not detected because the sample size was too small[189,194].

In light of these issues, i.e. hypothesis-driven analyses of pre-selected gene variants and the potential risk of running a type II error, the correction for multiple testing in the present analysis was substituted by an interpretation-based approach, focusing also on confidence interval values. Confidence intervals are more informative than p values because they provide a range of values, which is likely to include the true

population effect. They also indicate whether a non-significant result is or is not compatible with a true effect that was not detected because the sample size was too small.

4.6 Selection of Fibrinogen related Single Nucleotide Polymorphisms

SNPs used in the presented analysis were selected through a systematic literature search for gene-candidate and genome-wide association studies (GWAS) that identified genes and/or SNPs associated with circulating concentrations of plasma fibrinogen. Specific SNPs identified this way, together with tagging SNPs for any genes identified, were considered for inclusion in the final list of SNPs selected for subsequent meta-analysis. Secondary search was also carried out through The National Human Genome Research Institute database for potential additional references.

There is a possibility off non-random association of alleles located on different loci within the same chromosome; those associations can be calculated via measurement of linkage disequilibrium (LD)[304]. If LD is high, one way to explore genetic information across a given region or gene is to use a tagging SNP[8]. To identify fibrinogen-related tagging SNPs, The International Human Genome Project (HapMap) was searched, using the gene list derived from the literature search.

The final list of fibrinogen related SNPs and tagging SNPs that guided further stages of the genetic meta-analysis is presented as Appendix E.

4.6.1 Systematic Literature Search

The objective of the systematic literature search was to identify published articles in which gene variants had been found to be associated with circulating levels of fibrinogen.

The inclusion criterion for articles was the description of any human study which attempted to establish an association between plasma fibrinogen concentration and genetic variance (including gene candidate studies, GWAS and review articles). This allowed for the ultimate comprehensive selection of all published fibrinogen-related SNPs. Once articles have been identified, information on gene/SNPs which were reported as being statistically significantly associated with plasma fibrinogen levels (according to the criteria of that individual study) was extracted. Due to widely under-reported effect size in the majority of studies, the minimum effect size criterion for SNP-fibrinogen associations was omitted.

Search of Medline, Embase and PubMed

The primary search for relevant studies was carried out through the electronic databases Medline, EMBASE and PuBMed; search was run in parallel with adjusted terminology with 'mesh' terms (MEDLINE) equalled 'emtree' terms (EMBASE). 'Thesaurus' terms were adjusted accordingly to a particular database. Reference lists of retrieved articles were reviewed to identify any additional relevant studies. The

three types of studies that were used for to review according and according to inclusion criteria were:

- Gene-candidate studies
- Genome-Wide Association studies (GWAS)
- Review articles

Medline search strategy (1946 to May Week 4 2011)*

- exp Fibrinogen/ (78693)
- fibrin$.ti,ab. (185090)
- or 2 (207666)
- exp genetic variation/ (1024123)
- (gene$ adj3 varia$).ti,ab. (182571)
- genetic markers.mp. or genetic marker/ (89638)
- genes.mp. or gene/ (1775031)
- exp Polymorphism, Genetic/ (495502)
- (nucleotide adj3 polymorphism).ti,ab. (34449)
- (gene$ adj3 (epid$ or caus$ or determine$ or impact$)).ti,ab. (196855)
- SNP.ti,ab. (77591)
- (genetic adj3 marker).ti,ab. (9805)
- or/4-12 (2845630)
- 3 and 13 (9130)

*terms adjusted according to a particular database search terms

Inclusion criteria:

Research articles were included in they complied with the following criteria

- investigation of plasma fibrinogen concentration related genes/genetic variants
- human studies
- abstract available for an initial inspection
- were published in English language
- multiple publication of one study (in such cases, identified gene variance were assessed for a repletion with already documented evidence)

4.6.2 Search of the National Human Genome Research Institute database

To identify research articles which may have not been identified through the literature search of Medline, EMBASE and PubMed, The National Human Genome Research Institute (NHGRI) database was accessed. The search of NHGRI was guided by the list of all pre-identified genes that had been found through the literature search and implicated in altered plasma fibrinogen concentrations. However, after removing duplicates extracted from the above search, this yielded no additional articles.

4.6.3 Search of the International Human Genome Project (HapMap)

The primary aim of the HapMap organization is the sequencing of the human genome; the focus is on a broad range of studies concerned with the role of the human genome in health and disease[199]. For the present study, a HapMap search was undertaken for tagging SNPs in all genes pre-identified in the literature search (supplemented by information on genes implicated in altered plasma fibrinogen levels from experts working in the field) according to the criteria presented in the table 12:

Table 12: Parameters for tagging SNPs selection

Population	CEU	
Buffer zone	+/- 10 kb	where necessary (to get the full gene plus additional sequence on each side) this was adjusted accordingly
Pairwise method	Tagger Pairwise	
RSquared cut off	0.9	Altered in number of SNPs unhealthy either way
MAF cut-off	0.5	Altered in number of SNPs unhealthy either way

4.6.4 Results of search for fibrinogen relevant SNPs

The presented search identified 27 fibrinogen relevant genes, 131 fibrinogen-relevant SNPs and 530 fibrinogen relevant tagging SNPs.

Of the three fibrinogen genes themselves, the most studied was found to be the beta chain (FGB) polymorphisms, presumably due to evidence from in vitro research[213]. Variants in genes for alpha and gamma chains are also known to affect fibrinogen synthesis and production, albeit investigation into these was found to be less frequent. Several different polymorphisms were identified in each chain.

In addition to SNPs located within the fibrinogen genes themselves, the search also identified a number of polymorphisms located out-with the fibrinogen genes; these genetic variants were also included. There was a very small number of SNPs that were listed in other than 'rs' number format; to identify those, the 'dbSNP' database was utilized. This resolved all discrepancies in terminology, thus all SNPs were presented in the traditional format. Results of the search for SNPs are presented in Figure 7. The final list of fibrinogen related SNPs and tagging SNPs that were identified from the studies found in the literature search and from HapMap is presented as Appendix E.

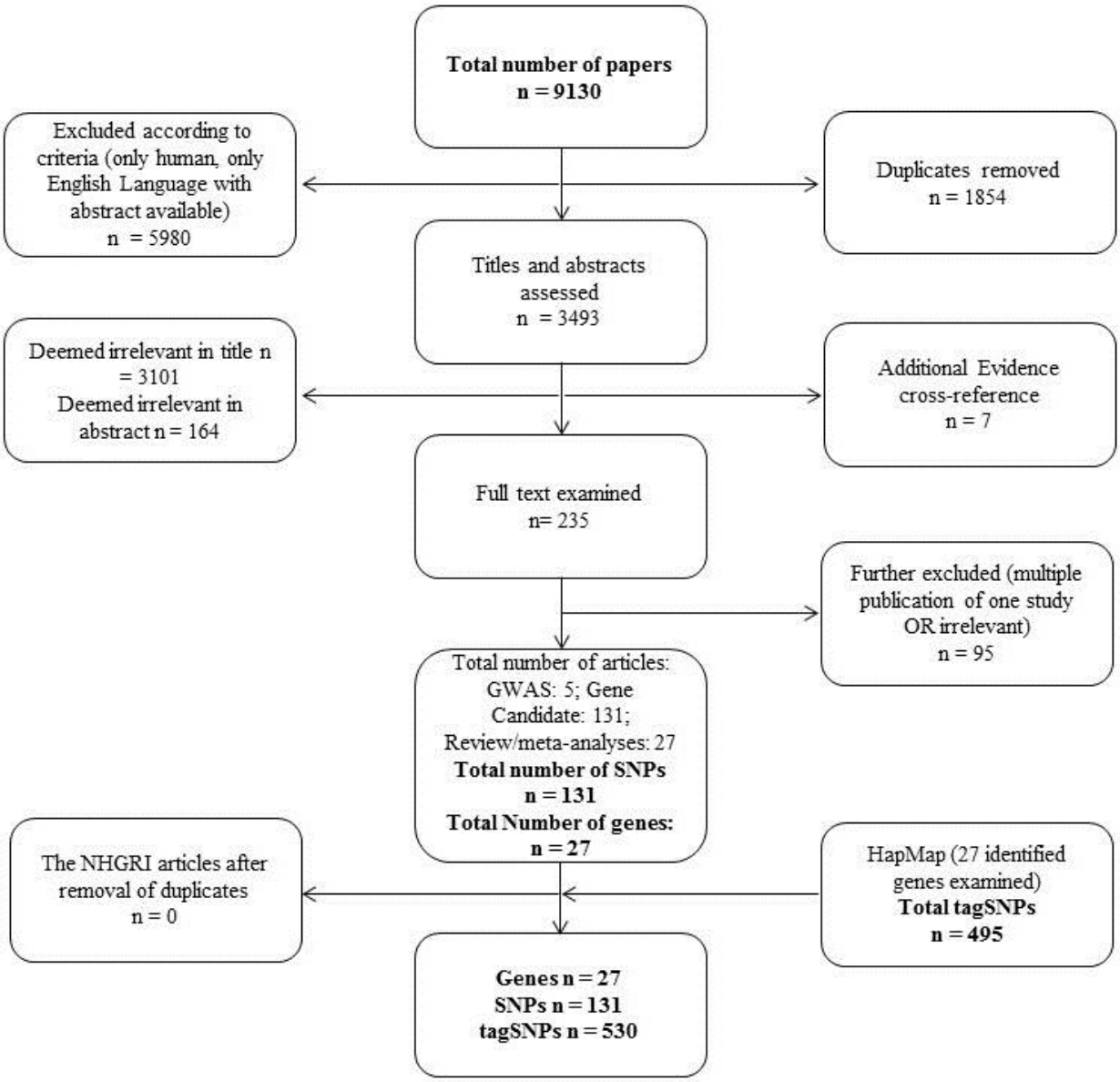


Figure 7: Results of fibrinogen relevant SNPs

4.6.5 Final stage of SNP selection

A list of SNPs as outlined above was sent to all collaborative centers with a request form to identify and forward all SNPs that were genotyped for each cohort.

After all files were converted to a mutually compatible format, further selection was carried out; each SNP was checked for occurrence in the remaining cohorts. SNPs were excluded if they occurred in 2 or fewer cohorts only. This resulted in further reduction, excluding 234 SNPs, bringing the total number of fibrinogen related SNPs to 61 SNPs.

Chapter 5: Results: Association of inflammatory markers with cognition in the follow up phase of the ET2DS

The chapter is concerned with the ET2DS. It begins with a description of the general baseline characteristics of the ET2DS cohort. Comparison between baseline characteristics of subjects who did and did not attend the follow up clinic is presented. This is followed by reporting bivariate correlation results, illustrating relationships between unadjusted (raw) data. Multivariate linear regression analyses form the last part of this section. It presents a set of models, first assessing association between four inflammatory markers and cognitive performance at the follow up wave only, i.e. cognitive impairment predicted by baseline inflammatory markers. Further adjustment for respective baseline scores provides an estimated 4-year cognitive change; adjustment for MHVS, test of a peak pre-morbid cognitive functioning, provides an estimated life-time cognitive change. Lastly, conventional cardiovascular risk factors and events were controlled for in order to assess the role of these in the inflammation-cognition relationship in people with T2DM.

All analyses were undertaken by myself, except for the imputation of missing cognitive data which was undertaken by the ET2DS team; imputed data were used in order remain consistent across multiple analyses on different topics conducted by the ET2DS researchers.

A summary of baseline measures of assessed risk factors and potentially confounding variables and baseline cognitive scores summary, available for both attenders and non-attenders of the follow up wave, are provided in table 13 and table 14, respectively. This allows for a direct comparison between these sub-groups and for an assessment of potential survival bias. A comparison of baseline and follow up cognitive scores specific to the *attenders* sub-sample is presented in Table 15.

In comparison to the entire ET2DS population, there were some, though predominantly modest, observed differences in clinical and demographic values of subjects cognitively tested at the follow up wave (*attenders)*. In percentile representation, gender representation remained approximately same between both groups; however, on average *attenders* were 1 year younger and generally healthier. They presented with consistently lower levels of plasma circulating biomarkers; the acute phase proteins, plasma fibrinogen and CRP and the cytokine IL-6 significantly lower (except for TNF-α levels). In terms of cardiovascular risk factors, systolic blood pressure and the mean reading of cholesterol level were significantly higher in the *non-attenders* group albeit still within the normal physiological range.

The same pattern was detected in diagnosed cardio-vascular disease history; *attenders* generally reported a lesser prevalence of cardiovascular events, with the exception of TIA. A*ttenders* were more likely to be either never or ex-smokers (ceased at least 6 months prior the clinic) and had a lower consumption of alcohol. *Attenders* also presented with a significantly lower score of depression and anxiety.

In terms of years spent in formal education and in the social deprivation status (SIMD), the observed pattern indicated (in percentage) that *attenders* generally had obtained higher educational attainment and there was a trend toward a higher rank in their social deprivation index, compared to *non-attenders* of the follow-up wave.

Table 13: Baseline characteristics of the ET2DS population

	Attenders (n=831)		Non-attenders (n=235)		
	Total Number	**Mean ± SD,** median (quartile range) or n (%)	**Total Number**	**Mean ± SD,** median (quartile range) or n (%)	**p-value for difference or trend**
Age (years)	831	67.69 ± 4.16	235	68.71 ± 4.27	0.001
Male sex	831	430 (51.7)	235	117 (49.8)	0.596
Duration of diabetes (years)	824	6 (3 – 11)	229	7 (3 – 12)	0.196
Current treatment					
Insulin +/-tablets	830	139 (16.7)	235	47 (20.0)	0.281
Tablets only	830	526 (63.4)	235	153 (64.8)	
Diet only	830	165 (19.9)	235	35 (14.9)	
HbA1c (%)	804	7.39± 1.13	224	7.41± 1.08	0.872
Plasma glucose	821	7.54 ± 2.09	228	7.66± 2.13	0.425
Plasma fibrinogen	828	3.61± 0.75	235	3.78 ± 0.83	0.001
CRP*	817	3.63	226	5.49	0.001
IL-6*	829	3.73	235	4.61	0.048
TNF-alpha*	828	1.33	238	1.46	0.535
Total cholesterol	826	4.34± 0.90	231	4.23 ± 0.91	0.100
Systolic BP (mmHg)	829	132.51± 15.87	235	136.09 ± 18.09	0.006
Diastolic BP (mmHg)	829	68.95±8.87	235	69.44 ± 9.50	0.457
BMI (kg/m^2)	831	31.29 ± 5.59	234	31.93 6.03	0.130
Macrovascular disease					
MI	831	111 (13.4)	235	39 (16.6)	0.207
Angina	831	222 (26.7)	235	76 (32.3)	0.090
Stroke	831	44 (5.3)	235	18 (7.7)	0.171
TIA	831	27 (3.2)	235	4 (1.7)	0.213
PAD	831	53 (6.4)	235	12 (5.1)	0.472
Any CVD	831	293 (35.3)	235	100 (42.2)	0.041
Alcohol					
Never	200	25.5	86	36.6	0.002
Light drinker (<7)	386	46.5	88	37.4	
Moderate drinker (7-15)	118	14.2	23	9.8	
Heavy drinker (>15)	84	10.1	20	8.5	
Smoking					
Current smoker	831	108 (13.0)	235	45 (19.1)	0.042
Ex-smoker	831	390 (46.9)	235	109 (46.4)	
Never smoked	831	333 (40.1)	235	81 (34.5)	

	Attenders (n=831)		Non-attenders (n=235)		
	Total Number	**Mean ± SD,** median (quartile range) or n (%)	**Total Number**	**Mean ± SD,** median (quartile range) or n (%)	**p-value for difference or trend**
Education					
University degree	831	145 (17.4)	235	26 (11.1)	
Professional qualification	831	239 (28.8)	235	68 (28.9)	0.110
Secondary school	831	442 (53.2)	235	139 (59.1)	
Primary school	831	5 (0.6)	235	2 (0.9)	
SIMD rank					
Least deprived	831	99 (11.9)	235	28 (11.9)	
Less deprived	831	143 (17.2)	235	65 (27.7)	<0.001
Deprived	831	143 (17.2)	235	45 (19.1)	
More deprived	831	146 (17.6)	235	48 (20.4)	
Most deprived	831	300 (36.1)	235	49 (20.9)	
HADS anxiety	831	5 (3 – 8)	234	6 (4 – 9)	<0.001
HADS depression	831	3 (1 – 5)	234	4 (2 – 6)	0.002

Values are mean (standard deviation), median (interquartile range)* or percentage**

HbA1c - glycated haemoglobin; **CRP** – C-reactive Protein; **IL-6** – Interleukin-6; **TNF-alpha** – Tumour Necrosis Alpha; **BMI** – Body Mass Index; **SIMD** – Scottish Index for Multiple Deprivation; **HADS (A)** – Hospital Anxiety Depression Scale (Anxiety); HADS (D) – Hospital Anxiety Depression Scale (Depression)

Cognitively, *attenders* presented with significantly higher baseline cognitive scores in all subtests as well as in terms of the general intelligence score '*g*'; they were also less likely to progress towards a diagnosis of dementia; Table 14 summarises individual data. This again suggests a possibility of a methodological issue common to longitudinal design, that the physically and cognitively healthier subjects were more likely to engage in the follow up wave of the prospective study[252].

Table 14: Comparison of cognitive baseline scores (Attenders & Non-Attenders)

	Attenders	**(N = 828)**	**Non-Attenders (N = 238)**		*Difference: Number*	*Range*
	Total (N)	*Mean (SD)*	*Total (N)*	*Mean (SD)*	*p value*	
MMSE	830	28.47 (1.6)	233	27.7 (2.5)	0.77 (< .000)	0 – 30
MMSE<24	830	13 (1.6)	233	17 (7.3)	4 (< .001)	0 – 30
MHVS	820	31.45 (5.1)	229	29.06 (5.4)	2.39 (< .000)	0 – xxx
LM	822	25.86 (7.9)	28	23.02 (8.6)	2.84 (< .000)	0 – 50
Faces	827	66.34 (7.7)	232	63.9 (8.4)	2.44 (< .000)	0 – 94
BVFT	828	37.77 (12.5)	232	33.88 (13.4)	3.89 (< .000)	ad infinitum
MR	822	13.36 (5.2)	230	10.86 (5)	2.5 (< .000)	0 - 26
DST	828	50.42 (14.3)	229	44.65 (15.5)	5.77 (< .000)	0 - 93
LNS	822	9.93 (2.7)	226	8.7 (2.9)	1.23 (< .000)	0 - 21
TMT-B*	823	101 (79-132)	229	140.76 (75.1)	- 45.76 (<.000)	in seconds
'g'	804	.12 (.93)	217	-.44 (1.1)	- .32 (< .001)	N/A
Dementia Diagnosis	831	4 (0.5%)	235	15 (6.4%)	N/A	N/A

Values are mean (standard deviation), median (interquartile range)* or percentage**

MMSE – Mini Mental State Examination; MMSE < 24 – score indicating early dementia onset; **MHVS** – Mill Hill Vocabulary Scale; **LM** – Logical Memory (combined immediate and delayed recall); **Faces** – Faces recognition (combined immediate and delayed recall); **BVFT** – Borkowski Verbal Fluency Test; **MR** – Raven's Matrices; **DST** – Digit Symbol Test; **LNS** – Letter – Number – Sequencing; **TMT**-B – Trial Making Test – B; **HADS (A)** – Hospital Anxiety Depression Scale (Anxiety); **HADS (D)** – Hospital Anxiety Depression Scale (Depression); Diagnosis of Dementia as confirmed by GP notes or Information Services Division (ISD)

Table 15 provides bivariate correlations between the baseline and follow up cognitive scores of *attenders*. Results indicate moderate to high correlation between

those two-time scores (r = 0.542 to r = 0.871; p < 0.001). The strongest relationship was observed in MHVS scores (r = 0.87; p < 0.001); this highly stable result was expected as it supports the notion of 'crystallised' ability, assessed by a vocabulary test, remaining relatively insensitive to the effect of ageing[17]. Mean scores of LM and Faces improved significantly, suggesting a possibility of a practice effect[232]. The remaining mean scores declined significantly, suggesting an overall cognitive decline.

Table 15: Baseline and Follow-Up cognitive performance of 'Attenders'

	Baseline Scores		**Follow-up Scores**	
	Number	Mean (SD)	Number	Mean (SD)
MHVS	820	31.46 (5.07)	818	30.71 (4.94)
'g'	827	0.17 (0.93)	823	0.01 (1.00)
BVFT	827	37.81 (12.52)	826	36.83 (12.75)
DST	828	50.43 (14.34)	823	50.01 (14.12)
TMT-B*	827	101.49 (1.44)	823	111.05 (1.51)
MR	828	13.36 (5.23)	825	11.5 (5.22)
LNS	827	9.92 (2.66)	824	8.86 (2.89)
LM	827	25.83 (7.97)	825	27.27 (8.25)
Faces	827	66.37 (7.65)	825	69.25 (8.38)
MMSE	827	28.48 (1.63)	824	28.33 (1.77)

*Means of TMT-B are geometric means, number indicates seconds needed to complete the task

** Significant at the p < 0.001 level

Lastly, inter-correlations between baseline cognitive test scores were assessed. All cognitive scores and *g* correlated significantly with all cognitive scores. Some of the strongest correlations were detected in tests assessing speed of information processing, TMT-B, DST. Unlike in the other test where the higher, positive value

represents better cognitive performance, the negative values in TMT-B results represents a worse performance (the higher number, the longer it takes to complete the test). Relatively weaker correlations were found for measures of verbal (LM) and non-verbal (Faces) memory. All values are presented in Table 16 below.

In terms of the follow up cognitive scores, the pattern seems to mirror the baseline correlations; whereas the weakest correlations were observed in tests assessing verbal and non-verbal memory (LM and Faces, respectively), the strongest values were detected in tests assessing speed of information processing (TMT-B and DST). All values are presented in Table 17, below.

Table 16: Bivariate correlations between baseline cognitive test scores

	LM	Faces	MR	DST	TMT-B	LNS	BVFT	*g**
MHVS	0.38	0.28	0.45	0.37	-0.37	0.40	0.44	0.28
LM	---	0.24	0.28	0.27	-0.28	0.31	0.25	0.54
Faces		---	0.24	0.29	-0.26	0.20	0.22	0.47
MR			---	0.38	-0.46	0.40	0.36	0.67
DST				---	-.063	0.40	0.40	0.75
TMT-B					---	-0.50	-0.39	0.80
LNS						---	0.46	0.72
BVFT							---	0.67

Table 17: Bivariate correlations between four years follow-up cognitive test scores

	LM	Faces	MR	DST	TMT-B	LNS	BVFT	*g**
MHVS	0.43	0.29	0.45	0.34	-0.33	0.40	0.42	0.55

	LM	Faces	MR	DST	TMT-B	LNS	BVFT	*g**
LM	---	0.26	0.32	0.37	-0.38	0.37	0.41	0.61
Faces		---	0.29	0.29	-0.31	0.22	0.27	0.51
MR			---	0.44	-0.48	0.46	0.29	0.69
DST				---	-.065	0.47	0.41	0.79
TMT-B					---	-0.53	-0.37	0.81
LNS						---	0.41	0.75
BVFT							---	0.62

RELEVANT TO TABLE 16 & 17: *Values for associations of g with the seven cognitive tests (except MHVS) are factor loadings on basis of imputed cognitive test data. Values for all individual cognitive tests are correlation coefficients from two-tailed Pearson correlations, $p<0.001$. MHVS, Mill-Hill Vocabulary Scale; LM, Logical Memory; MR, Matrix Reasoning; DSC, Digit Symbol Coding; TMT-B, Trail-Making- Test B; LNS, Letter Number Sequencing; BVFT, Borkowski Verbal Fluency Test

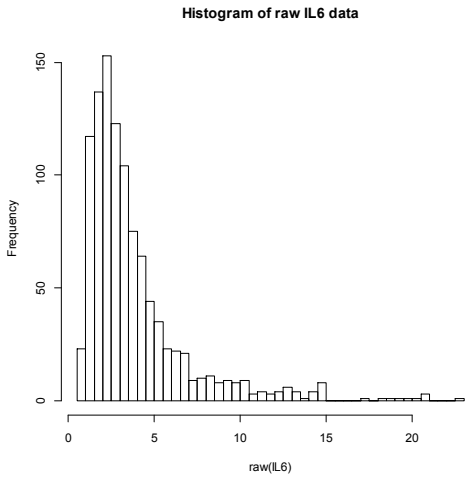
5.1.1 Model assumption

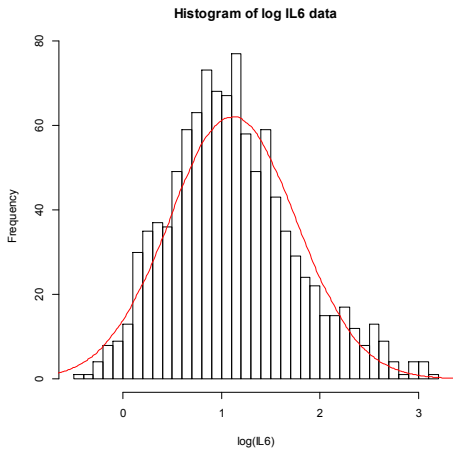
For the purpose of the multiple linear regression analyses, data were explored and inspected for normality, linearity and homoscedasticity.

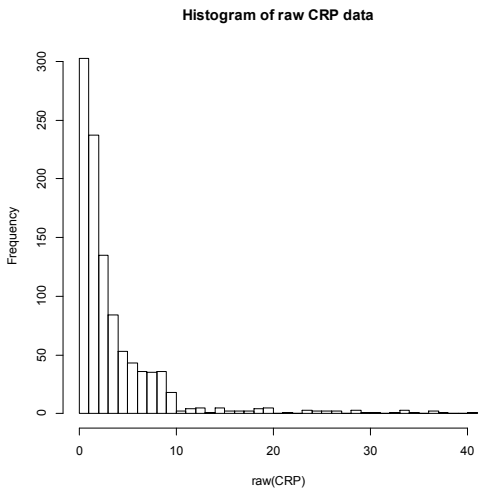
5.1.1.1 Data distribution

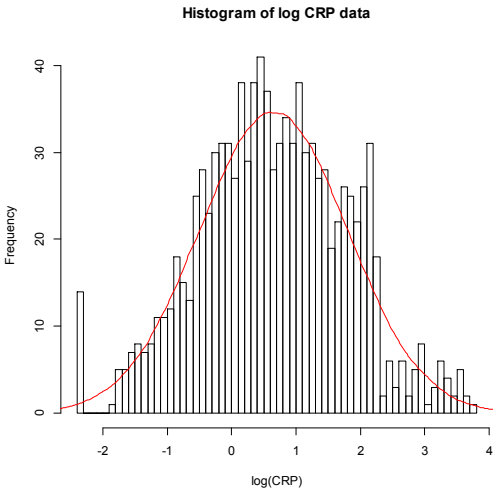
The distribution of all continuous variables were compared to the Normal distribution, both numerically and visually via histograms and Q-Q plots; obvious outliers were checked against a paper record and consequently either corrected or removed. All variables that presented as skewed (IL-6, CRP, TNF-alpha, TMT-B, smoking (N of packs/year), all right-skewed) were transformed to their natural logarithm. For those variables, medians and inter-quartile ranges for the raw values are presented. Histograms and Q-Q plots for these variables before and after log transformation are presented in Figure 8 below.

The remaining variables, deemed as normally distributed, were treated as continuous variables and summarized with means and standard deviations values along with p-values for difference between the two sub-samples of the baseline cohort (*attenders* and *non-attenders*). All categorical variables are reported as frequency distribution in total number and percentage.









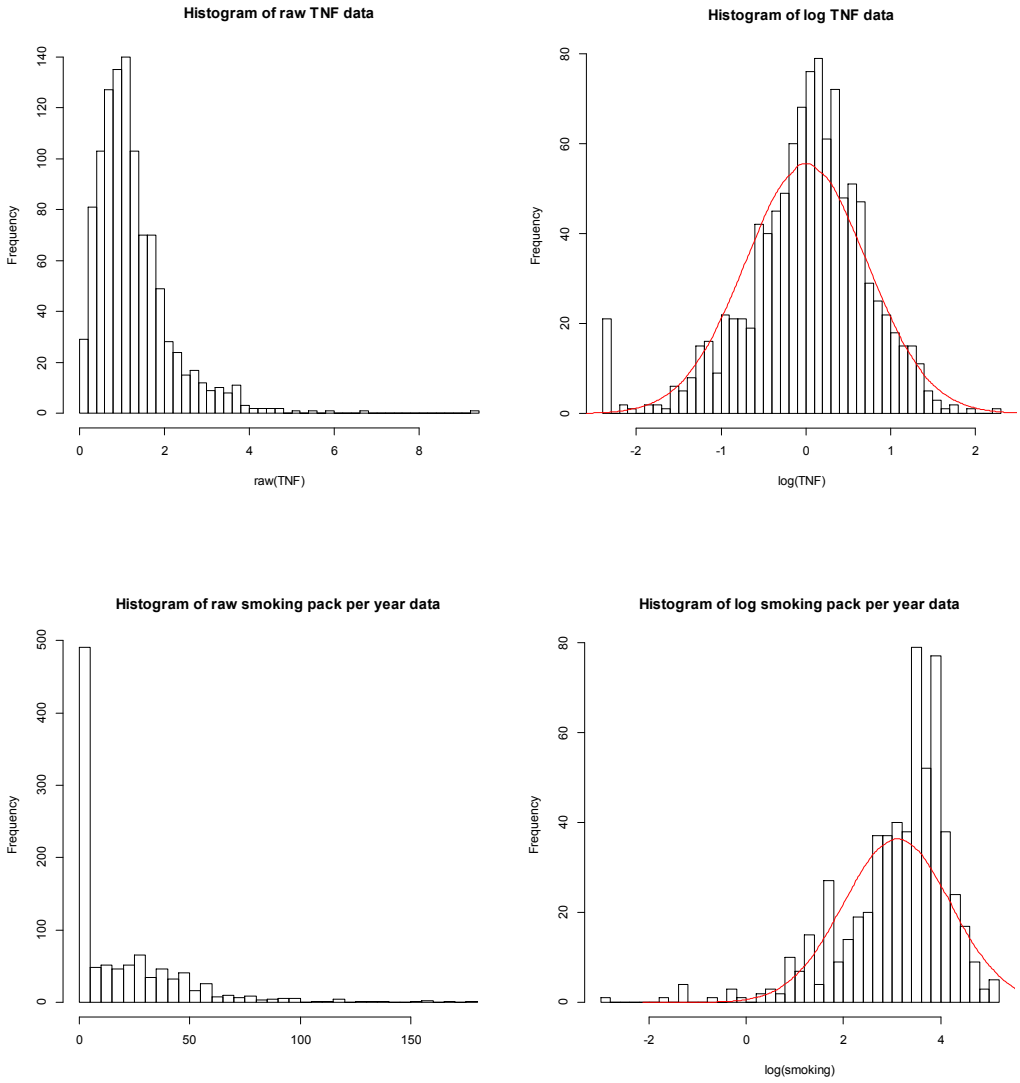


Figure 8: Histograms of the raw and natural log-transformed

5.1.1.2 Associations of dependent variables and potential confounders

A perfect (or near-perfect) linear relationship between two or more predictors/covariates may present the problem of collinearity, therefore a series of bivariate (Pearson) correlations were calculated to assess the relationship between all cognitive scores and potential confounding variables. One the major advantages of

this study was the large sample size that allowed for sufficient power to detect even small correlation coefficients. There was not a particularly large coefficient between predictors and/or covariates, ranging r= -.010 (cholesterol – smoking) to r= .521 (fibrinogen –C-reactive protein). In terms of correlation between predictors/covariates and cognitive scores, Pearson correlations on raw data were conducted for baseline cognitive scores and for follow up cognitive scores. All baseline correlation coefficients were reasonably small (ranged r= -.002 (Fib – LM) to r= .182, $p<0.01$ (IL6 – TMT). Equally, follow up cognitive test scores correlated with baseline predictive and confounding variables, with the lowest value r= -.010 (T2DM – LM) to the highest, r =0.148, $p<0.01$ (alcohol – MR). Overall, all correlation values ranged from low to moderate, suggesting that collinearity was unlikely to present in later analyses.

5.1.1.3 Homoscedasticity

Homoscedasticity is an assumption tested in multiple regressions. It predicts that at each level of the predictor value, the variance of the residual remains constant. Conventionally, this is examined visually via scatterplots and histograms where the residuals associated with the dependent variable shows what variance is left after accounting for independent variables. A scatterplot, showing the regressed standardised residual on to the standardised predictor value, needs to be visually examined. If the plot resembles a bird nest or a funnel plot, it can be assumed the presence of heteroscedasticity, meaning less accurate estimates when the values of

the dependent variable are increasing. In this study, based on a visual inspection of histograms and scatterplots, it was concluded that this assumption was not violated.

5.1.1.4 Removal of subjects with extreme inflammatory marker values, adjustment for mood status

Acute inflammation might bias the results between plasma biomarker levels and cognitive scores. To discount this, the dataset was also examined for the presence of extreme values in inflammatory markers. Biomarker levels detected above 1.5 interquartile range thresholds were deemed as indicative of an acute inflammation; all values above this level were regarded as being indicative of acute inflammation and abnormally large values may have obscured any potential relationships. However, this was detected only sporadically (4.7%). Analyses were repeated after exclusion of subjects with extreme measure and this made no significant change to the overall results that remained similar.

In terms of adjustment for mood evaluation scores, evidence indicates an association between prolonged depressive mood and accelerated cognitive decline in elderly people[305] and depressive mood and elevated inflammatory markers[306]. In the present study, adjustment for HADS scores, a validated measure of anxiety and depression status, resulted only in marginal alterations to a significance level in global cognitive function 'g'; no noticeable change was detected in analyses assessing individual tests. Table 18 shows standardised parameters, standard error values and significance values for '*g*' scores modeled as described above (*5.1.7.4 Multifactorial analyses*)

with HADS scores as covariates in following sequence: anxiety score, depression score and scores anxiety and depression scores in the final model.

Table 18: Inflammatory markers and 'g' adjusted for Hospital Anxiety and Depression Score

	Fibrinogen	C-Reactive Protein	Interleukin – 6	TNF - α	Inflammatory Factor
4-year change[1]	-.109 (.047)*	-.126 (.051)*	-.145 (.050)**	-.085 (.049)	-.194 (.051)***
Life-time change[1]	-.073 (.038)	-.068 (.042)	-.101 (.041)*	-.045 (.039)	-.126 (.042)**
4-year change[2]	-.105 (.046)*	-.117 (.051)*	-.138 (.049)**	-.083 (.048)	-.186 (.051)***
Life-time change[2]	-.067 (.033)	-.053 (.042)	-.090 (.040)*	-.043 (.039)	-.111 (.041)**
4-year change[2]	-.105 (.047)*	-.118 (.052)*	-.0139 (.050)**	-.083 (.049)	-.189 (.052)***
Life-time change[2]	-.066 (.038)	-.052 (.042)	-.089 (.041)*	-.041 (.039)	-.112 (.042)**

[1] Fully adjusted model (as above) + HADS (Anxiety score)

[2] Fully adjusted model (as above) + HADS (Depression score)

[3] Fully adjusted model (as above) + HADS (Anxiety & Depression scores)

5.1.2 Multiple regressions – baseline marker levels and follow-up (adjusted) cognitive results

Multiple linear regression analysis allows exploring an effect that 2 or more predictors (either continuous or categorical) have on a dependent variable, presented as a continuous outcome[278]. Given the type of data collected for the presented study, it was deemed as the most suitable analytical approach to determine strength of predictive value of inflammatory markers on a 4-year cognitive change and life-time cognitive change. At first, series of age and sex adjusted models were fitted to assess relationships between baseline inflammatory markers and follow up cognition; these associations were analyzed further through a series of linear regressions when either 4-year cognitive change (adjusted for respective baseline scores) or life-time estimated change (adjusted for MHVS score) was modeled. Analyses were repeated with cardiovascular risk factors and events as covariates. These were the baseline measures of: total cholesterol; myocardial infarction; angina; stroke; transient ischaemic attack; smoking; alcohol; HbA1c; duration of type 2 diabetes. Finally, all biomarkers and inflammatory factor predictive values were assessed in models adjusted for anxiety and depression (HADS) scores.

Individual biomarkers regression results

Plasma fibrinogen regression results:

Plasma fibrinogen level was significantly associated with a follow up decline in 'g' ($\beta = -0.114$; $p<0.001$), 4-year cognitive change ($\beta = -0.108$; $p<0.01$) and a life-time change ($\beta = -0.084$; $p<0.01$) and independently of risk factors at 4-years ($\beta = -0.093$; $p<0.05$) and life-time cognitive change ($\beta = - 0.066$; $p<0.05$). Follow up cognitive impairment across cognitive domains was detected in most cognitive scores,

standardised coefficient ranged between -0.073 to -0.113; $p<0.05$ to $p<0.001$); except for LM ($\beta = -0.042$; $p=0.215$) and Faces ($\beta = -0.52$; $p=0.141$).

After an adjustment for the respective baseline scores, the significant association remained at same level in Raven's Matrices ($\beta = -0.036$; $p<0.05$), BVFT ($\beta = -0.049$; $p<0.05$) and LNS ($\beta = -0.097$; $p<0.01$) scores. Significance of association decreased in DST scores ($\beta = -0.076$; $p<0.01$) and in TMT-B score ($\beta = 0.03$; $p<0.05$)

Life-time cognitive decline, as compared to the 4-year change analyses, showed a similar pattern. Fibrinogen predicted the same level of cognitive decline in DST ($\beta = -0.093$; $p<0.01$) and in TMT-B ($\beta = 0.074$; $p <0.05$) and slightly weakened in LNS ($\beta = -0.072$; $p<0.05$). Significance of association vanished in BVFT ($\beta = -0.049$; $p=0.124$) and in Raven's Matrices ($\beta = -0.051$; $p=0.175$).

Adjustment for covariates – as indicated in the preceding paragraph, weakened the magnitude and statistical significance of most associations; irrespective of other factors, fibrinogen was a significant predictor of a decline in DST both in terms of 4-year change ($\beta = -0.075$; $p<0.05$) and life-time change ($\beta = -0.071$; $p<0.05$), decline in LNS at 4 year decline ($\beta = -0.069$; $p<0.05$) and life-time decline ($\beta = -0.060$; $p<0.05$) and TMT-B at life-time decline ($\beta = 0.097$; $p<0.05$). Overall, the most effect of the plasma fibrinogen levels was detected in general cognitive ability, followed by performance on DST, LNS and TMT-B. No significant association was observed either LM or Faces scores.

Plasma CRP regression results:

Plasma CRP levels significantly predicted decline in general cognitive ability across the whole spectrum: follow up 'g' ($\beta = -0.123$; $p<0.001$), 4-year change ($\beta = -0.131$; $p<0.001$) and independently of covariates ($\beta = -0.105$; $p<0.05$). In terms of life-time ability, CRP predicted decline ($\beta = -0.084$; $p<0.01$); however, this significance vanished, albeit at borderline value, when adjusted for covariates ($\beta = -0.069$; $p=0.006$). In terms of the individual, follow up individual scores, CRP strongly affected performance in follow up DST ($\beta = -0.127$; $p<0.001$), slightly less so in TMT-B ($\beta = 0.102$; $p<0.01$) and LNS ($\beta = -0.090$; $p<0.01$) and marginally in Ravens Matrices ($\beta = -0.084$; $p<0.05$) and BVFT ($\beta = -0.074$; $p<0.05$).

Same level of significance was observed in 4-year cognitive change scores of DST ($\beta = -0.093$; $p<0.001$) and LNS and TMT-B ($\beta = 0.076$; $\beta = -0.074$; $p<0.05$, respectively). However, in models adjusted for respective baseline measure, significance has vanished in Raven's Matrices ($\beta = -0.043$; $p=0.138$) and BVFT ($\beta = -0.029$; $p=0.249$). Life-time estimated cognitive decline was observed in DST score ($\beta = -0.092$; $p<0.01$) and TMT-B score ($\beta = 0.078$; $p<0.05$) but no other significant association was found.

Independently of cardiovascular conditions, CRP levels was associated with 4-year decline in DST, LNS and TMT-B ($\beta = -0.066$; $\beta = -0.059$; $\beta = -0.059$, $p< 0.05$, respectively).

When the model was set to assess associations between baseline CRP levels and life-time change, independent of cardiovascular risk factors and events, only significant

relationship was observed in DST (β = -0.061) and TMT-B performance (β = 0.070; p<0.05); no other significant prediction was detected.

As with the plasma fibrinogen levels, the CRP levels did not predict any significant decline in LM and Faces scores.

Interleukin – 6 regression results

Interleukin-6 proved to be the strongest predictor across domains, independently of covariates. It strongly predicted a follow up decline in 'g' (β = -0.195; p<0.001), 4-year change (β = -0.163; p<0.001), life-time cognitive change (β = -0.153; p<0.001). Comprehensive adjustment weakened these associations a little at 4-year cognitive decline (β = -0.112; p<0.01) as well as a life-time decline (β = -0.121; p<0.01).

Compared to plasma fibrinogen and CRP levels, IL-6 significantly predicted a follow up cognitive impairment across all domains with standardised coefficient ranging from -0.083 to 0.137; p values <0.001 to <0.05. This association was maintained in terms of 4-year change (range β = -0.031 to -0.137; p values <0.001 to <0.05), except for the TMT-B (β = 0.039; p=0.166). Life-time cognitive change analyses revealed statistically significant association for all tests with standardized coefficients ranging from -0.086 to -0.140; p values <0.001 to <0.01, except for LM score (β = -0.147; p=0.453).

Full adjustment of the 4-year cognitive scores revealed a sufficient prediction of decline in DST score (β = -0.089; p<0.01), MR (β = -0.075; p<0.01) and LNS score (β = -0.10; p<0.01) and slightly in Faces scores (β = -0.056; p<0.05). In a fully-

adjusted model assessing a life-time cognitive change, the significance of association was slightly remained of a similar magnitude; it was detectable in DST score (β = -0.109; p<0.01), LNS score (β = -0.102; p<0.01), Raven's Matrices (β = -0.102; p<0.01) and TMT-B (β = -0.089; p<0.01). Slightly weaker relationship was detected between IL-6 levels and Faces score (β = -0.069; p<0.06).

TNF - α regression results

In comparison to the above results, level of TNF - α proved to be the weakest predictor. In terms of general cognitive ability, strong significant decline was detected at the follow up model (β = -0.116; p<0.001). However, the remaining analyses revealed only marginal significance at 4-year cognitive change (β = -0.069; p<0.05), life-time cognitive change (β = -0.066; p<0.05) and, independently of risk factors and events at 4-year follow up measure (β = -0.046; p<0.05).

However, no relationship was found between this biomarker and fully adjusted the life-time cognitive decline (β = -0.036; p=0.95).

In terms of the follow up scores, significance ranged from Faces (β =-0.111; p<0.01), MR (β =-0.103; p<0.01), TMT-B (β =0.089; p<0.01) and weaker for DST (β =-0.081; p<0.05), BVFT (β =-0.074; p<0.05) and LNS (β =-0.073; p<0.05). No significant association were found in LM scores. In comparison to other markers, TNF - α most strongly predicted decline in non-verbal memory assessed by Faces; this relationship is maintained across the spectrum; 4-year cognitive decline (β = -0.065, p<0.05), even when fully adjusted (β =-0.062; p<0.05) and life-time cognitive

decline (β =-0.084; p<0.05), independently of CVD factors and events (β =-0.079; p<0.05).

Apart from these associations, TNF - α was a predictor of a 4-year decline in DST (β =-0.068; p<0.01), independently of adjusted factors (β =-0.064; p<0.01) and a marginal significant predictor of life-time decline in MR (β =-0.605; p<0.05) and of a 4-year decline in LNS score (β =-0.042; p<0.05).

Otherwise no significant relationships were observed in regards to TNF - α levels. Overall, compare to other investigated biomarkers TNF-α, showed the weakest effect on cognitive performance, with the exception of non-verbal memory.

General inflammatory factor regression results:

General inflammatory factor, a component derived from levels of four biomarkers, showed a similar predictive pattern as each marker individually; especially, it was close to identical with the significance pattern detected in IL6 levels.

The strongest associations were detected in follow up '*g*' (β =-0.197; p<0.001), 4-year longitudinally (β =-0.193; p<0.001) and in terms of life time change (β =-0.137; p<0.001). Full adjustment did not affect these associations in 4-year decline model (β =-0.136; p<0.001) or life-time change model (β =-0.119; p<0.001). In general, follow up models showed significant impairment across all cognitive tests; the strongest in DST score (β =-0.179; p<0.001), TMT-B score (β = 0.151; p<0.001), LNS and MR scores demonstrating same level of impairment (β =-0.151; p<0.001). A slightly weaker impairment was observed in BVFT score (β =-0.106; p<0.01) and

Faces score (β =-0.108; p<0.01), followed up by scores in LM test (β =-0.077; p<0.05).

In terms of a life time change in cognitive performance, all scores declined significantly, except for the LM test. As in previous models, here too the highest decline, detected at p<0.001, was observed in measures of attention, information processing and mental flexibility (DST: β= 140; TMT-B: β = 0.117; and LNS: β = 0.109). Decline in non-verbal reasoning was also detected (MR: β =-0.100; p<0.01), followed by a marginally significant life-time decline in executive functioning (BVFT: β =-0.066; p<0.05) and non-verbal memory (Faces: β =-0.48; p<0.05). Comprehensive adjustment for cardiovascular risks and events affected those associations quite profoundly. In the 4-year cognitive change model, it remained significant strongly for DST score (β =-0.097; p<0.001) and slightly less so in LNS score (β =-0.097; p<0.01). Comparatively, adjustment attenuated associations slightly less in a life-time cognitive change models, where apart from the DST score (β =-0.106; p<0.001) and LNS score (β = 0.084; p<0.01), significant decline was also detected in TMT-B score (β = 0.022; p<0.01) and marginally in Faces score (β = 0.077; p<0.05) and MR score (β = 0.069; p<0.05).

Overall, these models generated the highest standardised Beta values, especially in models assessing a general cognitive decline.

5.1.2.1 General pattern of significant effect of baseline biomarkers levels on follow up cognitive scores

The results of the presented analyses indicate Interleukin – 6 levels as having the strongest predictive role in a 4-year as well as a life-time cognitive decline. Almost identical pattern of significance derived from models assessing the predictive power of the general inflammatory factor on general cognitive factor as well as individual sub-test scores. Unlike the associations predicted by the remaining circulating biomarkers, both IL-6 and inflammatory factor affected cognitive performance of verbal and non-verbal memory (assessed by Logical Memory and Faces). Majority of those associations were detected independently of common cardiovascular risk factors and events, suggesting a possible alternative biological pathway in which these biomarkers affect cognitive performance at advanced age.

In terms of individual cognitive scores, overview of the patterns of significance suggest that tests assessing non-verbal reasoning, information processing, attention and mental flexibility suffered far more severely of both 4 year and life-time cognitive decline than tests that aimed to assess domains when verbal skills are required (generally linked to crystalized intelligence). This is in line with established evidence[17]; however, possibility of limitation common to longitudinal design with ageing cohort, such as practice effect and survival bias, are plausible. This will be discussed in Chapter 7.

Age, sex and potential confounding variable adjusted results are presented in Table 19 below.

Table 19: Results: Inflammatory markers and cognitive change

	'g'	Logical Memory	Faces	Raven's Matrices	Verbal Fluency	Digit-Symbol Test	Letter-Number Sequence	Trail Making Test-B
Fibrinogen				***Standardised β (Standard Error)***				
Age, Sex	-.114 (.035)***	-.042 (.035)	-.052 (.035)	-.073 (.035)*	-.074 (.036)*	-.113 (.034)***	-.097 (.035)**	.092 (.035)**
Age, Sex, Baseline CF[1]	-.108 (.035)**	-.029 (.027)	-.016 (.028)	-.036 (.027)*	-.049 (.021)*	-.076 (.024)**	-.087 (.029)**	.038 (.026)*
Fully Adjusted[1]	-.093 (.037)*	-.015 (.029)	-.017 (.029)	-.014 (.027)	-.047 (.022)*	-.075 (.025)**	-.069 (.031)*	.027 (.027)
Age, Sex, MHVS[2]	-.084 (.028)**	-.017 (.032)	-.033 (.033)	-.051 (.031)	-.049 (.033)	-.093 (.032)**	-.072 (.032)*	.074 (.032)*
Fully Adjusted[2]	-.066 (.029)*	-.007 (.033)	-.033 (.035)	-.021 (.032)	-.053 (.034)	-.071 (.033)*	-.060 (.036)*	.063 (.033)*
C-reactive Protein								
Age, Sex	-.123 (.036)***	-.041 (.036)	-.049 (.035)	-.084 (.035)*	-.074 (.036)*	-.127 (.035)***	-.090 (.035)**	.102 (.035)**
Age, Sex, Baseline CF[1]	-.131 (.035)***	-.042 (.028)	-.036 (.028)	-.043 (027)	-.029 (.021)	-.093 (.024)***	-.076 (.029)**	.074 (.027)**
Fully Adjusted[1]	-.105 (.038)*	-.021 (.030)	-.027 (.031)	-.012 (.029)	-.005 (.023)	-.066 (.026)*	-.059 (.032)*	.059 (.028)*
Age, Sex, MHVS[2]	-.084 (.029)**	-.002 (.032)	-.023 (.034)	-.035 (.032)	-.015 (.033)	-.092 (.033)**	-.048 (.033)	.078 (.033)*
Fully Adjusted[2]	-.046 (.030)	.012 (.035)	-.025 (.036)	-.009 (.034)	-.009 (.036)	-.061 (.035)*	-.039 (.035)	.070 (.035)*
Interleukin-6								
Age, Sex	-.195 (.033)***	-.083 (.034)*	-.109 (.034)**	-.146 (.034)***	-.115 (.035)**	-.168 (.033)***	-.165 (.034)***	.137 (.034)***
Age, Sex, Baseline CF[1]	-.163 (.034)***	-.061 (.027)*	-.066 (.028)*	-.084 (.026)**	-.058 (.020)**	-.074 (.023)**	-.137 (.029)***	.039 (.026)
Fully Adjusted[1]	-.112 (.037)**	-.039 (.029)	-.056 (.029)*	-.075 (.028)**	-.034 (.022)	-.089 (.025)**	-.101 (.032)**	.024 (.028)
Age, Sex, MHVS[2]	-.153 (.027)***	-.049 (.031)	-.086 (.032)**	-.118 (.030)***	-.087 (.032)**	-.140 (.031)***	-.134 (.031)***	.111 (.031)***
Fully Adjusted[2]	-.121 (.029)**	-.047 (.034)	-.069 (.035)*	-.102 (.033)**	-.052 (.035)	-.109 (.034)**	-.102 (.034)**	.089 (.034)**
TNF - α								
Age, Sex	-.116 (.034)***	-.042 (.035)	-.111 (.034)**	-.103 (.034)**	-.074 (.035)*	-.081 (.034)*	-.073 (.035)*	.089 (.034)**
Age, Sex, Baseline CF[1]	-.069 (.034)*	-.011 (.027)	-.065 (.028)*	-.026 (.026)	-.0245 (.020)	-.068 (.023)**	-.042 (.029)	.040 (.026)
Fully Adjusted[1]	-.046 (.036)*	.011 (.028)	-.062 (.028)*	-.017 (.027)	-.015 (.021)	-.064 (.023)**	-.016 (.029)	.034 (.027)

	'g'	Logical Memory	Faces	Raven's Matrices	Verbal Fluency	Digit-Symbol Test	Letter-Number Sequence	Trail Making Test-B
Age, Sex, MHVS[2]	-.066 (.028)*	.005 (.031)	-.084 (.033)*	-.065 (.031)*	-.028 (.033)	-.042 (.032)	-.028 (.032)	.052 (.032)
Fully Adjusted[2]	-.036 (.028)	.018 (.032)	-.079 (.034)*	-.049 (.031)	-.008 (.033)	-.025 (.032)	-.004 (.033)	.041 (.033)
Inflammatory Marker								
Age, Sex	-.197 (.034)***	-.077 (.035)*	-.109 (.035)**	-.150 (.035)***	-.106 (.036)**	-.179 (.034)***	-.150 (.035)***	.151 (.035)***
Age, Sex, Baseline CF[1]	-.193 (.035)***	-.056 (.028)*	-.62 (.028)*	-.070 (.027)**	-.054 (.021)*	-.110 (.024)***	-.129 (.029)***	.067 (.027)*
Fully Adjusted[1]	-.136 (.38)***	-.028 (.030)	-.055 (.031)	-.045 (.028)	-.033 (.023)	-.097 (.025)***	-.097 (.032)**	.053 (.029)
Age, Sex, MHVS[2]	-.137 (.028)***	-.029 (.031)	-.048 (.034)*	-.100 (.031)**	-.066 (.033)*	-.140 (.031)***	-.109 (.032)***	.117 (.032)***
Fully Adjusted[2]	-.106 (.030)***	-.016 (.034)	-.073 (.036)*	-.069 (.034)*	-.046 (.036)	- .106 (.034)***	-.084 (.035)**	.022 (.349)**

[1]respective BL score; baseline measures of: Total cholesterol; MI; angina; stroke; TIA; smoking; alcohol; HbA1c; duration of T2DM

[2] Mill Hill Vocabulary Score, baseline measures of: Total cholesterol; MI; angina; stroke; TIA; smoking; alcohol; HbA1c; duration of T2DM

Significance code: *** p<0.001; ** p<0.01; *p<0.05

Chapter 6: Results: Genetic Association Meta-Analysis

This chapter presents the results of the meta-analyses conducted on data collated from a seven population-based studies, the aim of which to determine the associations of pre-selected single nucleotide polymorphisms (SNPs) with cognitive functioning. Data collection and initial data cleaning was carried out by each collaborative center independently and then shared, allowing for individual cohort analysis and meta-analysis using raw data. This required some preliminary calculations, corrections and analyses. The results of initial descriptive analysis performed on all demographic data and laboratory data and the subsequent calculation of the general cognitive factor '*g*' for each cohort separately, are presented. This was followed by a set of unadjusted and subsequently age and sex adjusted regression analyses assessing significance of relationship between plasma fibrinogen values and pre-selected SNPs, cognitive performance and the same SNPs and plasma fibrinogen values and cognitive performance. The results of the meta-analyses on the relationships of the pre-selected SNPs with plasma fibrinogen levels and with general cognitive factor 'g' are then reported.

6.1 Description of seven collaborative cohorts

Table 20 provides a summary of the demographic and clinical data provided by each collaborative cohort. The entire dataset for each collaborative cohort was not always available. Therefore, comparison of the characteristics of subjects included in the analysis with those of excluded subjects (i.e. subjects without available genotype and cognitive data) was not possible. On the other hand, demographic data on included subjects was available for most studies and for most variables of interest.

In terms of the age range of the study populations, there were some noticeable differences across cohorts. The difference between the mean age of the youngest (WHII) and oldest study (LBC 1936) study population was 18.3 years. Five of the seven cohorts comprised of population with mean ages ranging; chronologically youngest study was WHII (mean age 60.8; SD 5.94), followed by the AAA trial (mean 61.6; SD 6.54), then the ELSA cohort (mean 66.0; SD 9.64), followed by the ET2DS (mean age 67.9; SD 4.11) and the LBC 1936 (59.6; SD=0.83). The mean ages of the remaining two cohorts, both Scottish, fell within the 70 to 80 year age range with the EAS cohort being the younger of the two cohorts (mean age 73.9; SD 5.3) and the LBC21 being the oldest population of the 7 collaborating cohorts (mean 79.1; SD 0.57). The majority of the cohorts had a relatively equal gender distribution; exceptions were the WHII study where approximately three quarters of the cohort were male and the AAA trial with only one quarter approximately of male subjects.

Circulating plasma fibrinogen levels were within a very similar range for all cohorts. The majority of plasma fibrinogen vales were within the normal physiological range of 2.0 – 4.5 g/l[107] and examination for potential outliers (>1.5 interquartile range) detected only a very small number of outliers in the ELSA, WHII and LBC 1921 cohorts with the highest value of plasma fibrinogen was 8.9 g/l.

Examination of cardiovascular risk factors revealed that generally the cohorts with the 'healthiest' profile was the WHII population (in relation to blood pressure, serum cholesterol values and plasma HbA1c values and the presence of T2DM). There was a relatively consistent pattern observed for smoking status where the majority of subjects were either non-smokers (AAA Trial and WHII) or ex-smokers (EAS, ET2DS and LBC 21) and minority of subjects were current smokers. In the LBC 1936, there were numbers of subjects who either never smoked or gave up at least 12 months before the data collection. Comparable data were not available for the ELSA cohort. A similar pattern across studies was also observed for data on an average number of consumed units of alcohol; most subjects (across all cohorts) reported taking 1 to 2 units per in average week in last year. Data for the LBC1936 and WHII studies indicated an average alcohol intake of 10.59 and 11.9 units per week, respectively.

There were relatively similar distributions of socio-economic statuses between studies. Despite there was differences in the presentation of this information in each cohort, percentage values indicated that middle to higher social class was most commonly reported. Perhaps correspondingly, the majority of subjects in each collaborative cohort attained either a university degree or some form of post-school

qualification/training. Interestingly, the LBC 1921 cohort differed in this respect; here the majority (87.9%) of subjects completed university education.

Assessment of mood status was conducted by self-reported questionnaire (Hospital and Anxiety Depression Scale, HADS, described in details in Section in all the Scotland based cohorts. Studies showed a similar pattern with mean anxiety scores slightly exceeding the mean depression score. However, no sign of clinically disturbed mood was detected in neither of these cohorts. ELSA and WII mood assessment was carried out with the General Health Questionnaire (GHQ) for which data was not available.

Table 20: Demographic data of seven collaborative cohorts

	AAA	**EAS**	**ELSA**	**ET2DS**	**LBC 21**	**LBC 36**	**W II**
Total sample	3350	1592	12099	1066	550	1091	10308
Baseline Year	1998-2001	1987-88	2002-03	2006-07	2000	2004	1985-88
Total sample received (n)	2312	852	5606	1066	517	1005	3413
Wave/year used in this stud	1998-2001	1987-88	2008-09	2006-07	2000	2004	2002-04
Study design	Prospective	Prospective	Prospective	Prospective	Prospective	Prospective	Prospective
Sampling frame	General Practices	General Practices	Respondents to HSE	Diabetes Register	MHT survivors	MHT survivors	Workplace
Sub-Sample for presented analysis	**2061**	**534**	**5458**	**1045**	**517**	**1005**	**3413**
Age	61.6 (6.54)	73.9 (5.3)	66.01 (9.64)	67.9 (4.21)	79.1 (0.57)	69.6 (0.83)	60.78 (5.94)
Gender (male *n %*)	564 (27.5)	270 (50.7)	2558 (45.7)	535 (51.2)	215 (41.6)	509 (50.6)	2580 (75.6)
Plasma Fibrinogen (g/L)	3.48 (0.77)	3.24 (.70)	3.23 (0.73)	3.65 (0.7)	3.59 (0.9)	3.28 (0.6)	3.01 (0.6)
Systolic BP (mm Hg)	147.1 (21.3)	150.5 (21.8)	119.25 (47.2)	133.3 (16.4)	167.&& (26.7)	149.8 (19.2)	128.27 (16.9)
Diastolic BP (mm Hg)	83.7 (10.7)	79.14 (10.7	66.36 (26.6)	69.1 (9.01)	82.46 (12.9)	81.4 (10.3)	74.4 (10.5)
Total Serum cholesterol (mmol/L)	6.16 (1.1)	5.12 (1.4)[$]	5.39 (2.7)	4.31 (0.9)	5.66 (1.1)	5.44 (1.2)	3.89 (1.6)
T2DM *(n%)*	56 (2.7)	20 (3.8)		1066 (100)	24 (4.6)	87 (8.7)	153 (4.5)
HbA1c			4.99 (2.5)	7.40 (1.1)	5.69 (0.7)	5.93 (0.74)	5.35 (0.7)
BMI		26.1 (4.3)	26.5 (7.6)	31.4 (5.7)	26.18 (4.1)	27.81 (4.43)	26.7 (4.4)
CVD (MI and/or stroke; *n%*)	36 (1.7)	71 (13.3)	471 (8.6)	330 (31.5)	39 (7.5)	247 (24.6)	[$]89 (2.6) – stroke only

	AAA	EAS	ELSA	ET2DS	LBC 21	LBC 36	W II
Smoking status							
Current smoker (*n%*)	675 (32.8)	85 (15.9)	***Number of packs/year***	153 (14.4)	36 (7.0)	144 (13.2)	279 (8.2)
Ex-smoker (*n%*)	660 (32.0)	247 (46.3)	884 (14.4)	499 (46.8)	255 (49.3)	473 (43.4)	1477 (43.3)
Never (*n%*)	726 (35.2)	202 (37.8)		414 (38.8)	225 (43.5)	474 (43.4)	1610 (47.2)
Alcohol (unit/week/last year, *n%)**			Never – 475 (8.8) Daily – 804 (14.9) 5-6d/w – 293 (5.4) 3-4d/w (582 (10.8) 1-2/W – 1184 (22.0) 1-2/M – 589 (10.9) 1-2/2xM – 320 (5.9) 1-2/year – 398 (7.4) N/A – 546 (10.1) Missing – 267 (4.8)				
0 drinks		50 (9.4)		309 (29.0)	1 (0-100)*	10.59 (14.17) units per week	11.9 (13.3)
1-2 drinks		290 (54.4)		471 (44.1)	≥ 1: 265 (51.3)		
3-4 drinks		110 (20.7)		178 (16.7)	≤ 1: 252 (48.7)		
5-6 drinks		47 (8.8)		56 (5.3)			
7-9 drinks		20 (3.7)		21 (2.0)			
10+ drinks		15 (2.9)		7 (0.7)			
missing		2 (0.1)					
SIMD (*n%)**			[$]Prof – 301 (4.9) Managerial – 1852 (30.0) Skilled – 3554 (57.7) Unskilled – 346 (5.6) Armed forces: 12 (02) Never worked – 85 (1.4) Missing 19 (0.4)				
Most deprived	888 (26.5)	I – 79 (14.8)		127 (11.9)	125 (24.2)	High Social Class (I or II): 592 (55.3) Lowest two deprivation categories: 673 (62.1)	I – 375 (11.0)
Highly deprived	751 (22.4)	II – 199 (37.3)		208 (19.5)	173 (33.5)		II – 1399 (41.0)
Deprived	505 (15.1)	IIN – 117 (22.0)		188 (17.6)	200 (38.7)		IIIN – 327(9.6)
Low deprived	483 (14.4)	IIIM – 91 (17.1)		194 (18.2)	12 (2.3)		IIIM – 17 (0.5)
Least deprived	723 (21.6)	IV – 37 (6.9)		349 (32.7)	5 (1.0)		IV – 3 (0.1)
Missing		V – 8 (1.5)			2 (0.4)		V – 3 (0.1)
		VI – 3 (0.4)					Missing - 1289 (37.8)
Education (*n%)**							
University (n, %)		[$]110 (20.6)		[$]171 (16.0)	453 (87.6)	NA	<16 – 778 (22.8)[$]
Post-school Training (n,%)	NA	112 (21.0)	NA	307 (28.8)	51 (9.9)		17-18 645(18.9)
Secondary (n, %)		304 (57.0)		581 (54.5)	11 (2.1)		>18 - 1102 (32.3)
		6 (1.1)		7 (0.7)	2 (0.4)		Missing: 887

	AAA	EAS	ELSA	ET2DS	LBC 21	LBC 36	W II
Primary (n, %)		2 (0.2)					(26.0)
Assessment of Mood			*Depression*[$]*:* Medication: 60				
HADS/depression	2.94 (2.6)	3.81 (8.6)	(1.1)	3.85 (2.9)	5.13 (3.24)	4.88 (3.21)	General Health
HADS/anxiety	5.29 (3.6)	5.51 (9.7)	Counselling: 5 (0.1)	5.72 (2.9)	3.46 (2.28)	2.81 (2.25)	Questionnaire
Other measure			Both: 27 (0.4)				

***** If other measure, please indicate

[$] Only baseline data available for this measure

BP (S) – blood pressure; Ex-smoker - > 6 months ago before clinical assessment;HSE: HADS (A) & HADS (D) – Hospital Anxiety Depression Scale – anxiety and depression sore, respectively; MHT: Mental Health Testing; MI – Myocardial Infraction; SIMD – Scottish Index of Multiple Deprivations; NA – not available data

6.2 General Intelligence Factor 'g'

Although cognitive neuropsychological assessment batteries varied across studies, each battery contained mutually compatible tests that enabled calculation of a general intelligence factor *'g'*.

Significant correlation between individual tests assessing three main cognitive domains is required for successful calculation of principal components that account strongly for the variance between each test score[278]. Summary of all results derived from a set of principal component analyses is presented in table 21 below. Individual studies' details, such as contribution and loadings were discussed in relevant sections above. All PCAs were run in R 2.1.4.

Table 21: Principal Component Analysis: Cognitive scores, collaborative cohorts

	Factor Loading	Extraction (%)	Component	Variance (%)	Eigenvalue
AAA					
Auditory Verbal Test	0.640	41.0	1	55.8	2.79
Verbal Fluency Test	0.631	39.8	2	14.9	0.74
Raven's Matrices	0.805	64.8	3	13.3	0.67
Digit Symbol Test	0.821	67.4	4	8.7	0.43
- Trial Making Test-B (ln)	-0.821	66.2	5	7.3	0.36
EAS					
Logical Memory	0.768	59.0	1	49.6	1.96
Verbal Fluency Test	0.635	40.3	2	21.2	0.85
Raven's Matrices	0.703	49.5	3	16.9	0.67
Digit Symbol Test	0.705	49.8	4	12.5	0.50
ELSA					
Prospective Memory	0.528	33.8	1	45.7	1.83
Words Recall	0.768	58.9	2	20.4	0.82
Animals	0.716	39.0	3	19.3	0.77
Literacy score	0.624	51.3	4	14.5	0.58
ET2DS					
Logical Memory	0.530	28.1	1	44.0	3.08
Faces	0.456	20.8	2	13.0	0.91
Verbal Fluency	0.678	45.9	3	11.2	0.78
Ravens' Matrices	0.663	44.0	4	9.8	0.69
Digit Symbol Coding	0.753	56.6	5	9.3	0.65
Letter Number Seq.	0.710	50.4	6	7.8	0.54
- Trial Making Test-B (ln)	0.793	62.8	7	4.9	0.34
LBC 1921					
Logical Memory	0.732	53.6	1	52.1	1.56
Verbal Fluency Test	0.619	38.3	2	27.9	0.84
Raven's Matrices	0.802	64.3	3	19.9	0.59
LBC 1936					
Logical Memory	0.645	39.3	1	48.4	2.42
Verbal Fluency Test	0.707	42.4	2	15.5	0.78
Raven's Matrices	0.702	47.6	3	13.3	0.66
Digit Symbol Test	0.640	49.1	4	12.3	0.62
Letter	0.775	639	5	10.5	0.52
Whitehall II					
AH – 4	0.816	66.7	1	59.7	2.39
Word List	0.603	36.4	2	18.8	0.75
Verbal Fluency	0.809	65.5	3	11.8	0.47
Animals	0.838	70.3	4	9.7	0.39

6.3 Results from genetic-association meta-analysis

Table 22 summarizes all significant results detected in the meta-analyses of genotype and plasma fibrinogen values and genotype and general cognitive function, indexed as 'g'. Prior to the main meta-analyses, linear regressions were carried out, results of these were saved and subsequently used in the meta-analyses.

The first part of the table summarises results for the seven polymorphisms that were statistically significantly associated with elevated plasma fibrinogen level; the second part shows results of five SNPs that reached the nominal level of significance ($p<0.05$) for association with 'g'.

For each significantly associated SNP, information indicating location on the gene, total number of people in the analysis as well as the studies which had the SNP information available is presented in the table.

In total, 61 pre-selected SNPs in a total number of 14033subjects were selected for the presented meta-analysis. Nominally significant associations were detected between seven fibrinogen related SNPs and plasma fibrinogen levels; these were: rs4129267 (p= 0.00033); rs7518199 (p=0. 00419); rs8137951 (p=0. 00795); rs315952 (p= 0.04037); rs4537545 (p=0 .04117); rs9853387 (p=0.02108) and rs1279840 (p= 0.01123). These significant associations persisted the correction for age and sex. The magnitude of effect size remained within the same range before

adjustment. None of the significantly associated polymorphisms were in one of the fibrinogen genes *FGA, FGB* or *FGG*.

Meta-analysis investigating the relationship between the pre-selected 61 polymorphisms and the general cognitive factor 'g' revealed five SNPs were nominally associated with impairment in general cognitive factor 'g' at the significance threshold of $p<0.05$. These were rs1800497 ($p=0.00475$); rs2070016 ($p=0.03917$); rs4681 ($p=0.04259$); rs4251961 ($p=0.00578$) and rs1130864 ($p=0.04049$). Adjustment for age and sex did not attenuate these associations greatly except those for rs4681 ($p=0.10467$) and for rs1130864 ($p=0.09281$), which both became non-significant at the $p=0.05$ level.

In the case of the rs1130864 SNP (*CRP* gene), significant results were observed in both meta-analysis (assessing the relationship between plasma fibrinogen and the SNP and 'g' and the SNP); there was a weak, marginal level of significance in the age and sex corrected model investigating the plasma fibrinogen – SNP relationship and a marginally significant association between plasma fibrinogen SNP and the general cognitive factor 'g'. The magnitude of all associations was small.

Forest plots illustrating the relative strength and contributing to the overall significant results are presented in for the unadjusted and age and sex corrected models of SNPs and plasma fibrinogen levels and the same for the SNP and 'g' impairment Appendix F and G.

Summary

In this study sample, 7 out of the 61 pre-selected polymorphisms were significantly associated with plasma fibrinogen and 5 out of the 61 selected polymorphisms were associated with the general cognitive factor 'g'($p< 0.05$). The remaining two SNPs which were associated with 'g' were located within inflammation-related genes. These findings are discussed further in the text.

Only two associations were linked to fibrinogen genes directly: *FGA* (rs2070016) and *FGB* (rs4681); both significantly associated with impairment in general cognitive ability and only one polymorphism was significantly linked to both, elevated plasma fibrinogen levels and impaired general cognitive ability 'g'; this was the rs113084(*CRP* gene). Majority of the remaining significantly associated polymorphisms are located within inflammation-related genes. The results of the meta-analysis were therefore consistent with the assumption of my thesis that increased levels of circulating inflammatory markers play an important role in development of age-related cognitive impairment.

Table 22: Results: meta-analysis

SNP	Chr	Chr. Pos. on hg 18(bp)	Gene	Total No	Total No/study	SNP – Fib p-value		*Estimate-Slope*; 95% CI		SNP – 'g' p-value		*Estimate-Slope;* 95% CI	
						Non-adj.	Adjusted	Non-adj.	Adj.	Non-adj.	Adj.	Non-adj.	Adj.
rs4129267	1	154426264	*IL6R*	12139	EAS; ELSA; ET2DS; LBC21; LBC36; WII	.00033	.00046	.04 [.02;.06]	.04 [-.03; .01]	.88474	.99093	-.01 [-.04; .01]	-.01 [-.04; .02]
rs7518199	1	154407419	*IL6R*	10437	EAS; ELSA; ET2DS; WII	.00419	.00565	.03 [.01;.05]	.03 [.01; .05]	.93485	.92467	-.01 [-.04; .01]	-.01 [-.04; .01]
rs8137951	22	51165664	*SHANK3*	5967	ET2DS; LBC21; LBC36; WII	.00795	.02436	-.04 [-.06;-.01]	-.03 [-.06; .00]	.49051	.56749	-.04 [.00; .08]	-.03 [-.01; .07]
rs315952	2	113890304	*IL1RN*	6443	ELSA; LBC21; LBC 36	.04037	.02604	.03 [.00; .07]	.04 [.00; .07]	.38061	.36526	-.02 [-.06; .02]	-.02 [-.06; .02]
rs4537545	1	154418879	*IL6R*	6463	ELSA; LBC21; LBC36	.04117	.03033	.03 [.00; .06]	.03 [.00; .06]	.79670	.83619	-.01 [-.04; .03]	.00 [.04; .03]
rs9853387	3	136038988	*PCCB*	4979	EAS; ET2DS; WII	.02108	.03436	.03 [.00; .06]	.03 [.00; .06]	.95728	.77421	.02 [-.02; .06]	.03 [-.01; .07]
rs1279840	3	136006576	*PCCB*	4979	EAS; ET2DS; WII	.01123	.04230	-.04 [-.07; -.01]	-.03 [-.06; .00]	.45644	.86777	.02 [-.03; .06]	.00 [-.05; .04]
rs1800497	11	112776038	*ANKK1*	3106	AAA; ET2DS	.843794	.82026	.01 [-.03; .06]	.01 [-.03; .06]	.00475	.01302	.09 [.03; .15]	.08 [.02; .14]
rs2070016	4	155729764	*FGA*	4628	AAA; ET2DS; LBC21; LBC36	.218374	.18391	-.07 [-.11; -.02]	-.07 [-.11; -.03]	.03917	.01257	.07 [.01; .13]	.05 [-.01; .11]
rs4681	4	155710282	*FGB*	3106	AAA; ET2DS	.637909	.55063	.01 [-.03; .05]	.01 [-.03; .05]	.04259	.10467	.06 [.00; .11]	.04 [-.01; .09]
rs4251961	2	113590938	*IL1RN*	10437	EAS; ELDA, ET2DS; WII	.783693	.66315	-.01 [-.03; .02]	-.01 [-.03; .01]	.00578	.01698	-.04 [-.08; -.01]	-.04 [-.07; .01]
rs1130864	1	15794915	*CRP*	1579	EAS; ET2DS	.081974	.05092	.05 [-.01; .11]	.05 [.00; .11]	.04049	.09281	-.08 [-.16; .00]	-.07 [-.15; .01]

Adjustment: Age and Sex; Meta-analysis conducted on unstandardized values

Chapter 7: Discussion

In this chapter the findings of the work undertaken for this thesis are discussed. The main findings in relation to each of the four biomarkers are considered, including discussion of findings from the longitudinal analysis of the ET2DS, and in the case of fibrinogen, including results from the genetic analysis. Wherever possible, results are discussed and compared with existing evidence. Methodological issues are highlighted where appropriate as well as in sections specifically addressing the pros and cons of using longitudinal epidemiological studies and genetic studies respectively in the investigation of cognitive decline. This chapter also has a major focus on possible neuro-pathological mechanisms resulting in accelerated cognitive decline. The chapter concludes by highlighting the importance of the evidence and implications for further research.

7.1 Summary of main findings – the ET2DS

An association of plasma cytokines and acute-phase proteins with 4-year and life-time cognitive decline in older adults with type 2 diabetes has largely been supported by the analysis presented from the ET2DS. In particular, higher levels of all four measured markers of inflammation, as well as the general inflammatory factor, predicted greater decline in general cognitive ability, independent of major cardiovascular risk factors and events (with the exception of TNF-alpha in the adjusted life-time decline model). The overall pattern of results indicated the most consistent association across all cognitive domains for the general inflammatory levels and plasma IL-6 levels. The acute-phase proteins, plasma fibrinogen and CRP, were particularly associated with accelerated decline in measures of speed of information processing, attention and mental flexibility, working memory and information organization. The detected patterns of association with cognitive decline were consistent in terms of both late-life decline and life-time decline; in a majority of domains that were affected in the 4-year assessment, life-time decline was also observed. The exception to this was performance on the Logical Memory test, suggesting marginal or no decline in verbal memory performance. Overall, the direction and magnitude of associations were consistent with the available literature

Baseline measures of the circulating inflammatory cytokines IL-6 and TNF-alpha largely predicted significant decline in the whole range of cognitive domains tested. A great number of these associations were independent of conventional cardiovascular factors and events, suggesting a possible direct relationship of the

cytokines with brain function; perhaps through neuroinflammation. This was especially observed in models assessing the role of IL-6.

Similar results for IL-6 as were found in the ET2DS were also obtained in the MAAS study. Here, the highest tertile of IL-6 levels predicted the greater decline in a general cognitive score, irrespective of adjusted variables[236,237] and in orientation and working memory[237]. In the study by Schram[239], combining the Rotterdam and Leiden cohorts, IL-6 was significantly associated with general decline and decline in memory but only in the smaller, Leiden cohort; in the large sample used in the Rotterdam study, IL-6 predicted annual decline in general but only in participants who were also carriers of APOE ε4. Similar results were also detected in the PROSPER study, in which increased IL-6 concentration significantly predicted associateddecline in executive and memory functioning. Additionally, IL-6 genotype carriers performed significantly worse in an executive, functioning task[245].

Interestingly, in the ET2DS, TNF-alpha was the weakest predictor of cognitive decline overall, in terms of the smallest effect as well as the least number of associations that reached the $p< 0.05$ level of statistical significance. At all levels of modeling, higher TNF-alpha was associated with poorer scores in non-verbal memory, measured by the Faces test; however, in the remaining cognitive domains only sporadic associations were detected. In particular, TNF-alpha showed no significant association with decline in scores for neuropsychological tests that assessed executive function (despite an association with lower 'one-off' follow up scores). These results are opposed to findings from the Framingham study where TNF-alpha levels were significantly associated with decline in executive functioning

but no with scores at a single time point. While the Framingham cohort was largely comparable to the ET2DS population in terms of size, age, and sex, baseline TNF-alpha levels were slightly higher in the ET2DS (1.33 compared with 1.2 pg/ml). Also, compared with the diabetic population, only 12 percent of the Framingham cohort was diagnosed with T2DM and there was a slightly lower prevalence of CVD. On remaining descriptive variables of interest, however, these cohorts were comparable and the selection of covariates was largely identical[242]. One possible explanation for the conflicting results is the shorter length of the follow up in the ET2DS (4 years in the ET2DS and an average 6.3 years in the Framingham study), allowing less time for decline to have occurred. Also, possibility of methodological and other differences between cohorts must be considered. The Framingham study was commenced in 1948, that is almost six decades before the ET2DS baseline data collection. Perhaps due to a differing time points and associated differences in life style habits, most notably in smoking status (approximately 40% and 17% in Framingham and the ET2DS, respectively)[257,307]. A possibility of a cohort effect might be an additional explanation of the differences between each study results.

In the ET2DS, the acute- phase proteins, plasma fibrinogen and CRP, largely showed similar pattern in their associations with 4-year, as well as life-time decline in the various cognitive tests. Results from the Framingham study indicated an association of plasma fibrinogen with annual decline in executive functioning, attention and mental flexibility and a borderline significant relationship between CRP levels and naming and lexical retrieval[242].

In the AAA Trial, plasma fibrinogen and CRP levels significantly predicted decline in general cognitive ability as well as the remaining cognitive domains, except for memory functioning[251]. Results from the EAS also indicated a significant, CVD-independent, relationship between plasma fibrinogen and decline in general cognitive ability and verbal memory[241]. Owing to cross-over of research teams involved in the AAA, EAS and ET2DS, the latter two in particular have similar methodology, especially in the selection of neuropsychological tests and analytical approach but the remaining studies were also methodologically similar to the ET2DS. All were reasonable large cohort studies with at least 39 months of follow up and the selected covariates used in fully adjusted models were similar to those in the ET2DS. The only distinct difference was in the prevalence of T2DM that makes participants in the ET2DS cognitive functioning more vulnerable and susceptible to the effect of systemic inflammatory markers.

As for the cytokines, associations of the acute--phase proteins with cognitive decline were largely independent of all adjusted vascular covariates, again raising the possibility of a direct relationship between the proteins and brain function. Altogether, the epidemiological evidence suggests the possibility of a peripheral-to-brain system communication and subsequent neuro-inflammation as a plausible biological mechanism that eventually results in poorer cognitive performance. In this model, circulating levels of IL-6 and, to a lesser degree, plasma fibrinogen levels, would appear to have the strongest effects on the brain as they were the strongest predictors of cognitive decline.

7.1.1 Possible role of depression and vascular disease in the inflammation/cognition association

Although my findings would be consistent with a direct effect of inflammatory mediators on the brain, there are several other possible explanations for the associations detected. Two of these (depression and vascular disease as mediators of any effect) are considered here. Methodological issues which might have affected my results are considered later in the chapter.

Depression

Systemic inflammation and neuro-inflammation in particular, has been directly linked with the prevalence of depression, especially in elderly populations[81,85,308,309]. This is possibly due to integration of cytokines and acute phase proteins and the hypothalamic-pituitary-adrenal axis (HPA) and the locus coeruleus-norepinephrine (LC-NE)[85]. Increased levels of these biological markers of inflammation are linked with HPA hyperactivity that in turn is associated with central obesity, insulin resistance, high blood pressure and dyslipidaemia[85], factors directly relevant to T2DM. Therefore, especially in diabetic populations, it can be reasoned that depression might mediate the effect of inflammatory markers on cognitive performance. However, such findings were not detected in the presented analyses on the ET2DS. This might be explained by a number of factors. Depression is a complex condition, developing over a long period of time with a whole range of risk

factors and determinants[46]. In the ET2DS, depression status was assessed at a single time point using a 'screening' questionnaire, whereas the majority of the cited evidence is drawn from data on mental health status (i.e., doctor-diagnosed history of clinical depression, melancholic depression, etc.). Furthermore, subjects returning to a follow up wave of cognitive assessment are known to be generally healthier, including better psychological well-being, than their non-attending counterparts[61]. Nevertheless, given the plausible biological mechanism between elevated inflammatory markers and the risk of depression, especially in diabetic populations, in future waves of assessment this may become more evident in the ET2DS.

As expected with any complex condition, to determine the direction of the relationship between depression and cognition has proved troublesome. Evidence of whether depression incidence in adults diagnosed with mild cognitive impairment increases conversion into frank dementia is inconclusive[310]. Alternatively, it can be argued that people with clinically detectable cognitive decline are more prone to develop depressive symptoms; brain regions that are most affected by age-related neuro-degeneration mechanisms and are first affected in the early stages of Alzheimer's disease, are also directly involved in maintaining mental health status and thus making these adults more disease-vulnerable. Alternatively, systemic inflammation has been proposed in this pathological mechanism, but whether its role may be confounding or mediating is unclear. Psychological stress can cause an acute-phase response via activation of the HPA axis and the LC-NE system[90]. Equally, chronic low-grade inflammation associated with long-term medical conditions (diabetes, cardiovascular co-morbidity), can be a reason for poor mental health,

which in turn, might exacerbate production of dedicated cytokines and acute-phase markers. This is even more plausible in the light of evidence that the relationship between prevalence of depression and elevated inflammation seems to span across a person's life, even if in the case of just one depressive symptom and in otherwise healthy individuals[311].

Large and robust studies such as Whitehall II reported the role of IL6 and plasma fibrinogen, but not CRP, in11.8 years follow up assessment of depressive symptoms, including cognitive symptoms of depression, independent of common biological and social risk factors[312]. Despite the large size of this cohort, the purposeful sampling methods (only civil servant, much younger (mean age 50) may bias the results and must be considered when interpreting these in the light of relevant other evidence. Generally, depressive symptoms are more prevalent in representative population of women[313] but this was not reported by the Whitehall II study. Also, authors reported that there was no significant evidence of reverse relationships, i.e. depression measured at follow up did not significantly relate to baseline measures of inflammatory markers. This suggests a one-directional relationship with inflammation acting as a risk factor for depression. Nevertheless, further studies need to ascertain the direction and magnitude of this relationship. In particularly, focus on diabetic population may be clinically beneficial, provided a higher level of diabetes-related comorbidities, such as elevated systemic inflammatory markers.

Vascular Disease

Understanding the role of cardiovascular status in mechanisms underlying cognitive decline might lead to a better understanding of the shared patho-physiology of age-related cognitive decline and also the two most common forms of dementia, Alzheimer's disease and vascular dementia. People with diabetes have a comparatively higher prevalence of cardio-vascular disease compared with the non-diabetic population[314]. Furthermore, people with diabetes are more likely to suffer from chronic, low-grade inflammation[81], whilst the role of elevated inflammation in vascular disease[315], including stroke[316] has been well documented.

With regards to stroke, it is complicated to assess the exact extent to which this event contributes to cognitive change, as cognitive assessment pre- and post-stroke are very seldom available. In the ET2DS, pre-morbid cognitive ability was estimated with a vocabulary test, which is a great methodological advantage. The analysis included in this thesis did not include assessment of the relationship between cardiovascular disease and cognitive change. However, such analysis in the ET2DS has recently been completed. This showed a statistically significant association of baseline history of stroke with change in general cognitive ability 'g', determined by adjustment of late-life 'g' for the MHVT ($p=0.002$) and with change in executive functioning ($p=0.012$), attention and mental flexibility ($p<0.001$) and information processing and working memory ($p<0.001$). Also, scores on these tests contributed most to the overall cognitive ability factor 'g', in contrast to age and sex matched, non-diabetic population[317]. Successful performance on these particular cognitive domains depends on brain regions that generally show greater age-related neuro-

pathological changes and vulnerability to vascular events[33], suggesting a possible role of T2DM in acceleration of these age-related cognitive changes. Very similar results were found when the relationship between baseline history of myocardial infarction and estimated life-time change in cognitive performance was assessed; in addition to decline in general cognitive ability (p=0.017), information processing (p=0.013) and non-verbal reasoning (p=0.005) also were also significantly associated with the outcome of interest[318]. To our knowledge, this was the first study that reported results of relationship between stroke (as an isolated risk) and life-time change in cognitive performance. In light of the fact that cardiovascular co-morbidity is one of the highest risk factor for accelerated cognitive decline and the lack or inconsistency of prospective data, especially in diabetic population, the need for further prospective studies are warrant.

A role for TNF-alpha in the development of fronto-temporal dementia has been suggested[319], although rather than a direct effect on the brain in the form of neuro-inflammation, this may be due to the role of TNF-alpha in activation of an acute-phase response. Activation of acute-phase proteins (fibrinogen and CRP) results in altered blood viscosity with a consequent influence on brain functioning via reduced cerebral blood flow. Fronto-temporal dementia is usually associated with vascular events, most likely stroke or TIA[31], and a role TNF-alpha in carotid atherosclerosis has been reported[320], which makes this 'vascular-mediated' pathway more plausible. Nevertheless, further research is required to ascertain the mechanism by which TNF-alpha might contribute to age-related cognitive change, especially in diabetic populations exhibiting with higher concentrations of inflammatory markers.

It is relatively widely accepted that people with diabetes are more likely to present with declined cognitive performance as a result of a wide range of co-existing conditions, potentially including elevated inflammatory markers as well as the presence of cardiovascular co-morbidity. Whereas in the ET2DS plasma fibrinogen was significantly associated with the majority of cognitive tests in terms of 4-year and life-time change (except for verbal and non-verbal memory), this was not reported in the Caerphilly study, despite their robust neuropsychological assessment and follow-up of 5 years. Also, the Caerphilly study comprised of male subjects only, who are more likely than women to suffer from cardio-vascular conditions[321] thereby increasing the risk of impaired or altered cognitive performance. Whether the presence of T2DM in the ET2DS could account for the conflicting results is debatable but possible.

To identify the mechanism(s) by which inflammation, cardiovascular disease and T2DM contribute to age-related cognitive dysfunction is methodologically difficult, mainly due to the inter-relationship between the various vascular factors. The latter might, at least in part, explain current inconsistency in evidence and lack of diabetes-specific findings. Precise and detailed understanding of the mechanism(s) may help to guide further clinical work in terms of focus on specific types of dementia. For example, conflicting evidence exists in terms on the role of T2DM in the aetiology of Alzheimer's disease and vascular dementia. In a 5-year follow up study, MacKnight et al.[322], detected a link between T2DM and vascular cognitive impairment, however, this relationship did not significantly increase the prevalence of either Alzheimer's disease or vascular dementia. Overall, evidence suggests that the role of T2DM in the

ageing brain is complex with a range of possible biological mechanisms underlying the eventual change in cognitive ability.

7.1.2 Possible direct effect of inflammatory markers on the brain

Any causal role of inflammatory mediators in cognitive decline may involve a direct effect of the biomarker on the brain, especially if the cardiovascular pathology is being by-passed, as suggested by the independence of the biomarker-cognition association from cardiovascular risk factors and disease as was the case in the ET2DS. A role for increased levels of IL-6, particularly in impaired hippocampal activity, has been documented in epidemiological studies[323] and experimental, mainly animal models, such as reduced synaptic plasticity in rats[324] and a long-term potentiation[325]. The proposed rationale for the role of IL-6 in neuro-inflammation is that IL-6 is capable of penetrating the blood-brain-barrier directly, and subsequently altering the brain-immune response system [326]. Braida et al[100] provide further evidence, assessing the trajectory of cognitive ability of transgenic mice, where animals with over-expressed IL-6 presented a 63% reduction in cognitive ability, whereas those not expressing IL-6 improved their cognition (maze learning and reduced amnesic effect). Moreover, non-steroid and anti-inflammatory medication were shown to restore neurogenesis in the hippocampus of rats[84].

Currently, no comparable results are available for humans; the animal studies, however, provide promising evidence of a key role for IL-6 in overall cognition and

memory function specifically. Indeed , hippocampal grey matter volume atrophy has been inversely correlated with peripheral levels of IL-6 in otherwise healthy community-dwelling subjects, aged 30-54 years[327]; i.e., in a population sample two decades younger and T2DM-free compared with the ET2DS cohort.

Given this suggestive evidence and the potential clinical benefits in terms of development of risk-group's tailored prevention and an early intervention, there seems to be justifiable scope for further research conducted with humans, for example, providing periodic measures of cognitive scores in relation to plasma levels of IL-6. Of interest, cerebral over-expression of IL-6 in transgenic mice affected long-term potentiation (LTP)[325], an effect occurring in the hippocampus and the cerebral cortex and representing activities that underpin memory and learning utilization[31]. Strictly speaking, without a fully functional hippocampus people would suffer from impaired sense of identity, disorientation in place and time, dysfunction in everyday tasks and would find it difficult or impossible to identify where they are, where they intended to go and how to get there. All these difficulties seem to correspond with the clinical presentation of people diagnosed with Alzheimer's disease[31]; it is therefore reasoned that the hippocampus is one of the first brain regions to suffer damage in this condition[328].

The above observations are particularly relevant when it comes to diabetic populations. One of the possible mechanisms to account for decreased cognitive function in diabetic populations is linked to insulin resistance as a contributor to hyper-insulinaemia and with the consequence of accelerated cognitive decline[329]. Insulin might play a role in accelerated cognitive decline through a direct effect on

the brain[99]. Peripheral insulin is transported to the central nervous system through the blood-brain barrier. Insulin receptors are distributed around the brain, most notably in CA1 and the dentate gyrus, subfields of the hippocampal formation[330]. The principal function of the hippocampus is the transference of information from short-term into long-term memory and spatial navigation, episodic, autobiographical and anterograde memory[331], Clinically, impairment of these cognitive functions correspond with performance of the people with Alzheimer disease[332]. This is further supported by the evidence that abnormal insulin activities can play a role in associate learning and memory formation[333]. Conversely, the administration of insulin was shown to significantly improve cognitive performance, particularly in the memory functioning of patients diagnosed with Alzheimer's disease[334]. A possible explanation is the role of insulin in regulation of phosphorylation of tau protein[330] as well as affecting extracellular amyloid beta levels[335,336]. The results of the ET2DS would be consistent with exacerbation/acceleration of this age and diabetes related impairment and decline that is apparent through the effect of elevated circulating IL-6 levels exacerbating detectable decline in cognitive ability. This makes IL-6 a particularly important risk factor for age-related cognitive decline.

7.2 Summary of the main findings - Genetic associations

This section will discuss the results of the meta-analyses performed to assess the association between pre-selected fibrinogen-related SNPs and cognitive impairment. First, the relationship between plasma fibrinogen-related SNPs and levels of plasma fibrinogen are discussed; followed by discussion of the associations between the same fibrinogen-related SNPs and general cognitive ability 'g'.

The rationale for this study was based on previous epidemiological observations suggesting a possible role for raised plasma fibrinogen levels in the aetiology of age-related cognitive impairment. The work aimed to expand on recent evidence indicating a significant relationship of the plasma fibrinogen related SNP rs2227414 (a SNP in the *FGB* gene) with circulating fibrinogen levels and cognitive ability in three Scottish cohorts[337]. This aim was to investigate this intriguing finding further by:

1. Increasing statistical power through an investigation conducted on data from a larger sample derived from collating data on over 14,000 subjects data and

2. Testing a wider selection of SNPs previously associated with plasma fibrinogen levels, including SNPs out-with the three main fibrinogen genes *FGA, FGB* and *FGG.*

Meta-analysis of data collated from seven large, population based cohorts, was used to assess the significance of associations between plasma fibrinogen related gene variants and general cognitive ability, indexed by 'g'.

A previously intended approach, Mendelian randomisation, was eventually rejected due to potential the quality of the instrument as well as potentially confounding effect. The core principle of Mendelian randomisation is that genes are randomly assigned from parental alleles during a stage called meiosis[183]. This is implied across the whole population, making the genetic effect rather robust. Therefore it is reasoned that genes (gene variances) can be successfully used as an *instrumental variables* that help to by-pass the effect of potentially mediating or confounding factors and thus disentangle the potentially causal relationship between predictor and outcome of interest[188,338]. The three key assumptions that need to be satisfied for the gene variance to classify as an instrumental variables are[339]:

1. The *instrumental variables* (gene variant) must be unrelated to any of the potentially confounding factors;
2. The *instrumental variables* (gene variant) must be associated with the predictor of interest and
3. The *instrumental variables* (gene variant) must be independent to the outcome of interest

Following these fundamental principles of the Mendelian Randomisation approach would require reliable relationship between fibrinogen-related SNPs and plasma fibrinogen levels whilst no relationship between fibrinogen-related SNPs and either

any of potentially confounding factors or the outcome, i.e. performance on neuropsychological assessment, in this analysis the general cognitive factor *'g'*).

Whereas the assumption one and three cannot be statistically tested and merely rely on biological and other explanation, the second premise can be tested. As presented in the section 6.3, of the included 61 fibrinogen-related SNPs, only seven showed a significant relationship with elevated plasma fibrinogen levels. More so, these associations were not subjected to a correction for multiple testing that would further reduce the number of significant associations. The choice of selected fibrinogen-related SNPs has been discussed above (section 4.6). Nevertheless, this result suggests the lack of strength of the selected *instrumental variable*, i.e. fibrinogen-related SNPs, presenting a serious concern for the analysis run in Mendelian Randomisation fashion. Furthermore, the exclusion of all SNPs possibly related to factors and events associated with either plasma fibrinogen circulation and/or cognitive dysfunction would have greatly reduced selection and final number of gene variants eligible for the meta-analysis.

Therefore the Mendelian randomisation approach was rejected; instead a set of linear regression models and meta-analysis of collated unstandardized parameters, were undertaken. This approach is consistent with the recent Scottish study which identified fibrinogen-related gene variants that showed a significant association with cognitive performance[337]. First, the selected SNPs were modeled against plasma fibrinogen were modeled and second, the same SNPs were modeled against the global cognitive ability 'g' was modeled. Both models were carried out at two stages: unstandardized raw values and age and sex adjusted values.

7.2.1 Associations of plasma fibrinogen levels with pre-selected SNPs

Having selected SNPs from the literature on the basis of published associations with circulating plasma fibrinogen, my initial set of analyses explored the relationship between these SNPs and plasma fibrinogen measured in the participating cohorts. Results from this meta-analysis indicated that just seven of the SNPs were significantly associated with elevated levels of plasma fibrinogen at the 0.05 level of statistical significance. Adjustment for age and sex made only a very small difference to the level of significance. Neither did it greatly affect the magnitude of effect size detected for each SNP implying that the relationship between gene variants and plasma fibrinogen level were relatively independent of age and sex.

The seven SNPs indicated by this modeling of fibrinogen-related SNPs from a range of genes against plasma fibrinogen levels indicated these seven gene variants were: rs4129267 (*IL6R*), rs7518199 (*IL6R*), rs8137951 (*SHANK3*), rs315952 (*IL1RN*), rs4537545 (*IL6R*), rs9853387 (*PCCB*) and rs1279840 (*PCCB*). Interestingly, none of these gene variants lies within the fibrinogen genes *FGA, FGB* or *FGG*. However, the majority of these genes lie on the inflammatory pathway; specifically, *IL6R* and *IL1RN*, interleukin-6 receptor and interleukin 1 receptor antagonist, respectively. Both IL-1 and IL-6 cytokines are known to be powerful markers of the liver acute-phase response; this includes increase in plasma fibrinogen in the circulation[81]. Indeed, the strongest level of significance and the largest effect size was observed in the relationship between plasma fibrinogen and rs4129267 (*IL6R*). With regards to

the remaining associated SNPs/genes, the relationship with plasma fibrinogen is biologically less obvious. The *SHANK3* gene is primarily responsible for making a protein that plays a role in functioning of synapses and also a formation and maturation of dendritic spines, essential for the transmission of nerve impulses. Previously, *SHNAK3* mutations have been found in people suffering from an autistic spectrum disorder[340] and also in individuals with intellectual disability and/or delayed intellectual development[341] . The *PCCB* gene is responsible for forming a functioning enzyme propionyl-CoA that plays a key role in the normal processing of proteins, particularly in a breakdown of several amino acids. Approximately 55 various mutations in the *PCCB* gene are prevalent in individuals who suffer from propionic academia, condition where the body is only partially/not able to process specific types of proteins and lipids[342].

The lack of significant associations between the remaining 54 SNPs and plasma fibrinogen can be explained by several reasons. Firstly, although SNPs were identified from published literature included in an electronic database, according to clearly defined inclusion/exclusion criteria, a considerable degree of heterogeneity exists between the studies populations identified in this way and those included in this project; there is therefore a high possibility that the original findings simply did not translate into the presented replication populations. Furthermore, depending on allele frequency, selected SNPs might contribute less to plasma fibrinogen concentration than was initially implied. A larger sample size would clarify this possibility. In order to account for an issue linked to LD, the HapMap database was searched for tagging SNPs in genes known to influence plasma fibrinogen levels.

Nevertheless, a possibility exists that SNPs significant in this analysis are more proximate and thus linked to the true variant than the tagging SNPs identified by the search. Lastly, although all studies clearly report all criteria applied to conditions of blood withdraw and details of laboratory measures, the possibility of inconsistency in plasma fibrinogen measures between studies affecting the final results cannot be ruled out fully. There is also the possibility that the original findings were false positive findings and/or that my own negative findings were false negative findings.

7.2.2 Associations of plasma fibrinogen SNPs and global cognitive ability

The main aim of the presented meta-analysis was to determine the association between fibrinogen-related SNPs and global cognitive ability. Again, analysis was conducted in two steps: unadjusted and age and sex adjusted models.

Of the 61 modelled SNPs, five were significantly associated with 'g' at the level of $p< 0.05$: rs1800497 (*ANKK1*), rs2070016 (*FGA*), rs4681 (*FGB*), rs4251961 (*IL1RN*), and rs1130864 (*CRP*). None of these SNPs were found to be significant predictors of plasma fibrinogen levels, apart from the last mentioned SNP rs1130864 (*CRP*). A very weakly significant association was detected between these SNPs and 'g'. However, this was only observed independently of age and sex covariant, with the effect size above average within the scale of magnitude detected in this study; for the

SNP-plasma fibrinogen relationship, it was 0.5 (95% CI -0.01 – 0.11), for the SNP – 'g' association -08 (95% CI -0.16 – 0.00). The negative value of effect size values in SNP and 'g' associations indicate the minor allele frequency.

An overlap of significant associations was observed only in two out of the seven participating cohorts (the EAS and ET2DS) a total sample of 1579 subjects. These results therefore did not benefit from collating the large dataset that was carried out in order to increase the possibility of reaching enough statistical power to detect a true association. In fact, except for the rs4251961 (*IL1RN*) polymorphism that was also genotyped in the ELSA and Whitehall II (beside the ET2DS), the remaining SNPs that were significantly associated with impairment in 'g', were only genotyped and detected in Scottish cohorts.

All selected SNPs were modelled directly in unadjusted and age and sex adjusted analysis; all but two of the 5 SNPs which were significantly associated with 'g' ($p<0.05$), were significantly associated with impairment in 'g' independently of age and sex. The exception was the rs4681 (*FGB*) variant; the magnitude of effect sizes differed slightly between unadjusted and adjusted models at 0.6 (95% CI 0.00 - 0.11) and 0.4 (95% CI -0.01 – 0.09), respectively. The second exception was the rs1130864 (*CRP*); here also there was only a slight difference between magnitude of the effect size between models at -0.08 (95% CI -0.16 – 0.00) and -0.7 (95% CI -0.15 – 0.01), respectively.

The most interesting finding is the overlap of significant associations between plasma fibrinogen levels, fibrinogen related rs1130864 (*CRP*) and impaired global

cognitive ability. A previous Mendelian randomisation study of the CRP gene did implicate this biomarker in a whole range of conditions, including inflammation, insulin resistance and diabetes[343], coronary heart disease[344] and, quite recently, in impairment of late-life cognitive ability[345]. The latter study involved considerable overlap with the current study in terms of the included cohorts; four Scottish cohorts that formed the total study sample (i.e., the AAA, EAS, ET2DS and LBC 1936) were also investigated in the present analysis ANCOVA results indicated significant prediction of impairment in the verbal fluency score (in AAA), and attention and mental flexibility, information processing speed and non-verbal memory (in ET2DS). Other associations did not reach the nominal significance level of $p<0.05$[345]. In the present study, the rs1130864 SNP was genotyped in 5 of the7 collaborating cohorts and showed a significant level of prediction of impaired general cognitive ability only in the EAS and the ET2DS. Somewhat surprisingly, association between tagging SNP rs2227412 (*FGB*) was not statistically significant, despite a previous findings[337].

The Rotterdam study investigated the relationship between three *CRP* gene variants, including rs113084 and incidence of dementia. In comparison with the Scottish cohort study[337] and my own study, the Rotterdam study was based on prospective follow-up (mean 9.2 years) for frank dementia and a large sample of just under 6000 subjects, mean age 68.9 years (SD 8,7). Whereas 607 cases of dementia were diagnosed during the follow up period, there was no significant association detected between the rs1130864 SNP and risk of dementia. In my study, the association was only detected in data collated from the EAS and the ET2DS cohorts; these cohorts

comprised of comparatively older adults that suffered from a high prevalence of cardiovascular comorbidity (the EAS) and also type 2 diabetes (the ET2DS). Inconsistency in findings of an association between the CRP gene variants and cognitive impairment may be due to variation in the study population, differences in measures of phenotype (especially cognitive) data or it might indicate results obtained in my study were due to chance, highlighting the need for replication of this finding in further, larger studies with measures of both cognitive impairment/dementia and cognitive decline.

The fibrinogen gene SNP rs2070016 (*FGA*) that determines clot structure[213], has been implicated to contribute to prevalence of hypertension (MONICA/KORA study[346]) and to cerebral small vessel disease. The Rotterdam Study[347], perhaps due to a very small study sample, failed to detect significance association of rs2070016 with severe carotid artery disease in predominantly male subjects[348], which alongside the rs4681 failed to predict coronary heart disease[130] but managed to support the role of this gene variants in plasma fibrinogen synthesis[130]. Overall, the involvement of increased plasma levels of fibrinogen in these pathological mechanisms is leading to a decreased cognitive performance, as documented by a number of epidemiological observations[16,239,241,317].

As with the previously reported analysis, here too the majority of markers that were significantly associated with 'g' lie on the inflammatory pathway of the bodily mechanism. Above that, two out of the five SNPs lie within the fibrinogen gene. Additionally, there is a strong biological interplay, primarily linked to hepatic response and activated immune system that influences production and synthesis of

plasma fibrinogen circulation. These were the rs4251961 (*IL1RN*) and rs1130864 (*CRP*) SNPs. The strongest relationship, in terms of the magnitude of the detected effect size, was between 'g' and the rs1800497 (*ANKK1*) SNP. *ANKK1* gene is linked to dopamine mechanism in brain[349], neuro-psychiatric disorder[350], alcohol and tabacco smoking dependency[349,351]; additionally, A1 allele is linked to prevalence of addition, antisocial-type of behaviour and attention-deficit hyperactivity disorder[352].

Additionally, the role of the *IL1RN*, the IL-1 receptor gene that was significantly linked to plasma fibrinogen concentration in this study (rs315952) and to impairment in global cognitive ability (rs4251961) is of interest. Biologically, IL-1 has the primary role of a mediator of non-infections inflammation and B-cell maturation as well as further cytokines release[82]. Together with TNF-alpha, it induce expression and secretion of IL-6 and all these cytokines are implicated in the development of atherosclerotic plaque and in acute coronary conditions[353]. Furthermore, IL-6 is known to regulate expression of IL-1by release of IL-1 receptor antagonist and TNF-alpha[85]. The fundamental role of IL-6 in activation of acute-phase proteins, such as plasma fibrinogen and CRP, associated with increased blood viscosity, has been documented[106] as well as the relationship between blood viscosity and a late-life cognitive performance[241].

IL-1 also has a central role in neuro-inflammation; this multi-potent protein is known to accompany age-related neuro-degenerative changes[354]. For example, in AD patients' brain, overexpression of *IL1RN* was detected, directly relevant to AD typical brain neuropathological changes[355]. Therefore there is an implied possibility of the role of this gene's variants within *IL1RN* gene that directly affects brain

efficiency through a process of neuro-inflammation, masking the true effect of plasma fibrinogen. Equally though, there is a possibility of a reverse relationship where elevated plasma fibrinogen levels might cover up the true causal factor, in this case the IL1 interleukin 1 receptor antagonist. Determining the exact nature of the bio pathological relationship of modifiable risk factors is beyond the scope of this investigation. Nevertheless, it is reasonable to assume that, despite the weak association signals and small effect sizes of these associations, the *CRP* and *IL1RN* genes can be implicated in with a reasonable degree of certainty.

7.3 Evaluation

7.3.1 Longitudinal analysis - ET2DS

Longitudinal epidemiological studies on cognitive decline present a number of problems, weaknesses and challenges in interpretation, including those shared with any other chronic health condition as well as those specific to cognitive ageing. Methodological obstacles characteristic of longitudinal observational studies on cognitive decline will be discussed in relation to the design of the ET2DS. This critique draws on an article published by Allen[173] which highlights the main obstacles in longitudinal studies investigating the relationship between cognitive performance and diabetes.

Epidemiological studies that encapsulate the complexity of the ageing process must deal with the complex and dynamic multifactorial and multidimensional relationship between a wide range of potentially important variables which may be causal, confounding, mediating or co-incidental in terms of their association with cognitive decline. The majority of biomarkers that are thought to contribute to neuropathological mechanisms that affect the speed and trajectory of cognitive ageing in diabetic populations are also likely to be associated with cognitive decline in epidemiological observations of general populations and it is difficult to distinguish diabetes-specific risk factors for accelerated cognitive decline. Since differences in age-related cognitive profiles exist between individuals in a given

population, longitudinal cognitive data that report a change in cognitive scores within individuals over time are increasingly reported.

It is well recognized that the selection of participants for epidemiological studies of cognitive ageing can introduce a set of substantial biases that need to be considered when interpreting the results[252]. This applies equally to studies of diabetic[173] as well as general populations. Perhaps inevitably, the portion of the population that can be considered as suitable for cognitive ageing investigations as well as the profile of those who are willing to engage in such studies, is skewed towards subjects with higher childhood cognitive scores[59]. Generally, people with higher childhood intelligence scores are more likely to have higher educational attainment, leading to white-collar jobs and a higher social and socio-economic status[59,61]. There is also a significantly greater chance of engaging in a healthier lifestyle (i.e., diet, exercise, etc.) with a consequently lower prevalence of medical risk factors likely to be associated with accelerated cognitive decline[356].

Use of birth cohort data allows assessment of the possible effect of pre-morbid intelligence on age-related (patho)biological risk factors, including levels of peripheral and central levels of circulating inflammatory markers (which themselves show age-related increase concentration[357,358]). Luciano et al reported evidence of 'reverse causation' where a childhood (age 11) intelligence score significantly predicted circulating levels of CRP at the age 70[234]. Of interest, CRP levels are known to be higher among people with conventional cardiovascular risk factors and co-morbidities, such as diabetes and metabolic syndrome, increased blood pressure, obesity and dyslipidaemia[82,359]. To explain their findings, Luciano et al postulated

that the lower levels of childhood IQ measures are associated with less advanced health awareness, lower socio-economic status and less-healthy life-style[360] and consequently determine physical health at older age. Individuals with higher childhood IQ score are also more likely to remain engaged in cognitively demanding exercise activities (i.e., problem solving puzzles, reading, etc.) and psychological wellbeing may also be greater with enhanced mental health strategies and social networks for necessary support[61]. The transfer and sharing of both the genes and environments involved in developing cognitive traits from parent to off-springs may also play a role.

Increased mortality rates have also been linked to poorer childhood intelligence[59,361]; since most neurodegenerative changes, associated with clinical manifestation of cognitive difficulties, commence and develop at an advance age, there is a strong possibility of survival bias. This methodological limitation is heightened in prospective studies where not only study participants are likely to have generally healthier cognitive, physical and mental health status at baseline, but also, healthier subjects are more likely to attend for follow up assessment. As a consequence, study results are often drawn from samples which may not be representative of true rates and patterns of cognitive decline in the general or targeted population[252].

To complicate matters further, the more able participants are also more likely to exhibit a practice effect in the result of their cognitive testing[232]. In longitudinal studies of ageing, the same battery of neuropsychological assessment is often repeatedly administered to assess trajectories and patterns of change in cognitive performance. This provides the possibility of encoding information into long-term

memory with potential subsequent retrieval. There are some types of memory, for example episodic memory (memory for event and personal information) or working memory (storage and processing at the same time) which show faster, age-related progressive decline[362] than other, such as prospective memory (memory for future events) and semantic memory (factual memory)[40]. Moreover, there are some people who can actually manifest a clinical improvement on some (particularly memory) tests, as they get older, performing significantly better than their younger counterparts[363]. There is some evidence to suggest that this is due to a more efficient and effective use of external cues[364]; the selective drop out of frail and less-able participants results in re-testing of the relatively healthier survivors who are therefore more likely to benefit from practice[365]. This is may be further complicated by a potential 'ceiling' effect for the most able and the a 'floor' effect for the least able[232]. Ability to apply compensatory strategies and/or draw on pre-existing cognitive patterns and cues would be consistent with the notions of the cognitive reserve[38,366] and 'successful ageing'[367]. The latter argues that contrary to traditional views (that cognitive decline is an integral, inevitable part of the ageing process), cognitive/mental decline occurs due to one or more identifiable, often preventable and modifiable risk factor[367]. Nevertheless, individual difference is this 'gain' tends to diminish gradually with greater age, possibly being balanced by neuropathological brain ageing processes[282].

In addition to the limitation listed above, prospective studies assessing cognitive health in people with diabetes diagnosis, including the ET2DS, may have specific, diabetes-related issues. The incidence of severe hypoglycaemia and chronic

hyperglycaemia have been proposed to influence cognitive ability in type 1 and type 2 diabetes, respectively[172,180] and these factors need to be considered in multivariate analysis (as well as other potential confounders which are not specific to diabetes). In terms of the problem of evidence of reverse causality and the medical and psychological conditions that co-exist with diabetes presents a particularly important issue in the selection of representative diabetes-specific study populations. In addition, non-cognitive changes in personal ability, for example sensory loss or affected vision due to diabetic retinopathy (that itself is associated with speedy cognitive decline[368]), might affect the persons' practical skills and ability to complete the cognitive tasks.

An important aim of epidemiological studies on cognition is to identify aetiological mechanisms underlying age-associated cognitive decline. The latter involves identifying potential risk factors' and their inter-relationship and, ideally, establishing the causal nature of particular determinants. However, as with any condition, the most that can be determined from an observational epidemiological study such as the ET2DS is association (or correlation) between putative risk factors. Importantly, for studies on cognitive decline, the relationship between cognition and physical health may well be bi-directional and a wide range of with variables, whether measured or unmeasured, may either confound or mediate any association between risk factors and cognitive ability. These factors must be given careful consideration in the analysis and interpretation of study findings.

Lastly, in the presented study, regression analyses were conducted using the individual marker and finally using an inflammatory factor that was derived through

four inflammatory markers-based PCA. Decision for this approach was discussed in the section 3.8.1.2. In an alternative approach, inflammatory markers would be used as covariates in each of the model. This approach would allow assessing the association between predicting inflammatory markers whilst controlling for each of the three other biomarkers. It can be argued that including three more covariates might weaken the power of the regression analysis. On the other side, this approach might have been more valuable as it would provide standardised coefficients that would allow assessing the strength of relationships in flexible way. Therefore further analysis shall also explore this approach.

7.3.2 Genetic Association Study

Previous results of epidemiological observation and genetic association studies indicated the role of plasma fibrinogen in a whole range of conditions that were subsequently significantly associated with age-related cognitive impairment and decline, including a significant relationship between plasma fibrinogen levels and accelerated decline in cognitive performance. Therefore a genetic association study was carried out with the attempt to ascertain more robustly the role of plasma fibrinogen in aetiology of cognitive decline. The next section will discuss the strength and limitation of this methodological approach, and then conclude with summary and implication for further research work.

The work undertaken in this thesis represents the first large-scale examination of the relationship between specifically selected gene variants implicated in altered plasma

fibrinogen levels and age-related cognitive change. It was done through the collation of datasets from multiple well-defined, population based cohorts; applied meta-analysis of this type can reduce false positive and false negative findings[369]. Most studies assessing genetic associations do so through already published results. The most common approach is to extract each study's' effect size and/or p values and through statistical analyses arrive at conclusions on the role of the particular gene variant in phenotypes of interest[369]. Inevitably, this brings up issues linked to the quality of published studies, and one of the major issue linked to genetic-association meta-analysis, that publication is in favour of large effect size, statistically significant results[194]. One of the main issues in meta-analysis of secondary data is the major difference in choice of covariates that might affect the genotype-phenotype relationship[370]; subsequent large inconsistencies in reported results might skew the results of meta-analysis.

In my study, individual subject data were available; this allowed for calculation of the required values that were subsequently collated in the meta-analysis. Having such raw genetic and cognitive data available, including control over determination of the general cognitive factor 'g', meant that it was possible to conduct both uncorrected models as well as models corrected for age and sex in a considerably robust manner. Furthermore, each study was checked independently for population stratification, reducing one of the major obstacles in genetic association studies. All results were derived from data from prospective population-based cohort studies; these included large study populations with well-defined selection criteria and susceptibility to study bias was limited.

The majority of gene-association studies suffer from small sample sizes that do not allow for sufficient power to detect of subtle differences and weaker associations. Here the combination of reasonably homogeneous cohorts resulted in a large overall total number of included subjects, which were treated as it from a single cohort, thereby increasing power to detect associations. Despite this, the power may have still been limited to detect particularly small effect sizes, therefore there is a possibility of false negative results.

Evaluation of measures of cognitive function across collaborating cohort

One of the major weaknesses of the presented meta-analysis is the cross-sectional nature of the extracted data on cognition, i.e. data were only available for cognitive ability at a single time point. Although this should not have had an effect on the relationship between SNP and cognitive ability, it would have been interesting and potentially more informative to investigate the association of genetic variants with late-life cognitive decline as the phenotype most amenable to subsequent modification. However, between the seven selected cohorts, there were far too many inconsistencies in the time scale of serial cognitive assessments to enable a valid and comparable measure of cognitive decline to be created and after careful consideration of the role this adjustment was ruled out. There is a possibility that lack of association was not due to true absence of an effect of a particular SNP on cognition, but rather due to methodological issues such as measurement discrepancies, differences and/or errors in measurement of cognitive phenotype, which may have

weakened potentially observed effect sizes. Methodological constrains that affected the reliability and validity of the '*g*' values is discussed below.

For the purpose of the meta-analysis, the general intelligence factor '*g*' was calculated per individual cohort. The seven individual '*g*' values were derived from tests that were available for the three main cognitive domains – memory, executive functioning and information processing. All neuropsychological tests that were administered in each cohort were standardised with a high degrees of sensitivity to detect potential subtle changes in cognitive performance. However, the neuropsychological test batteries that were used to assess individual cognitive domains differed between studies. There was a substantial overlap in the administered batteries in the Scottish cohorts, own to collaboration between research teams. Cognitive scores from the two large English cohorts, ELSA and Whitehall II, differed considerably from the Scottish cohorts. This is specifically relevant to assessment of memory functioning. In the Scottish cohorts, verbal memory tests (Adult Verbal Learning Test and Logical Memory) assessed retrospective memory[4]. Verbal memory functioning heavily relies on the function of the medial temporal lobe[7] and this region, critical for consolidation of short-term to long-term memory, is known to be the first affected by neurodegenerative changes lined to MCI and early onset of Alzheimer disease[48,58]. On the other side, in the English cohorts prospective memory was assessed by 'word list' tasks. Prospective memory functioning is generally associated with the frontal lobe region[371]. Whereas this area of the brain is particularly vulnerable to the effect of age-related neuroanatomical changes[26], clinical symptoms are compatible with localized damage, such as fronto-temporal

dementia or vascular dementia[7]. Unlike the Alzheimer's disease, variation and reduction of a cerebral blood flow has been implicated in the pathogenesis associated with clinical symptoms of dementia of vascular type. Neuroimaging studies support this view; despite divergence of functionality linked to particular brain region, there is no evidence of a one, same, biological network that would modulate general intelligence functioning[372]. This discrepancy means that general intelligence factors, calculated for individual study, were derived from scores indicating different types of memory performance. Results of the meta-analyses indicated that all but one significant association that was detected between gene variances and the general intelligence factor, were observed only in the Scottish cohorts (the exception was in the SNP *IL1RN*). Therefore it seems that one of the possible reasons why the meta-analysis did not benefit from the large collated data set, was the inconsistency in measurement of specific cognitive function.

Furthermore, collated data from seven independently organized cohorts meant that it was not possible to apply clearly defined standardizes operational procedures that would be consistently adhered to through the entire process of cognitive data collection. Therefore the test scores might have been influenced by issues, such as measurement error, participants' motivation to testing and precision of test administration. Moreover, further variability exists in terms of the length of time differences between each wave of data collection in each cohort, which affects homogeneity of each cohort's '*g*' value. Also, despite the meta-analysis presented in this study was conducted on '*g*' that was derived from cross-sectional data, these tests scores were drawn from prospective-based data sets. Therefore there is a

possibility of a selective attrition and practice effect[173,252]. This lack of control over each study designs might have affected otherwise robust measures of cognitive ability 'g'[277]. Consequently, this might have affected the robustness of results of the meta-analysis and lead to the lack of association between genotype data and cognitive performance indexed by '*g*'.

Causal Inference and Mechanisms

The results of this study are consistent with involvement of plasma fibrinogen level in accelerated age-related cognitive decline. The genotypes that were significantly associated with impaired overall cognitive ability have been shown previously to influence circulating plasma fibrinogen levels; albeit such association was not detected in the present analysis.

The rs4681 (*FGB*) has been shown to be directly involved in fibrinogen synthesis and the rs2070016 (*FGA*) to determine clot structure[213,373]. This suggests that fibrinogen may alter age-related cognitive ability via its haemostatic and rheological properties. This is in line with mounting evidence indicating the role of elevated plasma fibrinogen level in Alzheimer's disease and with ischaemic stroke and vascular dementia. In this study, both of these genes were implicated in age-related cognitive impairment in the general cognitive factor 'g'.

Alternative pathways have also been implicated by the results of this study. The identified genes are also known to contain gene variants that are either indirectly

linked to biological mechanisms that activates the acute-phase response (*IL1RN* gene), or are acting in parallel with other inflammatory markers that are also activated through the hepatic response (*CRP* gene). Plasma fibrinogen levels also are elevated indirectly via biological mechanisms that are linked to alcohol dependency, substantial cigarette smoking or mental health issues and neuro-psychiatric disorders, mechanisms associated with the *ANKK1* gene.

The hypothesis of a direct brain effect in the form of neuro-inflammation also needs to be considered, especially in the light of close biological interplay between cytokines IL-1 and IL-6. These are known to penetrate the blood-brain barrier and via the activation of glial cell, to trigger the whole cascade of immune-response processes. This hypothesis is in line with Reiner et al. (CARDIA study[374]). They reported a reasonably strong statistically significant relationship of the rs4251961 (*IL1RN gene*) and CRP, IL-6 and plasma fibrinogen levels. Overexpression of IL-1 in the brains of patients with Alzheimer's' disease and its involvement in neuro-degeneration is also evident. In addition, the rs315952 (*IL1RN gene*) may affect levels of plasma fibrinogen through an indirect biological mechanism whilst contributing significantly to cognitive dysfunction directly through a process of neuro-inflammation, exacerbating the overall mechanism resulting in age-related cognitive impairment in a large, population, community-dwelling citizens.

Although the presented evidence can be seen as a consistent with causal involvement of plasma fibrinogen in cognitive impairment, there are also issues affecting the validity and strength of the overall results from the meta-analysis. Genetic association studies often require large datasets of few a thousand participants in order

to reach sufficient statistical power due to small effect sizes of individual genetic variants[194]. In order to address this issue, the presented meta-analysis was conducted on a large, collated dataset. Nonetheless, there is a possibility that the overall small magnitude of expected effect sizes might reflect the need for a larger study population.

The instrumental variable, i.e. the pre-selected individual fibrinogen-related SNPs may have not been sufficiently strong. The possibility of a high allele frequency might have resulted in a disruption between the (potentially) causal SNP and the one pre-selected for this analysis (from the literature search or the HapMap database), and therefore the one that was requested from the collaborative centers and subsequently subjected to linear regressions and meta-analysis. Indeed, it has been demonstrated that truly causal SNPs do not necessarily do not necessarily show the strongest associations[199].

Determination of the phenotype of interest, that is the general cognitive ability factor 'g', was conducted from raw cognitive scores received from each cohort independently. However, since data collection was carried out in each collaborative center independently, no control was possible in terms of assuring a standardised operational procedure common to all seven cohorts; although the same cognitive domains were tested in each study, the precise tools differed, especially between the Scottish and English cohorts.

Interestingly, in the SNs- cognition meta-analysis all but one of the statistically significant gene variants came from the Scottish based studies that share the almost

the same psychology cognitive neuropsychological battery (in the SNPs-plasma fibrinogen meta-analysis all seven studies were evenly represented. This has raised the possibility that the benefit of collating data from different cohorts into one large dataset might have been lost due to the heterogeneity in cognitive assessment and scores available to calculate the general cognitive factor.

The possibility of a confounding effect linked to population stratification[21,375] is reasonably unlikely considering that a request was made only for data from a European-origin only subjects and each study investigators conducted a thorough examination and cleaning prior providing data for the presented analysis.

7.4 Conclusion and future direction

The primary aim of this study was to investigate associations between circulating inflammatory biomarkers and cognitive impairment and cognitive decline in advanced age. The aim was addressed in two steps. The first part of this thesis focused on prospective data derived from a diabetic ageing population, the ET2DS.

In this relatively unique, large-scale epidemiological study that focuses entirely on older, diabetic population, plasma levels of cytokines IL-6 and TNF-alpha and acute phase proteins plasma fibrinogen and CRP were modelled in a set of linear regressions to determine change in cognitive ability over 4-years and estimated life-time cognitive decline. In the second stage of the thesis, pre-selected fibrinogen-related SNPs and fibrinogen-related tagging SNPs were modelled against plasma fibrinogen and against the general cognitive ability factor 'g' that was derived through principal component analysis in each study individually. Linear regressions and meta-analysis assessing the strength of genetic associations was carried out on a large data set, consisting of seven, population based cohorts; five Scottish and two English cohorts.

Modelling baseline plasma biomarkers levels against follow up cognitive scores, adjusted either for a respective baseline score (determining four-year change in cognitive performance) or for scores of a vocabulary test estimating peak cognitive ability (determining estimated life-time cognitive change) revealed an overall decline in the general cognitive factor 'g' associated with the baseline levels of all

investigated biomarkers, at all stages of the models (the lowest magnitude was observed on the TNF-alpha models).

The strongest and most statistically significant associations were detected in analyses modeling the general inflammatory maker and IL-6 levels. These associations were most noticeable for tests measuring information processing speed, attention and mental flexibility and working memory. Only slightly weaker relationships were detected in analyses that modeled levels of acute phase proteins (plasma fibrinogen and CRP) with a very similar pattern across cognitive domains. The majority of associations persisted after adjustment for conventional cardiovascular factors and events.

Results of the ET2DS longitudinal analysis have made a contribution to the body of evidence on the systemic inflammation and late-life cognitive ability relationship. Specifically, the presented results add by providing a prospective data analysis, suggesting that increased levels of circulating inflammatory markers might directly affect the brain, possibly through penetrating the blood brain barrier and by their role in neuroinflammation, thereby accelerating age-related cognitive decline. This suggests that patients' risk of accelerated age-related cognitive decline can potentially be reduced though a clinical intervention, focusing on management of systemic inflammation. Further epidemiological studies can compare risk factors associations with cognitive decline between people with and without type 2 diabetes to determine if the ET2DS results are specific only to diabetic older people or general ageing population. This would further clarify whether elevated inflammatory

markers are, and if so, how strongly, risk factors affecting the trajectory of cognitive ageing

With regards to the genetic association analysis, it was hypothesised that polymorphisms that have been shown previously to be determinants of circulating plasma fibrinogen may significantly affect general cognitive ability in a large data set of non-demented, community-dwelling citizens in Scotland and England. I also investigated statistical significance of the relationship between pre-selected SNPs and plasma fibrinogen levels.

Whereas there were 7 SNPs that were associated with levels of plasma fibrinogen at the statistical level of $p<0.05$, only 5 SNPs were significantly associated with impairment in the general cognitive factor 'g'. Polymorphisms rs1130864 (*CRP gene*) seem to affect plasma fibrinogen level, however, this was a marginally significant associations, only just reaching the $p<0.05$ level; also, the effect size magnitude was small. Number of methodological issues might have accounted for this finding, most notable relatively weak predicting variable (plasma fibrinogen-related SNPs were extracted from published literature, therefore there was a substantial lack of control) and relatively weak outcome variable (own to differences between the selection and data collection of cognitive tests that formed the general intelligence factor '*g*'). It is therefore not clear whether these results were false positives, whether they indicate a role of plasma fibrinogen in accelerated decline in general cognitive ability via its hemostatic and rheological properties or whether other inflammatory markers, known to have a strong patho-biological relationship

with plasma fibrinogen have an important role, affecting the quality of cognitive performance directly through the brain neuro-inflammatory mechanisms.

The overall aim of this thesis, (both the prospective analysis of change in cognitive performance in the ET2DS as well as the genetic association analysis) was to explore a possible causal relationship between plasma fibrinogen and impaired general cognitive ability. The results suggest that inflammatory pathways are indeed implicated in accelerated cognitive impairment and decline in ageing populations. The relatively small effect sizes found in the multivariate model might be also accounted for by a methodological issues and nature of research into cognitive ageing.

Strength of this thesis is that it provided evidence of a longitudinal change in cognitive performance in a clearly defined ageing cohort with comprehensive cognitive and biomedical/laboratory data. Modelling baseline measures of biologically related inflammatory markers against different measures of cognitive performance (4-year and life-time) showed results consistent with a relationship between peripheral and central inflammation leading to accelerated cognitive decline. Review of the literature suggested that this was the first large scale study in older people with type 2 diabetes to assess such a wide range of inflammatory markers and to address change over time prospectively.

Further novel findings were generated through a genetic association study conducted in a large dataset collated from well defined, population based prospective studies. Despite the limitations, such as limited strengths of the instrumental variable and

difficulties defining measured phenotype more precisely, meta-analysis of approximately 14 300 subject demonstrated that fibrinogen-related SNPs located in a wide range of genes seem to represent significant determinants of age-related impairment in general cognitive ability, supporting the findings of the prospective ET2DS in terms of an effect of inflammation has on the speed of age-related changes in cognitive performance.

It is still premature to declare a causal nature of the inflammation-cognition relationship. However, the findings have important implications for further studies. In terms of the epidemiological evidence on the role of systemic inflammation in age-related cognitive decline in T2DM, a further follow up wave in the ET2DS, providing serial cognitive assessment scores, would be ideal for modelling the pattern and speed of decline in general cognitive ability. It would also allow observation of the pattern of decline in specific cognitive domains. Such study design could also allow for measurement of biomarker trajectories (i.e. serial laboratory measures of systemic inflammatory markers). Results could further inform subsequent investigations in the form of a randomised control trial testing an evidence-based clinical intervention in a diabetic population.

Furthermore, randomised control trial (RCT), one of the highest-rated designs in epidemiological research, might focus on an investigation of modification of levels of circulating inflammatory markers though a pharmaceutical treatment. However, methodological constrains of RCT, such as requirement of participants' motivation to adhere to allocated type of treatment for a prolonged period of time may lead to samples that are not typical of the general population as well as target (risk group)

population. Also, in comparison to observational cohort studies RCT commonly suffer from a higher attrition rate as well as the reduced duration of follow-up. Alternative approach to assess potential causal role of the risk factors is the use of gene variance as a proxy variable in regression analysis. This, indeed, was the aim of the second part of the presented thesis.

The genetic association study, a number of directions can be taken further. This study focused on plasma fibrinogen related polymorphisms in the three fibrinogen genes themselves as well as genes and variants that showed a significant association in with plasma fibrinogen, despite the fact that their primary role might be through alternative mechanisms. Results of the meta-analysis support and suggest that the role of neuro-inflammation in the aetiology of age-related cognitive impairment is certainly plausible. Further study, modelling a wider range of inflammation-related genetic variants, in particularly those related to IL-1, IL-6 and TNF-alpha would certainly add to the existing evidence. Use of stronger 'instrument' (in terms of association with the relevant circulating biomarker) would also advance knowledge further. In my study, I found only 7 out of 61 pre-selected SNPs to be significantly associated with plasma fibrinogen levels; most likely due to variability of study populations upon which my information was drawn. Recent research suggests that a 'multi-SNPs' instrument may be a stronger instrument for modelling potentially causal associations between biomarkers and outcomes.

The presented thesis has demonstrated the importance of research and clinical attention to age-related cognitive functioning, with emphasis on identification of protective as well risk factors, allowing identification of most vulnerable

populations. Findings of the presented longitudinal analysis (the ET2DS) have been disseminated at Diabetes UK conferences and published in the peer-reviewed journal[171,318]. At the time of completing this thesis, further manuscript focusing directly in the outcome of this thesis, is under review.

This thesis has added to the body of current evidence some highly important results, albeit these represent a fraction of the aetiology of the complex phenomena of cognitive ageing. Future studies could extend this investigation, focusing on a wider array of risk factors and their direct role on the neuro-inflammatory processes within the human brain. Such analyses would ultimately lead to identification of biological and pathological mechanisms responsible for the pattern and speed of cognitive decline and consequently inform further research investigation and clinical intervention.

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Appendices
Appendix A: Results of studies reviewed in Chapter 2

Table 23: Literature Review: Results

Author/ year	Covariates (adjusted analyses)	Results (p-values, OR, CI)				Interpretation/limitations/ implications
Weaver, JD, et al., 2002	A&S; household income, education level, marital status, ethnicity and health (smoking, alcohol, physical activity, BMI, HbA1c, total cholesterol, BL (sys, dias), T2DM, cancer, MI, stroke, hip and/or bone fracture)	Decline (2.5ys): general cognition: diff btw low to mid tertile p=0.002; and diff between mid to high p=0.006 Decline (7ys): general cognition: diff btw low to mid tertile p=0.45; and diff between mid to high p=0.01				Reported baseline characteristics of differences between yes/no follow-up survivors; suggesting survival bias.
Alley, DE., 2008	A&S; household income, education level, marital status, ethnicity and health (smoking, alcohol, physical activity, BMI, HbA1c, total cholesterol, BL (systolic, diastolic), T2DM, cancer, MI, stroke, hip and/or bone fracture)	Abstraction: Impairment: IL6 (>3.8): OR:1.52; 95% CI 1.01-2.27 Global cognition: Impairment 3rd IL6 (>3.8): OR: 1.62; 95% CI: 1.07-2.45) SPMSQ: decline 3rd IL6 (>3.8); OR:1.88; 95% CI: 1.20-2.94 ADJUSTED: SPMSQ: IL (>3.8): OR 1.67; 95% CI: 1.04-2.67) NO significant associations with CRP at any level				Cross-sectional/IL 6 analyses: generally linear negative relationship (equal to ET2DS).
Dik et al., 2005	A&S; education, APOE, depressive symptoms, rheumatic diseases, use of non-steroid anti-inflammatory drugs, aspirin, smoking and alcohol, physical activity, CVD (atherosclerosis, stroke, TIA, T2DM)		1st CRP	2CRP	3CRP	CRP or IL-6 not associated with decline
		µg/mL	0.2-1.7 (326)	1.8-4.8 (342)	4.9-171.0	
		Info Processing	1.0 (reference)	0.87 (0.85-1.34)	1.11 ()0.73-1.68	
		Immediate Recall	1.0 (reference)	1.13 (0.76-1.68)	1.10 (0.73-1.64)	
		Delayed Recall	1.0 (reference)	0.70 (0.48-1.04)	0.82 (0.56-1.21)	

Author/ year	Covariates (adjusted analyses)	Results (p-values, OR, CI)				Interpretation/limitations/ implications
		Fluid Intelligence	1.0 (reference)	1.03 (0.70-1.35)	1.21 (0.82-1.80)	
		MMSE	1.0 (reference)	0.85 (0.50-1.35)	1.26 (0.79-2.01)	
			1st IL6	2IL6	3rd IL6	
		pg/mL	<5.0 pg/mL		5.0-58.3	
		IPS	1.0 (reference)	No data	0.70 (0.39-1.24)	
		IR	1.0 (reference)	No data	1.04 (0.63-1.70)	
		DR	1.0 (reference)	No data	0.74 (0.44-1.26)	
		FI	1.0 (reference)	No data	1.16 (0.71-1.89)	
		MMSE	1.0 (reference)	No data	1.03 (0.60-1.78)	
Schram, MT., et al., 2007	sex, and education level, BMI, diabetes mellitus, and prevalent CVD, APOE e4, CVD, and atherosclerosis	*Rotterdam Study*		*Leiden 85+ Study*		
		CRP (n=2433): R^2; p-value		CRP (n=440): R^2; p-value		
		MMSE	0.02; 0.88	MMSE	0.01; 0.30	
		Global Ability	0.03; 0.43	Global Ability	0.00; 0.69	
		Executive Function	0.02; 0.21	Executive Function	0.02; 0.24	
		Memory (delayed)	0.00; 0.59	Memory (delayed)	0.01; 0.74	
		IL-6 (n=304): R^2; p-value		IL-6 (n=440): R^2; p-value		
		MMSE	0.04; 0.22	MMSE	0.01; 0.15	
		Global Ability	0.03; 0.26	Global Ability	0.01; 0.06	
		Executive Function	0.06; 0.49	Executive Function	0.02; 0.52	
		Memory	0.01; 0.60	Memory	0.02; 0.03	

Author/ year	Covariates (adjusted analyses)	Results (p-values, OR, CI)				Interpretation/limitations/ implications
		(delayed)		(delayed)		
Luciano, M., et al., 2009	BMI, BP (D), Tot chole, T2DM presence, HbA1C, smoking status					All associations accounted for by childhood IQ, CVD risk factors or both. Most interesting result: IQ at 11 years predicted elevation of CRP and fib
Marioni, RE, et al., 2009	ABI, BP (D), Tot cholesterol, smoking status.					Baseline CRP and fibrinogen levels were associated negatively with age and sex-adjusted follow-up scores on the majority of the cognitive tests, and the general cognitive ability factor (correlations= -0.054 to 0.105, $p<.05$). In analyses adjusting for baseline cognitive scores, asymptomatic atherosclerotic disease, and cardiovascular risk factors, both markers predicted decline in several cognitive domains (excluding memory). Baseline plasma viscosity, but not hematocrit, was associated negatively with follow-up test scores for general cognitive ability, information processing speed, and mental flexibility (correlations= -0.050 to -0.098, $p<.05$) and with decline across the same domains ($p<.05$).
Rafnsson, S., et al., 2010	A&S; depression, smoking, alcohol, diabetes/glucose intolerance, major cardiovascular disease from baseline up to 6 months prior to cognitive testing in 2002/03 either respective BL cognitive score or NART		4-years decline		Life-time decline	In a fully adjusted models, plasma fibrinogen associated with a greater 4 year decline in verbal memory and in no-verbal reasoning, but only with verbal memory in life-time cognitive decline
			Beta	SE	Beta	SE
		LM	-1.94	.92*	-3.28	1.52**
		MR	-.97	.55*	-.20	.71
		VF	-.45	.60	-.54	.95
		DST	-.18	.53	-.29	.82
		'g'	-.02	.04	.04	.07
Jefferson, A.L., et al, 2011		CRP - LM-D		.02	-.02-.06	.297
		CRP- TMT-B		.00	-.01-.01	.629
		CRP- visual reproduction		-.01	.00-.03	.648

Author/ year	Covariates (adjusted analyses)	Results (p-values, OR, CI)					Interpretation/limitations/ implications
		CRP – Boston Naming test	**-.02**	**-.04-(-.00)**		**.044**	
		IL6 - LM-D	.02	-.03-.08		.388	
		IL6- TMT-B	.01	-.01-.02		.342	
		IL6- visual reproduction	-.02	-.07-.02		.316	
		TNF-a - LM-D	-.02	-.11-.07		.606	
		TNF-a- TMT-B	**.04**	**.01-.07**		**.004**	
		TNF-a - visual reproduction	.00	-.07-.08		.941	
		TNF-similarities test	**-.08**	**-.16-(-.00)**		**.046**	
Mooijaart, SP., et al., 2011	A+S Country, APOE, adjusted for test applicable to cohort/country						
Mooijaart, SP., et al., 2013		**BL**	Lowest	Middle	Highest	p-values*	**Cross-sectional (5653 subjects):** Higher concentration associated with impaired EF, independent of CVD. No association with memory. **Longitudinal:** IL6 predicted decline in EF and memory, both independent of CVD *Adjusted for co-varieties

Appendix B: ET2DS: Cognitive Testing Pack

PART 3 – Cognitive function testing

Tester: CM CR MK IF Other……. *(circle initials)*

Preliminary tests:

BM ☐☐.☐ *(If < 4.0, re-arrange appointment)*

Cognitive Test Battery:

Time tests started _____:_____ (24hr clock)

Tick box if complete	If test incomplete/difficulty encountered, specify	SCORE 1*	SCORE 2*
☐ Hospital Anxiety & Depression Scale			
☐ Mini-Mental State Examination			
☐ Logical memory 1 (WMS-III)			
☐ Trail-Making Test B (TMB)			
☐ Faces 1 (WMS-III)			
☐ Matrix Reasoning (WAIS-III)			
☐ Digit symbol test (WAIS-III)			
☐ Borkowski Verbal Fluency Task			
☐ Letter-number sequencing (WAIS-III)			
☐ Logical memory 2 (WMS-III)			
☐ Faces 2 (WMS-III)			
☐ Visual reaction test			

* Circle final score for data entry

☐ Mill Hill test returned from subject, **checked** and **labelled**?

Appendix C: Data request forms from the collaborating cohorts

Collaborator's request to access data and/or biological samples from the English Longitudinal Study of Ageing

1. Name of all applicants, affiliations and contact details

a) Ms Markéta Keller, PhD Researcher, University of Edinburgh, Public Health Sciences, Teviot Place, Edinburgh EH8 9AG, m.keller@sms.ed.ac.uk, Tel: 0131 650 6964

b) Dr Jackie Price, Clinical Senior Lecturer in Epidemiology, University of Edinburgh, Public Health Sciences, Teviot Place, Edinburgh EH8 9AG, Jackie.Price@ed.ac.uk, Tel: 0131 650 3240

2. Title of project (less than 30 words): Evaluation of fibrinogen as causal risk factor for cognitive impairment in older age

start date: 01. 05. 2010 end date: 31. 08. 2012

3. Brief description of project (no more than 1-2 sides A4 with up to 10 key references)

(This must include a) a list of SNPs to be genotyped, b) pre-existing data on genotype frequency and c) reference any of the applicant's previous experience in this area)

Background

An increasing body of evidence suggests that circulating plasma fibrinogen may have a role in cognitive decline in ageing populations. A number of large-scale epidemiological studies have demonstrated statistically significant associations

between raised plasma fibrinogen levels and poorer cognitive ability [1] and some have demonstrated association between raised fibrinogen and cognitive decline [2, 3, 4]. There are also patho-physiological mechanisms which might explain a direct relationship between circulating fibrinogen levels and reduced cognitive ability, including reduced cerebral blood flow associated with raised plasma viscosity as a result of elevated fibrinogen levels in the blood. However, associations between plasma fibrinogen and cognition reported in epidemiological studies may be the result of confounding, for example by vascular diseases (although vascular disease may also be a mediating factor). It is therefore important to investigate the potential causal nature of such epidemiological associations. One method of doing this is to use fibrinogen genotype, known to affect fibrinogen levels, as proxy for plasma fibrinogen levels (the instrumental variable approach) and to test the association between fibrinogen genotype and cognitive ability directly. In our own research, on a diabetic cohort, we have demonstrated an association between fibrinogen gene variants (*FGA* and *FGB*) and cognitive ability, but findings were not replicated in other populations. However, the total sample size of the included studies was relatively small and genetic variants were not selected systematically to best reflect variation in fibrinogen levels.

Aim

The aim of the project is to determine the association between pre-selected SNPs (those which are known or suspected to affect plasma fibrinogen levels) and cognitive phenotypes in meta-analysis of large cohort studies, including those based in Edinburgh (AAA Trial, ET2DS, EAS and LBC 1936), ELSA and Whitehall II.

The Project

Systematic review and discussion with experts in the field to identify SNPs associated with altered fibrinogen levels (and possibly fibrinogen structure), including review of the results of recent GWAS studies. Please see the attached Excel sheet for the final results of a literature search for fibrinogen related SNPs (115) and for fibrinogen related tagSNPs (identified through the International

HapMap Project website (495). This list of SNPs will help to determine which SNPs are available in each of the included studies and which SNPs are common to more than one study.

Collation of data from the 6 included studies and discussion around the potential for additional genotyping where individual studies do not already have fibrinogen-related SNP data. J Price has some limited funds which could potentially support this additional genotyping if necessary, or funds may become available through UCLEB/metabochip.

Analysis of association between pre-selected SNPs and plasma fibrinogen levels in each study.

Analysis of association between pre-selected SNPs and cognitive phenotypes, where possible, as meta-analysis (depending on study homogeneity and ability to derive a common cognitive outcome across studies).

References:

1. Marioni, R., E., et al., *Genes, Brain and Behaviour, Vol. 9 Issue 3*, Pages 348 - 352

2. Rafnsson, S., B., Deary, I., J., Smith, S., B., et al., *JAGS 2007;55:700-7*

3. Schram, M., T., Euser, S., M., de Craen, A., J., M., et al., *JAGS 2007;55:708-16*

4. Van Oijen, M., Witteman, J., C., Hofman, A., et al., *Stroke. 2005;36:2637*

4. Rationale for undertaking this project with the ELSA rather than other datasets

Availability of detailed cognitive function testing on a large, elderly cohort and data on biomarkers of interest.

5. If DNA is being requested, please state amount and rationale for request for

DNA

At this stage in the collaboration, no additional genotyping is being requested as we will first determine which fibrinogen-related SNPs are already available in each study before proceeding further

6. Sample size calculation N/A

7. Funding: Has/will the project be(en) peer reviewed and funded?

The project is part of an ESRC PhD studentship awarded to Marketa Keller

8. Analyses: What key variables will be required for analysis?

SNP data – please refer to the attached Excel file

Plasma fibrinogen (measured at as many phases of the study as possible)

Cognitive phenotypes – measures for tests of each cognitive domain and where available, the general intelligence factor '*g*'

Estimate of pre-morbid cognitive functioning (measured by MHVS or NART).

Assessment of anxiety and depression

Demographic data – age, age at the data collection time, sex, socio-economic background, smoking history, history of alcohol consumption, history of type 2 diabetes

9. Electronic data: Will the project require deriving or producing new variables from existing data?

No

10. DNA samples: Are there any special requirements for the DNA extraction or potential problems with using pre-amplified rather than genomic DNA?

N/A

11. Feasibility and quality control: Do(es) the applicant(s) have sufficient experience and expertise to carry out the analysis?

Although forming part of the PhD project, the analysis will be closely supervised by Dr Jackie Price (epidemiologist) and Dr Niall Anderson (senior lecturer in genetic statistics, University of Edinburgh). Additional advice on analysis will be available from Professor Paul McKeigue (expert in Mendelian Randomisation analyses). Professor Ian Deary (psychologist) and other members of the MRC centre for Cognitive Ageing and Cognitive Epidemiology in Edinburgh. We also foresee that analyses will be discussed with ELSA collaborators as part of efforts towards joint publication of results.

12. Collaborative input: Will the applicants be working work collaboratively with the original investigators and/or a member from the ELSA team

Yes, with Dr Meena Kumari

13. **Statistical expertise: Will the analysis require statistical input from ELSA?**
See above

14. Agreement:

I confirm that I have read the above and am happy to comply with the terms and conditions.

Signature M. Keller Date: 17. 05. 2011

Name Markéta Keller

LBC1921/36 Data request form

Provisional title of study: Evaluation of fibrinogen as causal risk factor for cognitive impairment in older age – study of 6 ageing cohorts
Principal researcher (include institution address, email & phone) Markéta Keller College of Medicine and Veterinary Medicine The University of Edinburgh Teviot Place Edinburgh; EH8 9AG Email: m.keller@sms.ed.ac.uk Tel: 0784 343 2017
Provisional author list Dr Jackie Price Dr Mark Strachan Dr Niall Anderson Prof Ian Deary Ms Insa Feinkohl

Dr Stela Masle

And representatives from collaborating centres

Brief rationale for study

Background

An increasing body of evidence suggests that circulating plasma fibrinogen may have a role in cognitive decline in ageing populations. A number of large-scale epidemiological studies have demonstrated statistically significant associations between raised plasma fibrinogen levels and poorer cognitive ability [1] and some have demonstrated association between raised fibrinogen and cognitive decline [2, 3, 4]. There are also patho-physiological mechanisms which might explain a direct relationship between circulating fibrinogen levels and reduced cognitive ability, including reduced cerebral blood flow associated with raised plasma viscosity as a result of elevated fibrinogen levels in the blood. However, associations between plasma fibrinogen and cognition reported in epidemiological studies may be the result of confounding, for example by vascular diseases (although vascular disease may also be a mediating factor). It is therefore important to investigate the potential causal nature of such epidemiological associations. One method of doing this is to use fibrinogen genotype, known to affect fibrinogen levels, as proxy for plasma fibrinogen levels (the instrumental variable approach) and to test the association between fibrinogen genotype and cognitive ability directly. In our own research, on a diabetic cohort, we have demonstrated an association between fibrinogen gene variants (*FGA* and *FGB*) and cognitive ability, but findings were not replicated in other populations. However, the total sample size of the included studies

was relatively small and genetic variants were not selected systematically to best reflect variation in fibrinogen levels.

Aim

The aim of the project is to determine the association between pre-selected SNPs, known or suspected to affect plasma fibrinogen levels, and cognitive phenotypes in meta-analysis of large cohort studies, including those based in Edinburgh (AAA Trial, ET2DS, EAS and LBC1936), ELSA and Whitehall II.

1. Marioni, R., E., et al., *Genes, Brain and Behaviour, Vol. 9 Issue 3*, Pages 348 - 352

2. Rafnsson, S., B., Deary, I., J., Smith, S., B., et al., *JAGS 2007;55:700-7*

3. Schram, M., T., Euser, S., M., de Craen, A., J., M., et al., *JAGS 2007;55:708-16*

4. Van Oijen, M., Witteman, J., C., Hofman, A., et al., *Stroke. 2005;36:2637*

Main variables to be analysed

Data required for analysis

Demographic data:

socio-economic background

smoking history

history of alcohol consumption

history of type 2 diabetes

Data for statistical analysis: age, gender age at the data collection fibrinogen related SNPs (please refer to attached Excel Sheets) fibrinogen (plasma level, when collected) history of cardiovascular events (MI, angina, stroke) current (and previous where available) cognitive functioning values gained for individual testing, general intelligence factor 'g' where available estimate of pre-morbid cognitive functioning (measured by MHVS or NART). assessment of anxiety and depression
Which journal(s) are you considering? Genes Brain Behaviour Age and Ageing Psychosomatic Medicine

Signed: Markéta Keller M. Keller Date: 31. 10. 2011

(Applicant)

Signed: _____ Date:_____

(Ian Deary, study director)

Whitehall II Data Application Form

Preferred member of the WII team to be the WII Contact Researcher (if any):

Dr Meena Kumari

1. PRINCIPAL INVESTIGATOR

Title, forename, surname: Ms Markéta Keller

Employing Organisation: The University of Edinburgh

Position in organisation: PhD student

Address of organisation: Teviot Place; College of Medicine and Veterinary Medicine; Edinburgh; EH8 9AG

Telephone: 0131 6506964

Email: m.keller@sms.ed.ac.uk

Dr Jackie Price

Employing Organisation: The University of Edinburgh

Position in organisation: Clinical Senior Lecturer in Epidemiology

Address of organisation: Teviot Place; College of Medicine and Veterinary Medicine; Edinburgh; EH8 9AG

Telephone: 0131 650 3240

Email: Jackie.price@ed.ac.uk

Please attach the Principal Investigator's Curriculum Vitae.

2. RESEARCH TEAM / CO-APPLICANTS

Details of each Research Team member involved in the proposed project.

Research Team members / Co-applicants Employing Organisation Position in organisation Contact details (Email address/Telephone no)

Dr Jackie Price; The University of Edinburgh

Clinical Senior Lecturer in Epidemiology and Public Health Tel: 0131 650 3240

Jackie.Price@ed.ac.uk

Ms Markéta Keller The University of Edinburgh PhD student Tel: 0131 6506964

m.keller@sms.ed.ac.uk

3. PUBLICATIONS OF THE RESEARCH TEAM MEMBERS

List of the main publications of each Research Team member involved in the project.

Dr Price:

Lind PA, Luciano M, Horan MA, Marioni RE, Wright MJ, Bates TC, Rabbitt P, Harris SE, Davidson Y, Deary IJ, Gibbons L, Pickles A, Ollier W, Pendleton N, Price JF, Payton A, Martin NG. No association between cholinergic muscarinic receptor 2 (CHRM2) genetic variation and cognitive abilities in three independent samples. Behavior Genetics 2009;39:513-23.

Marioni RE, Stewart MC, Murray GD, Deary IJ, Fowkes FGR, Lowe GDO, Rumley A, Price JF. Peripheral levels of fibrinogen, C-reactive protein, and plasma viscosity predict future cognitive decline in non-demented individuals. Psychosom Med 2009; 71:901-6.

Riccardo E. Marioni, Ian J. Deary, Gordon D. Murray, Gordon D. O. Lowe, Snorri B. Rafnsson, Mark W. J. Strachan, Michelle Luciano, Lorna M. Houlihan, Alan J. Gow, Sarah E. Harris, Marlene C. Stewart, Ann Rumley, F. Gerry R. Fowkes, Jackie F. Price. Genetic Variants Associated With Altered Plasma Levels of C-Reactive Protein are not Associated With Late-Life Cognitive Ability in Four Scottish Samples. Behav Genet. 2010;40:3-11.

Marioni R, Strachan MWJ, Reynolds R, Deary IJ, Lowe GDO, Rumley A, Fowkes FGR, Frier BM, Lee A, Murray GD, Price JF. Association between raised inflammatory markers and cognitive decline in elderly people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. Diabetes 2010 Mar;59(3):710-3.

Marioni RE, Lowe GDO, Rumley A, Murray GD, Deary IJ, Strachan MWJ, Price JF, on behalf of the ET2DS investigators. Blood rheology and cognition in the Edinburgh Type 2 Diabetes Study. Age Aging 2010;39:354-9.

Riccardo E Marioni, Ian J Deary, Gordon D Murray, F Gerry R Fowkes, Jackie F Price. Associations between polymorphisms in five inflammation-related genes and cognitive ability in an elderly population. Genes Brain Behav. 2010;9:348-52.

Bolton JL, Marioni RE, Deary IJ, Harris SE, Stewart MC, Murray GD, Fowkes FGR, Price JF. Association between Polymorphisms of the Dopamine Receptor D2 and Catechol-o-Methyl Transferase Genes and Cognitive Function. Behavior Genetics 2010:40:630-8.

Houlihan LM, Wyatt ND, Harris SE, Hayward C, Gow AJ, Marioni RE, Strachan MW, Price JF, Starr JM, Wright AF, Deary IJ. Variation in the uric acid transporter gene (SLC2A9) and memory performance. Hum Mol Genet. 2010;19:2321-30.

Riccardo Marioni, Ian Deary, Gordon Murray, Gordon Lowe, Mark Strachan, Michelle Luciano, Lorna Houlihan, Alan Gow, Sarah Harris, Ann Rumley, Marlene Stewart, F. Fowkes, Jackie Price. Genetic Associations Between Fibrinogen and Cognitive Performance in Three Scottish Cohort. Behavior Genetics (In Press - available on line).

Aspasia Angelakopoulou, Tina Shah, Reecha Sofat, Sonia Shah, Diane Berry, Jackie Cooper, Jutta Palmen, Ioanna Tzoulaki, Andrew Wong, Barbara J Jefferis, Nikolas Maniatis, Fotios Drenos, Bruna Gigante, Rebecca Hardy, Ross Laxton, Karin Leander, Anna Motterle, Iain Simpson, Liam Smeeth, Andy Thomson, Claudio Verzilli, Diana Kuh, Helen Ireland, John Deanfield, Mark Caulfield, Chris Wallace, Nilesh Samani, Patricia B Munroe, Mark Lathrop, F Gerry Fowkes, Michael Marmot, Peter H Whincup, John Whittaker, Ulf de Faire, Mika Kivimaki, Meena Kumari, Elina Hypponen, Chris Power, Steve E Humphries, Philippa Talmud, Jackie Price, Richard Morris, Shu Ye, Juan Pablo Casas, Aroon D Hingorani. Integration of GWAS signals for lipids, diabetes

and coronary heart disease: A strategy to map causal pathways. Cardiovascular Biomarker Genetics Collaboration. European Heart Journal (under re-submission Feb 2011).

Ms Keller:

Neal, R., Keller, M., Belcher, J., and Wilkinson, C., 2099, 'How are men with prostate cancer followed up in the UK? Analysis of data from primary care case-notes and the general practice research database', Copenhagen SAPC abstract.

Member of the Prostate Cancer research team for the following:

Watson E, O'Brien R, Campbell C, Weller D, Neal RD, Wilkinson C, Rose P. The push towards primary care? Views of health professionals on the role of primary care in the follow-up of men with prostate cancer. Family Practice (in press)

O'Brien R, Rose P, Campbell C, Weller D, Neal RD, Wilkinson C, McIntosh H, Watson E. "I wish I'dtold them": a qualitative study examining the unmet psychosocial needs of prostate cancer patients during follow-up after treatment. Patient Education and Counselling 2010 doi:10.1016/j.pec.2010.07.006

O'Brien R, Rose PW, Campbell C, Weller D, Neal RD, Wilkinson C, Watson E. Prostate cancer patients' experiences of follow-up after treatment: a qualitative study. BJU International 2010 DOI:10.1111/j.1464-410X.2010.09292.x

McIntosh HM, Neal RD, Rose P, Watson E, Wilkinson C, Weller D, Campbell C. Follow-up care for men with prostate cancer and the role of primary care: a systematic review of international guidelines. Br J Cancer 2009;100:1852-1860

4. PREVIOUS APPLICATIONS

Have you or any of the Research Team members/Co-applicants applied for Whitehall II data in the past? No, we have not.

5. FUNDING

Do you already have funding to carry out this project? No – part of a PhD studentship

If you are planning to seek funding to carry out this project and the grant application is to be partially or totally based in the use of Whitehall II data, please give details about the funding application.

Synopsis of application (max 100 words)

6. RESEARCH PROJECT

6.1 PROJECT TITLE

Causal Risks for Age Related Cognitive Impairment

6.2 SUMMARY

The aim of the project is to determine the association between pre-selected SNPs (those which are known or suspected to affect plasma fibrinogen levels) and cognitive phenotypes in meta-analysis of large cohort studies, including those based in Edinburgh (AAA Trial, ET2DS and EAS), ELSA and Whitehall II.

6.3 CONTEXT

N/A

6.4 PROJECT DESCRIPTION

Background

An increasing body of evidence suggests that circulating plasma fibrinogen may have a role in cognitive decline in ageing populations. A number of large-scale epidemiological studies have demonstrated statistically significant associations between raised plasma fibrinogen levels and poorer cognitive ability 1 and some have demonstrated association between raised fibrinogen and cognitive decline 2, 3, 4. There are also patho-physiological mechanisms which might explain a direct relationship between circulating fibrinogen levels and reduced cognitive ability, including reduced cerebral blood flow associated with raised plasma viscosity as a result of elevated fibrinogen levels in the blood. However, associations between plasma fibrinogen and cognition reported in epidemiological studies may be the result of confounding, for example by vascular diseases (although vascular disease may also be a mediating factor). It is therefore important to investigate the potential causal nature of such epidemiological associations. One method of doing this is to use fibrinogen genotype, known to affect fibrinogen levels, as proxy for plasma fibrinogen levels (the instrumental variable approach) and to test the association between fibrinogen genotype and cognitive ability directly.

In our research, on a diabetic cohort (ET2DS), we have demonstrated an association between fibrinogen gene variants (FGA and FGB) and cognitive ability, but findings were not replicated in other populations. However, the total sample size of the included studies was relatively small therefore our aim is to carry out this analysis on large number of participants.

Scientific hypothesis

In line with the principle of Mendelian Randomisation, our aim is to determine the potential causal relationship between the circulating plasma fibrinogen level and cognitive functioning in ageing population.

Objectives

We propose to examine the relationship between pre-selected fibrinogen related SNPs and:

- plasma fibrinogen level;
- current cognitive functioning;
- pre-morbid cognition;
- cognitive decline (determined by discrepancy between pre-morbid and current cognitive function

Methodology and planned statistical analyses

1. Literature review identified SNPs associated with altered fibrinogen levels (and possibly fibrinogen structure), including review of the results of recent GWAS studies. Please see the attached Excel sheet for the final number of SNPs (115) and tagSNPs (495).

2. Data collection (ET2DS); follow up after 4 years, anticipated the end of May, 2011. This dataset will contribute to the meta-analysis.

3. Meta-analysis of 6 large cohort

Planned statistical analysis

The details of the statistical analysis plan are still to be decided. The primary analysis will concentrate on the association between SNPs and either (i) tests of cognitive function common to all cohorts or (ii) a summary cognitive score derived from each cohort.

Additionally, the following analyses will be carried out:

- Descriptive analysis to determine population characterises
- Principal component analysis to generate the 'g' (general intelligence factor)

Competing interests

None known.

References (max 10)

1. Marioni, R., E., et al., Genes, Brain and Behaviour, Vol. 9 Issue 3, Pages 348 - 352

2. Rafnsson, S., B., Deary, I., J., Smith, S., B., et al., JAGS 2007;55:700-7

3. Schram, M., T., Euser, S., M., de Craen, A., J., M., et al., JAGS 2007;55:708-16

4. Van Oijen, M., Witteman, J., C., Hofman, A., et al., Stroke. 2005;36:2637

6.5 PLANNED SCIENTIFIC OUTPUTS

Intended outputs/publications arising from the use of these data, including abstracts, posters and research papers.

1. PhD thesis
2. number of publications in peer reviewed journals

7. DATA REQUESTED

Data required for analysis:

Demographic data:

- socio-economic background
- smoking history
- history of alcohol consumption
- history of type 2 diabetes

Data for statistical analysis:

- age
- age at the data collection time

- sex
- fibrinogen related SNPs (please refer to attached Excel Sheet)
- fibrinogen (plasma level)
- current cognitive functioning; values gained for individual testing, general intelligence factor 'g' where available
- estimate of pre-morbid cognitive functioning (measured by MHVS or NART).
- assessment of anxiety and depression

1) Download the WII data dictionary from the Whitehall II website (www.ucl.ac.uk/whitehallII). It is an Excel file containing the exact variables names of the data items held in the WII database.

2) Highlight all of the variables needed. The list of highlighted items must be consistent with the project proposal.

3) Attach the highlighted WII data dictionary file to this application form.

Whitehall II Data User's Agreement

To be signed once the data sharing application has been approved.

Title, forename, surname: Markéta Keller, MSc

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Telephone: 0131 6503124

Email: m.keller@sms.ed.ac.uk

Project title: Systemic Inflammation and Late-Life Cognitive Ability

I agree that my project will use the requested Whitehall II data and is to be conducted according to the guidelines specified in the document entitled "Whitehall II Policy on Data Sharing". I confirm that I have read this document and agree to abide by the terms and conditions outlined there within.

I accept that my access to the Whitehall II resource is limited only to what is relevant for completion of the above named project.

Signature of WII data user: M. Keller

Name in block capitals: MARKÉTA KELLER

Date: 11. 01. 2011

Signature of research members Names in block capitals Date

Signature of the WII Contact Researcher:

Name in block capitals: .

Date: .

Signature of Prof. Sir Michael Marmot/ Prof Mika Kivimaki:

Name in block capitals: .

Date: .

Please sign, date and return to the WII Contact Researcher for counter-signature.

Appendix D: fibrinogen-related SNP; literature search

Table 24: Literature search – fibrinogen-related SNPs; GWAS

Study	Sample	Outcome	Design	ES/CI/ etc.	Gene/Loci/ Position	SNP	Stats	Results/Comments
Danik, JS, et al., 2009 [Catalogue,all other]	17686 healthy women	GWAS	337343 SNPs associated with plasma fibri level The Women's Genome Health Study		IL6R IL6R IL6R IL6R IL6R CPS1 FGA,FGB, FGG SLC22A5, SLC22A4 (same loci[SL]) IRF1 CD300LF, SLC9A3R1(SL) NAT9	rs6684439 rs4845623 rs4537545 rs4129267 rs8192284 rs7422339 rs1388070 rs4482740 rs7654425 rs7698829 rs1800790 rs6056 rs4220 rs1044291 rs2070016 rs1049636 rs1800788 rs1016988 rs10479002 rs10512597 rs1037170		19 SNPs were associated-all associations in paper. Fibrinogen and CRP level correlate (r=0.4), these genes were assessed. IL6R (8192284) and CD300LF SNP associated with CRP level. **LEPR!!!**
Dehghan, A., et al., 2009 [all]	GWA analysis on 6 population based studies (N=22116), European Ancestry	CV risk factors, used WHGS to replicate genome wide sign findings	RS (N=2068) FHS (N=7022) CHS (N=1993) ARIC (N=8051) MONICA/KORA (N=1523)	2661766 SNPs from 22096 subjects SNP call rate:	FGB exon7 IRF1 PCCB intron1 NLRP3	rs1800789 rs2522056 rs51154 rs1539019	$P=1.8x10^{-30}$ $P=1.3x10^{-15}$ $P=5.94x10^{-10}$ $P=1.04x10^{-8}$	4 loci marked by SNPs showing GW sign $P<5.0x10^{-8)}$;3 novel loci and 1 SNP in FGB **NB: *rs1800787*know to directly affect gene**

Study	Sample	Outcome	Design	ES/CI/ etc.	Gene/Loci/ Position	SNP	Stats	Results/Comments
			BBC 1958 (N=1459)	<90% >95% <95% <90% <95%				**transcription in basal and IL-6 stimulated condition**
Reiner, et al., 2006 [18]	DNA sample was available (*n* = 3788). USA	association btw common SNPs in the fibri genes and circulating levels of both 'functional' fibri and total fibri in a large, multi-center, bi-racial cohort of young US adults.	CARDIA STUDY A total of 59 polymorphic sites had a minor allele frequency of ≥ 5% in either the EA or AA SNP discovery sample		FGB FGA FGG 	rs1800791 rs1800790 rs1800788 rs6058 rs2227421 rs2227425 rs2070006 rs2070011 **rs2070016** rs2070017 rs2070018 rs6050 rs2070033 rs2070022 rs1800792 rs2066874 rs2066860 rs1049636 rs2066880 rs2066865	*All values in article, p.*	A common haplotype tagged by the A minor allele of the well-studied *FGB*−455 G/A promoter polymorphism (*FGB* 1437) was confirmed to be strongly associated with increased plasma fibrinogen levels. Two non-coding variants specific to African-American chromosomes, *FGA* 3845 A and *FGG* 5729 G, were each associated with lower plasma fibrinogen levels. In European-Americans, a common haplotype tagged by *FGA* Thr312Ala

Table 25: Literature search – fibrinogen-related SNPs; gene-associations/meta-analyses studies

Study	Sample	Outcome	Design	ES/CI/ etc.	Gene/Loci/ Position	SNP	Stats	Results
Bis, J.C., et al., 2009 [all]	6 GWAS; total 22096	CHARGE consortium	Meta-analysis if 6 GWAS (ARIC, CHS, FHS, RS, MONICA/KORA, Ausburg Study and BBCS 1958)		FGB FGB (exon 7) IRF1		$P<5.0 \times 10^{-8}$ $P<1.8 \times 10^{-30}$ $P<1.3 \times 10^{-15}$	All 4 loci confirmed by WGHS (Danik et al, 2009). Details of these GWAS below, also summery presented in Deghan, 2009
Chen X. , et al., 2008 [E+M]	1488 cases; 1234 control 1023 cases 1081 controls Chinese	CAD	Meta-analysis	95%; 0.94-1.84, 95% 1.24-2.46	FGB FGB	-148C/T -455G/A	P = 0.11 P=0.001	Absence of an association between the beta-fibrinogen gene -148C/T polymorphism and susceptibility to CAD and the possibility that -455G/A polymorphism (in particular, allele A) increases susceptibility to this disease in the Chinese population.
Gohil R. 2009 [E+M] Only abstract available	126,525 cases and 184,068 controls derived from 173 case-control studies	comprehensive meta-analysis on all candidate genes to assess their genetic contribution to the aetiology of venous thromboembolism	Not reported	CI 95% for each study	FGB	None included		Other than fibri gene variants also identified
Humphries SE., 1995 [M]	Review - genetic regulation of	Not reported	Not reported	Not reported	FGB FGB	-455 -148		Accounting for 3.1% variances of plasma fibri level

Study	Sample	Outcome	Design	ES/CI/ etc.	Gene/Loci/ Position	SNP	Stats	Results
	fibrinogen							
Iacoviello, L., et al., 2001 [ref]	Review of 23 studies	Fibri-gene interaction Gene CVD/MI association	Total number not included	No info	FGB	-455		18 significant association 3 non 1 +/- association
Morgan, TM., et al., 2007 [M]	811 patients 650 cont., Kansas City, white only	An extensive validation of putative genetic risk factors for Acute coronary syndrome	Replication of 85 variance in 70 genes		FGB	-455	P=0.03	Only the -4ff promoter was Any of the 85 varia nominally sign, otherwise nces were significant
Smith et al., 2005 [M+E]	12,393 cases; 21,649 controls	Association of gene variance with plasma fibri and CHD	Meta-analysis of 19 studies		FGB	-148C/T -455G/A		Fibri robustly related to genetic variants BUT unrelated to CHD
Voetsch B. Loscalzo J., 2004 [M]	Review (132 references)	Review of the most common genetic variations involved in the pathogenesis of arterial thrombosis	Review (132 references)		FGB	-455 G/A -854 G/A		Incosistently associated with plasma fibri level and with phenotype
Iacoviello, L., et al., 2001 [ref]	Total number not included	Fibri-gene interaction Gene CVD/MI association	Review of 23 studies (1991-2001)	No info	FGB FGB	-455A 0854G/A	Present in +/-20% of population and associated with .30g/L fibri	18 significant association 3 non 1 +/- association
De Maat, MP., et al., 2001 [p]	Review	Effects of diet, drugs and genes on plasma fibri	Not reported			-455G/A -148C/T	None reported	Both associated with plasma fibri level but not with risk of CV event. 148C/T good as close to IL-6 receptor
Keavney,	4685	MI	A meta-analysis	FGB	rs1800790	P=0.015C/C		Genotype also mild sign

Study	Sample	Outcome	Design	ES/CI/ etc.	Gene/Loci/ Position	SNP	Stats	Results
B., et al., 2006 [all]	confirmed MI 3460 controls all UK		of ISIS (International Studies of Infarct Survival) and 19 other studies of beta-fibrinogen genotypes (12 220 coronary disease cases and 18716 controls)	FGB	1148C/T Both in complete LD	P=0.022C/T P=0.064T/T; trend value p<0.001		associated with apoliprotein B/A Not major determinant in coronary disease

Appendix E:

Table 26: Final list of pre-selected fibrinogen-SNPs

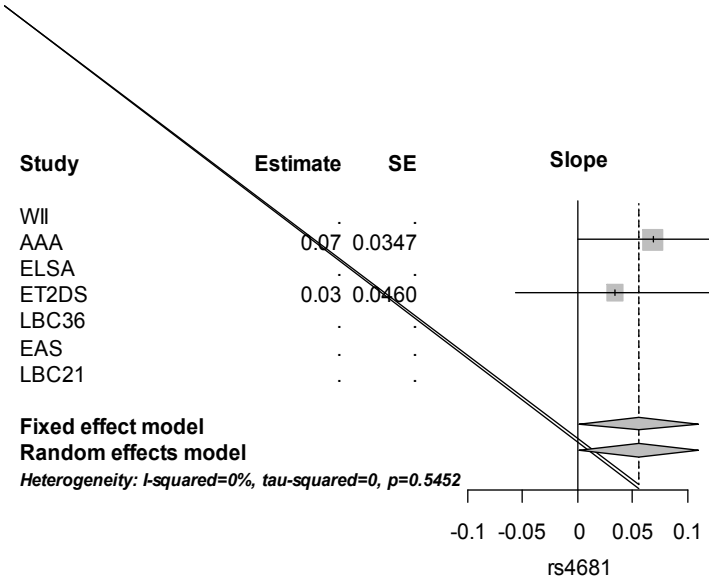
SNP name	AAA	EAS	ELSA	ET2DS	LBC21	LBC36	WII	p value	re-coding	Alleles
	2061	533	5600	1045	517	1005	3413			
rs4129267		x	x	x	x	x	x	0.0603	GG=2; AG=1; AA=0	AG
rs7518199		x	x	x			x	0.076	AA=2; CA=1; CC=0	AC
rs8137951				x	x	x	x	0.0158	GG=2; AG=1; AA=0	AG
rs315952			x		x	x		0.903	AA=2; AG=1; GG=1	AG
rs4537545			x		x	x		0.284	GG=2; AG=1; AA=0	AG
rs9853387		x		x			x	0.1307	AA=2; GA=1; GG=0	AG
rs1279840		x		x			x	0.5474	GG=2; AG=1; AA=0	AG
rs1800497	x			x				0.7733	CC=2; CT=1; TT=0	CT
rs2070016	x			x	x	x		0.2774	TT=2; CT=1; CC=0	CT
rs4681	x			x				0.5452	AA=2; GA=1; GG=0	GA
rs4251961		x	x	x			x	0.6098	AA=2; GA=1; GG=0	AG
rs1130864	x	x		x	x	x		0.7189	CC=2; CT=1; TT=0	CT
rs10889550		x		x	x	x	x	0.0493	AA=2; AG=1; GG=1	AG
rs9436297		x		x	x	x	x	0.2231	AA=2; GA=1; GG=0	AG
rs11208660		x		x			x	0.0364	GG=2; AG=1; AA=0	AG
rs1137101		x		x	x	x	x	0.7488	AA=2; GA=1; GG=0	AG
rs17415296		x		x			x	0.0443	CC=2; AC=1; AA=0	AC

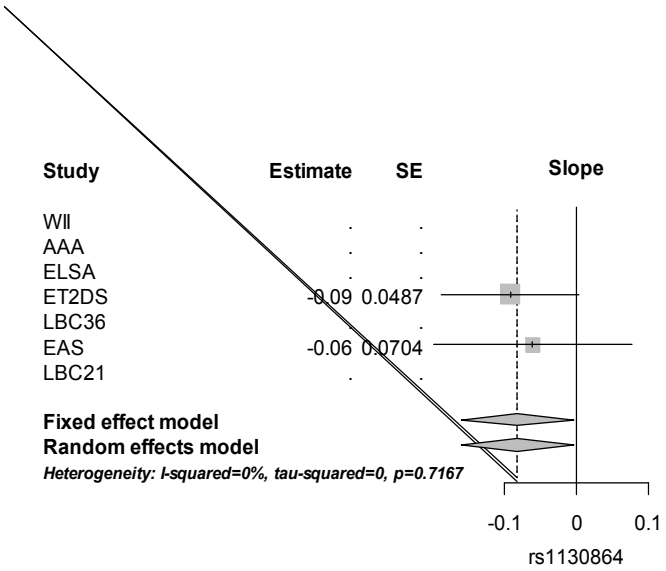
SNP name	AAA	EAS	ELSA	ET2DS	LBC21	LBC36	WII	p value	re-coding	Alleles
	2061	533	5600	1045	517	1005	3413			
rs4845625		x		x			x	0.0187	GG=2; AG=1; AA=0	AG
rs6689393		x		x			x	0.0361	GG=2; AG=1; AA=0	AG
rs4072391		x		x			x	0.0411	GG=2; AG=1; AA=0	AG
rs2287049				x	x	x	x	0.0002	AA=2; GA=1; GG=0	AG
rs3171845		x		x	x	x	x	0.7729	GG=2; AG=1; AA=0	AG
rs315921		x		x			x	0.1593	GG=2; AG=1; AA=0	AG
rs12613336		x		x	x	x	x	0.0177	AA=2; GA=1; GG=0	AG
rs7607205		x		x	x	x	x	0.5386	CC=2; AC=1; AA=0	AC
rs6750325		x		x			x	0.7207	CC=2; AC=1; AA=0	AC
rs6748782		x		x	x	x	x	0.2946	CC=2; AC=1; AA=0	AC
rs2887915		x		x	x	x	x	0.6406	GG=2; AG=1; AA=0	AG
rs10490320		x		x	x	x	x	0.6606	AA=2; GA=1; GG=0	AG
rs7574001		x		x			x	0.0111	AA=2; GA=1; GG=0	AG
rs9844666		x		x	x	x	x	0.3895	GG=2; AG=1; AA=0	AG
rs1153877		x		x			x	0.1598	GG=2; AG=1; AA=0	AG
rs556788		x		x			x	0.1276	AA=2; GA=1; GG=0	AG
rs16844401		x		x	x	x	x	0.4095	GG=2; AG=1; AA=0	AG
rs9282763		x		x			x	0.0853	AA=2; GA=1; GG=0	AG
rs1148		x		x	x	x	x	0.2261	AA=2; CA=1; CC=0	AC
rs2069827		x		x				0.1983	CC=2; AC=1; AA=0	AC
rs2069840	x	x		x				0.004	GG=2; CG=1; CC=0	CG

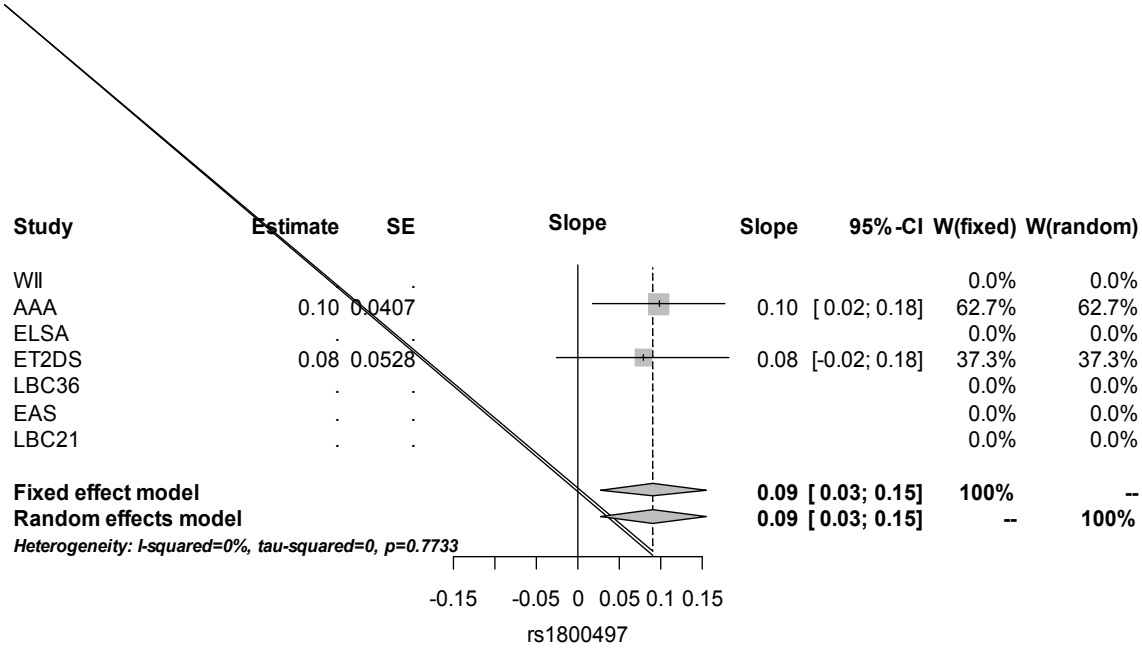
SNP name	AAA	EAS	ELSA	ET2DS	LBC21	LBC36	WII	p value	re-coding	Alleles
	2061	533	5600	1045	517	1005	3413			
rs10733789		x		x	x	x	x	0.3696	AA=2; GA=1; GG=0	AG
rs6479891		x		x			x	0.9282	GG=2; AG=1; AA=0	AG
rs7081614		x		x			x	0.0193	AA=2; GA=1; GG=0	AG
rs10822152		x		x			x	0.4884	AA=2; GA=1; GG=0	AG
rs10761741		x		x			x	0.4467	CC=2; AC=1; AA=0	AC
rs7923609		x		x	x	x	x	0.4105	AA=2; GA=1; GG=0	AG
rs10995530		x		x			x	0.0221	AA=2; GA=1; GG=0	AG
rs1169288		x		x			x	0.679	AA=2; CA=1; CC=0	AC
rs2464196	x	x		x	x	x	x	0.7093	GG=2; AG=1; AA=0	AG
rs4817986		x		x			x	0.0905	CC=2; AC=1; AA=0	AC
rs715586		x		x	x	x	x	0.3007	GG=2; AG=1; AA=0	AG
rs2285395		x		x	x	x	x	0.3904	GG=2; AG=1; AA=0	AG
rs11265618			x		x	x		0.1934	GG=2; AG=1; AA=0	AG
rs1386821			x		x	x		0.5904	AA=2; AC=1; CC=0	AC
rs2069832	x		x					0.6302	GG=2; AG=1; AA=0	AG
rs2070011	x		x					0.597	GG=2; AG=1; AA=0	AG
rs4240872			x		x	x		0.2002	AA=2; AG=1; GG=1	AG
rs2287047					x	x	x	0.0011	GG=2; AG=1; AA=0	AG
rs2070022	x				x	x		0.5964	CC=2; CT=1; TT=0	CT
rs17070145	x			x				0.7733	CC=2; CT=1; TT=0	CT
rs2227412	x			x				0.0449	AA=2; GA=1; GG=0	AG

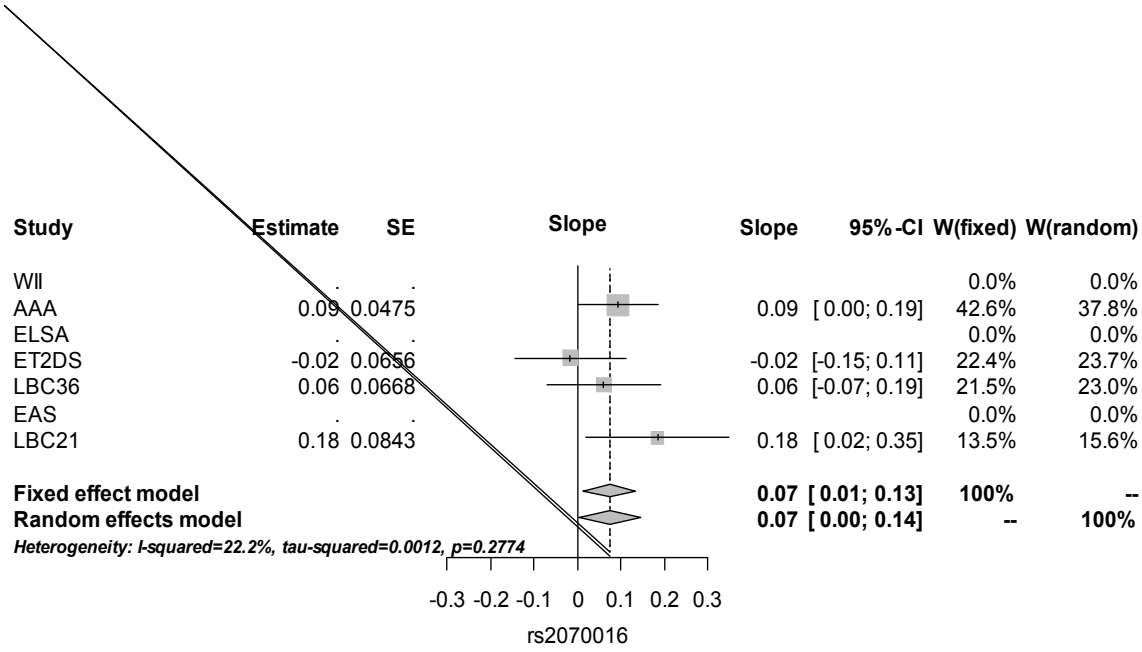
SNP name	AAA	EAS	ELSA	ET2DS	LBC21	LBC36	WII	p value	re-coding	Alleles
	2061	533	5600	1045	517	1005	3413			
rs1205		x		x				0.4722	CC=2; TC=1; TT=0	CT
rs4220	x			x	x	x		0.8438	GG=2; GA=1; AA=0	AG

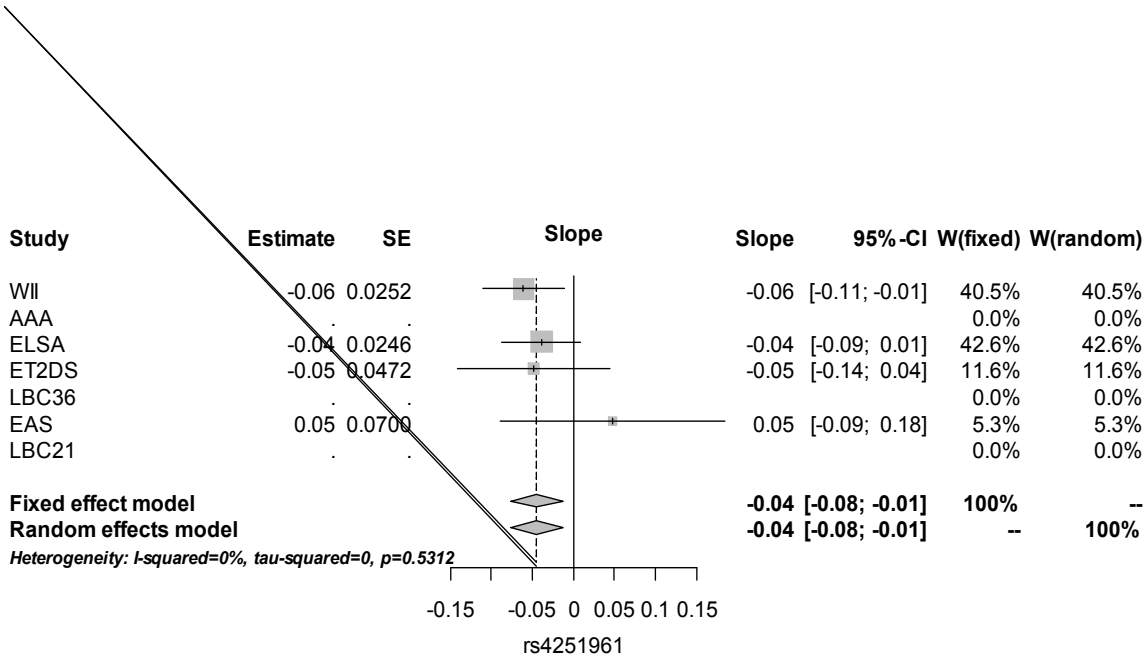
Appendix F: SNPs- 'g'/unadjusted associations



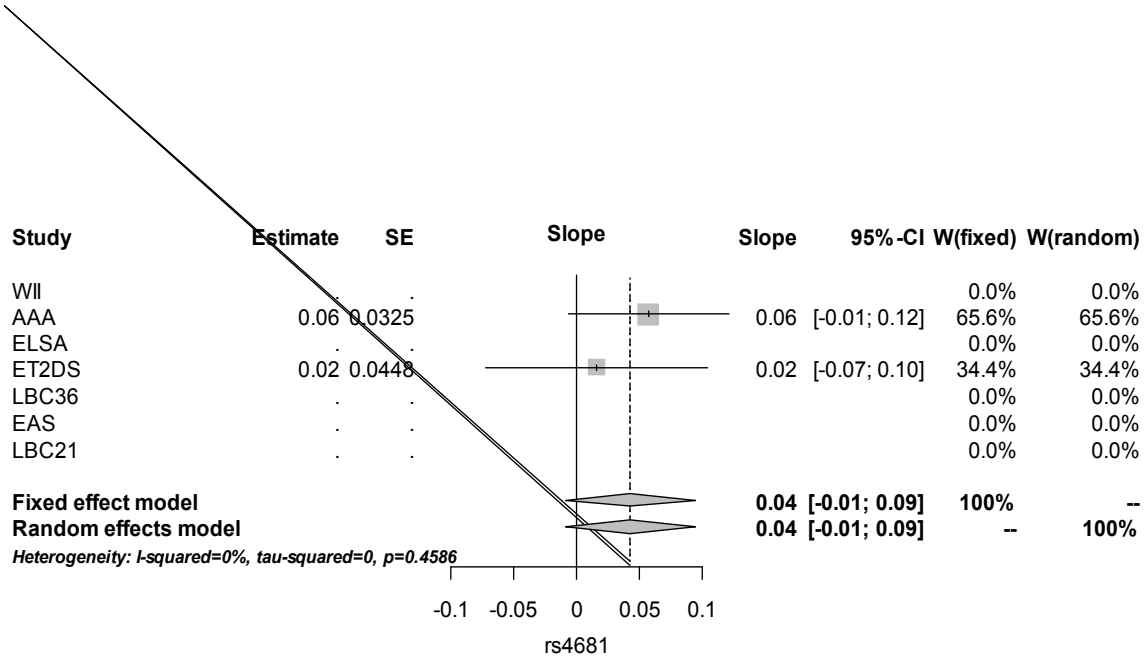


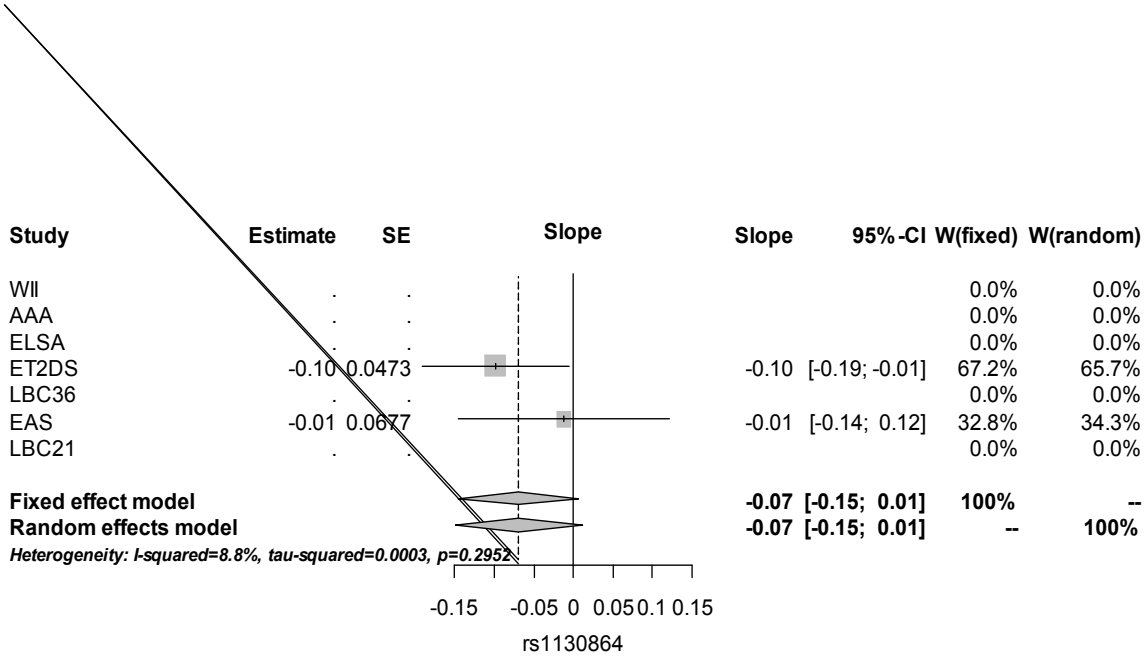

Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII . 0.0% 0.0%
AAA 0.10 0.0407 0.10 [0.02; 0.18] 62.7% 62.7%
ELSA . . 0.0% 0.0%
ET2DS 0.08 0.0528 0.08 [-0.02; 0.18] 37.3% 37.3%
LBC36 . . 0.0% 0.0%
EAS . . 0.0% 0.0%
LBC21 . . 0.0% 0.0%
Fixed effect model 0.09 [0.03; 0.15] 100% --
Random effects model 0.09 [0.03; 0.15] -- 100%
Heterogeneity: I-squared=0%, tau-squared=0, p=0.7733
-0.15 -0.05 0 0.05 0.1 0.15
rs1800497

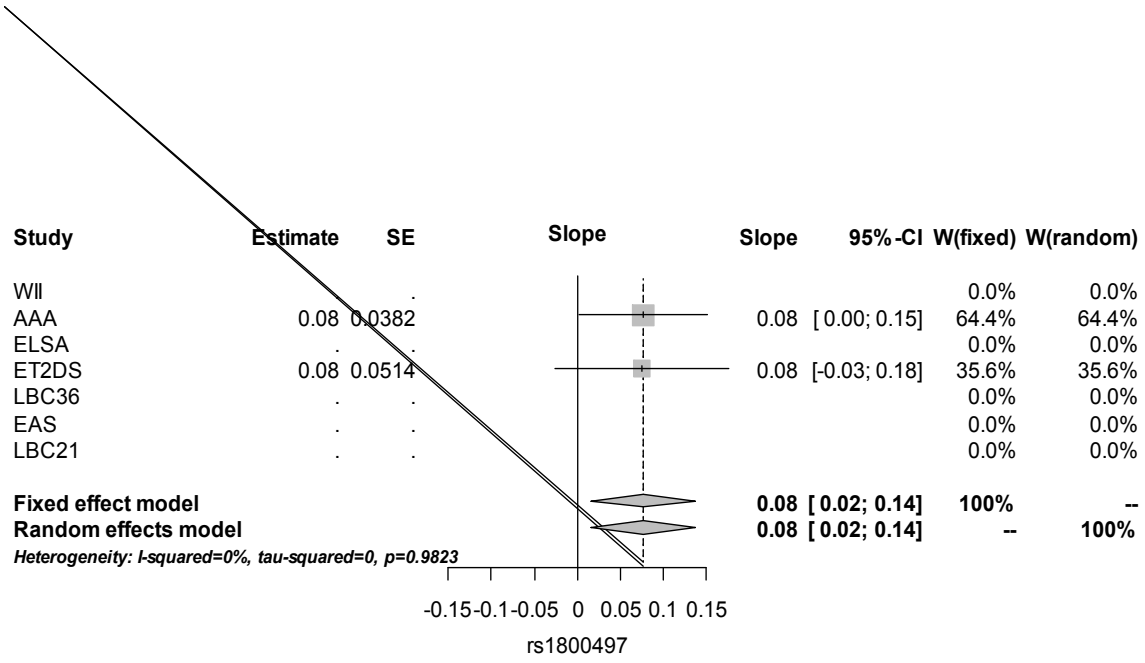

Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII . . 0.0% 0.0%
AAA 0.09 0.0475 0.09 [0.00; 0.19] 42.6% 37.8%
ELSA . . 0.0% 0.0%
ET2DS -0.02 0.0656 -0.02 [-0.15; 0.11] 22.4% 23.7%
LBC36 0.06 0.0668 0.06 [-0.07; 0.19] 21.5% 23.0%
EAS . . 0.0% 0.0%
LBC21 0.18 0.0843 0.18 [0.02; 0.35] 13.5% 15.6%
Fixed effect model 0.07 [0.01; 0.13] 100% --
Random effects model 0.07 [0.00; 0.14] -- 100%
Heterogeneity: I-squared=22.2%, tau-squared=0.0012, p=0.2774
-0.3 -0.2 -0.1 0 0.1 0.2 0.3
rs2070016

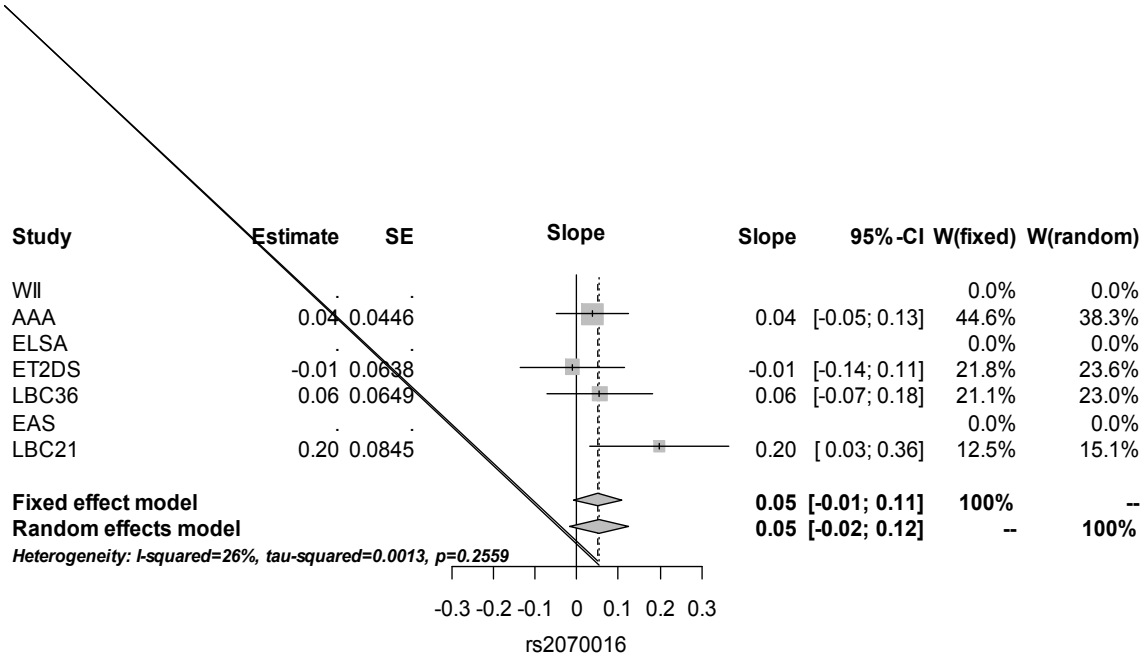

Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII -0.06 0.0252 -0.06 [-0.11; -0.01] 40.5% 40.5%
AAA . . 0.0% 0.0%
ELSA -0.04 0.0246 -0.04 [-0.09; 0.01] 42.6% 42.6%
ET2DS -0.05 0.0472 -0.05 [-0.14; 0.04] 11.6% 11.6%
LBC36 . . 0.0% 0.0%
EAS 0.05 0.0700 0.05 [-0.09; 0.18] 5.3% 5.3%
LBC21 . . 0.0% 0.0%
Fixed effect model -0.04 [-0.08; -0.01] 100% --
Random effects model -0.04 [-0.08; -0.01] -- 100%
Heterogeneity: I-squared=0%, tau-squared=0, p=0.5312
-0.15 -0.05 0 0.05 0.1 0.15
rs4251961

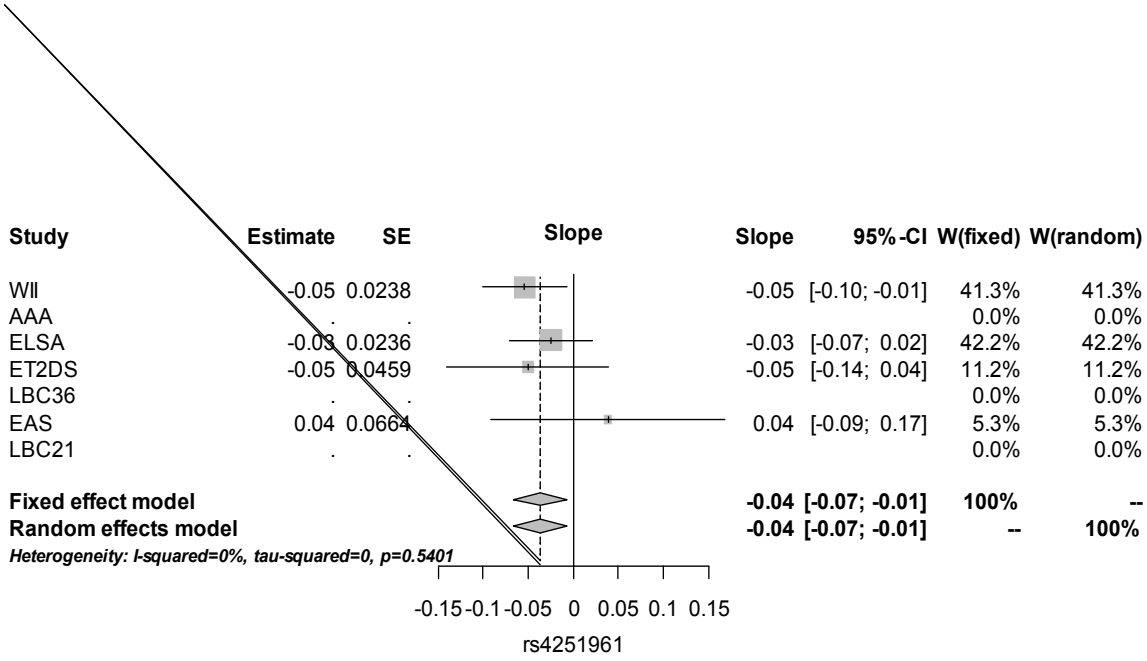
Appendix G: SNPs- 'g'/age and sex adjusted associations






Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII . . 0.0% 0.0%
AAA 0.08 0.0382 0.08 [0.00; 0.15] 64.4% 64.4%
ELSA . . 0.0% 0.0%
ET2DS 0.08 0.0514 0.08 [-0.03; 0.18] 35.6% 35.6%
LBC36 . . 0.0% 0.0%
EAS . . 0.0% 0.0%
LBC21 . . 0.0% 0.0%
Fixed effect model 0.08 [0.02; 0.14] 100% --
Random effects model 0.08 [0.02; 0.14] -- 100%
Heterogeneity: I-squared=0%, tau-squared=0, p=0.9823
-0.15 -0.1 -0.05 0 0.05 0.1 0.15
rs1800497


Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII . . 0.0% 0.0%
AAA 0.04 0.0446 0.04 [-0.05; 0.13] 44.6% 38.3%
ELSA . . 0.0% 0.0%
ET2DS -0.01 0.0638 -0.01 [-0.14; 0.11] 21.8% 23.6%
LBC36 0.06 0.0649 0.06 [-0.07; 0.18] 21.1% 23.0%
EAS . . 0.0% 0.0%
LBC21 0.20 0.0845 0.20 [0.03; 0.36] 12.5% 15.1%
Fixed effect model 0.05 [-0.01; 0.11] 100% --
Random effects model 0.05 [-0.02; 0.12] -- 100%
Heterogeneity: I-squared=26%, tau-squared=0.0013, p=0.2559
-0.3 -0.2 -0.1 0 0.1 0.2 0.3
rs2070016


Study
Estimate
SE
Slope
Slope
95%-CI
W(fixed)
W(random)
WII -0.05 0.0238 -0.05 [-0.10; -0.01] 41.3% 41.3%
AAA . . 0.0% 0.0%
ELSA -0.03 0.0236 -0.03 [-0.07; 0.02] 42.2% 42.2%
ET2DS -0.05 0.0459 -0.05 [-0.14; 0.04] 11.2% 11.2%
LBC36 . . 0.0% 0.0%
EAS 0.04 0.0664 0.04 [-0.09; 0.17] 5.3% 5.3%
LBC21 . . 0.0% 0.0%
Fixed effect model -0.04 [-0.07; -0.01] 100% --
Random effects model -0.04 [-0.07; -0.01] -- 100%
Heterogeneity: I-squared=0%, tau-squared=0, p=0.5401
-0.15 -0.1 -0.05 0 0.05 0.1 0.15
rs4251961