



**Investigating blood plasma levels of cytokines and amyloid- $\beta$  as potential diagnostic biomarkers for preclinical Alzheimer's disease**

Mitra Elmi

MBBS

Principal Supervisor: Prof. Ralph N. Martins

Department of Biomedical Sciences

Faculty of Medicine and Health Sciences

Macquarie University

NSW, Australia

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## Abstract

Alzheimer's disease (AD) is the most common form of dementia, but to date there is no effective treatment. Therefore, preclinical diagnosis is necessary to prevent or improve therapeutic approaches for disease management. Mounting evidence shows that inflammatory processes involving various pro-inflammatory cytokines including interleukin (IL)-1 $\beta$ , IL-6 and tumour necrosis factor (TNF)- $\alpha$  contribute to AD pathogenesis. While inflammatory biomarkers have been extensively studied in clinically diagnosed AD subjects, there has been a paucity of information on inflammatory biomarkers in cognitively normal elderly subjects with a high neocortical amyloid- $\beta$  load (NAL).

Based on the findings of increased pro-inflammatory cytokines in clinical AD, I hypothesised that plasma levels of these cytokines are also increased in preclinical AD, and these increased cytokine levels may have diagnostic value, and functionally play a role in impairing cognition.

In this study, approximately 100 plasma samples from healthy elderly adults with either high or low NAL were assessed for pro-inflammatory cytokines using conventional enzyme-linked immunosorbent assay (ELISA) kits. Furthermore, since plasma beta amyloid (A $\beta$ ) levels have recently been demonstrated to be altered in clinical AD, I investigated the levels of this peptide in preclinical AD. The A $\beta$  results, which were expressed as the ratio of amyloid beta (A $\beta$ ) 40/42 were compared between low and high NAL subjects.

The data collected were analysed using the statistical package for social sciences (SPSS).

No significant differences in the plasma levels of all the pro-inflammatory cytokines were observed between the two groups, although there was a consistent trend of increased levels in the high NAL group. However, the ratio of A $\beta$  40/42 was significantly higher in the high NAL group compared to the low NAL, and receiver operating characteristic (ROC) curves were created to evaluate predictive models. The accuracy of prediction was increased when A $\beta$  40/42 was added to the base model.

In conclusion, while the pro-inflammatory cytokines do not show promise as preclinical AD biomarkers based on the conventional ELISA assays used, the ratio of A $\beta$  40/42 is a potential candidate that merits inclusion in a diagnostic panel of biomarkers for preclinical diagnosis of AD.

## Statement of originality

I declare that this thesis has not been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution, and it is an original piece of research that it has been written by me. Any assistance which I have received in my research work has been appropriately acknowledged. I also certify that all sources of information are indicated in the thesis, and I have permission from original authors and publishers to use their images (Fig. 1, 2, 3, 5 and 21) in my thesis.

The research presented in this thesis was approved by the Macquarie University Human Research Ethics Committee, reference number for the original application: 5201701078 and for amendment: 5201826144153.

This thesis has been presented as a poster at the 3rd Annual EnCouRage Research Symposium, Macquarie University, 26/10/2018.

MRes 2, which is a direct pathway (in my case) to year two of Master of Research towards PhD is about a 10-month course at Macquarie University. This thesis provides partial fulfilment of the conditions of the course. It has also been affected by twice project alteration. The first project which was examining the effect of coconut oil on clinical factors related to health, cognition, quality of life, and Alzheimer's disease, was approved on 13 August when it was late to be my project. The second project, which was measuring plasma lipoprotein subfractions in the "virgin coconut oil for the treatment of Alzheimer's dementia study" cohort in Sri Lanka was not approved by the ethics committee at Macquarie University. Therefore, the third project, which was investigating plasma cytokines as potential diagnostic biomarkers for preclinical Alzheimer's disease started from the 3rd of September, from which a modification by adding some data related to plasma amyloid- $\beta$  occurred on 3/10/2018.

*Mitra Elmi*

Mitra Elmi

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## Abbreviations

AAV2	Adeno-associated-viruses-2
ABCA7	Adenosine triphosphate binding cassette subfamily A, member 7
AChEI	Acetylcholinesterase inhibitors
AD	Alzheimer's disease/dementia
ADAS-Cog	Alzheimer's disease assessment scale-cognitive scale
ADNI	Alzheimer's disease neuroimaging Initiative
AIBL	The Australian imaging biomarkers and lifestyle
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APP $\alpha$	Secreted APP-alpha
ATP	Adenosine triphosphate
AUC	Area under curve
A $\beta$	Amyloid beta/ Beta amyloid
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
$\beta$ HB	$\beta$ -hydroxybutyrate
BMI	Body mass index
BP	Blood pressure
CAP	Computerised analysis of positron emission tomography
CD	Classification determinant
CDR SB	Clinical dementia rating scale sum of boxes
CHD	Coronary heart disease

CNS	Central nervous system
COX-2	Cyclooxygenase-2
CPiB	Pittsburgh compound B
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computerised tomography
CVA	Cerebrovascular accident
DASS	Depression, anxiety, stress scales
EDTA	Ethylenediamine tetraacetic acid
ELISPOT	Enzyme-linked immune spot assays
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
ESR	Erythrocyte sedimentation rate
FBB	Florbetaben
FDA	Food and drug administration
FDG	Fluorodeoxyglucose
G6PD	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMF	Glia maturation factor
HDL	High density lipoprotein
HFD	High fat diet
HLA	Human leukocyte antigen
HMG-CoA	hydroxy-3-methylglutaryl-coenzyme A



hs-CRP	High sensitivity-C-reactive protein
HSP	Heat shock protein
IDE	Insulin-degrading enzyme
IGF	Insulin-like growth factor
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
INF	Interferon
iNOS	Inducible Nitric oxide synthase
KARVIAH	Kerr Anglican retirement village initiative in ageing health
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
MAC	Macrophage receptor
MAC-Q	Memory assessment clinic-questionnaire
MAPK	Mitogen-activated protein kinase
MCFA	Medium chain fatty acid
MCI	Mild cognitive impairment
MCT	Medium chain triglyceride
MIP-1 $\alpha$	Macrophage inflammatory protein-1 $\alpha$
MMSE	Mini mental state examination
MoCA	Montreal cognitive assessment
MRI	Magnetic resonance imaging
NAL	Neocortical amyloid- $\beta$ load
NF- $\kappa$ B	Nuclear factor-kappa beta
NFT	Neurofibrillary tangle

NGF	Nerve growth factor
NLRP	Nucleotide-binding oligomerisation domain-like receptor family pyrin
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
PET	Positron emission tomography
PICALM	Phosphatidyl-inositol binding clathrin assembly protein
PS	Presenilins
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SIMOA	Single-molecule array
SMC	Subjective memory complainer
SNP	Single nucleotide polymorphism
SPSS	Statistical package for social sciences
STM	Seven-transmembrane-spanning protein
SUVR	Standard uptake value ratio
T2DM	Type II diabetes mellitus
T3DM	Type III diabetes mellitus
TGF- $\beta$	Transforming growth factor- $\beta$
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
TOMM 40	Translocase of outer mitochondrial membrane 40
VCO	Virgin coconut oil
wr -CRP	Wide range-C-reactive protein

# Literature Review

## 1. Alzheimer's disease/dementia

### 1.1. Introduction

The world's population is rapidly aging, and the number of people with dementia is expected to rise to 65 million by 2030 [1]. Globally, four million and six hundred thousand new cases occur annually, and this number doubles every 20 years [2]. Dementia is a clinical syndrome, which involves progressive deterioration of intellectual abilities including memory, language, reasoning, decision making, visuospatial function, attention, and orientation as well as changes in personality, emotional balance and social behaviours [1,3].

Dementia has reversible and irreversible causes. Reversible dementias, also called pseudodementia, are relatively rare, and occur secondary to other medical conditions such as nutritional deficiencies (e.g. vitamin B12), endocrine disorders (e.g. hypothyroidism), space-occupying lesions (e.g. brain tumours), normal hydrocephalus, depression, and drug abuse. Irreversible (primary) dementias include neurodegenerative dementias mainly Alzheimer's disease (AD) with a frequency of 83.5% in global population followed by vascular dementia, dementia related to Parkinson's disease, dementia with Lewy bodies, frontotemporal dementia, and other rare types [1,3].

A German psychiatrist and neuropathologist named Dr. Alois Alzheimer described AD for the first time. In his prominent conference speech in 1906, and his ensuing article published the following year, he described a case (Auguste D), who was a 51-year-old woman with a peculiar disease of the cerebral cortex whose symptoms were disorientation, progressive memory loss, language and psychosocial impairment as well as psychiatric symptoms such as delusion, hallucination and paranoia [1,4].

AD is the most common neurodegenerative disorder for which there is no effective treatment. Due to the multifactorial nature of this disease, single therapeutic approaches are unlikely to lead to effective disease management [5–7]. It has been predicted that about 150 million of the global population will be afflicted with AD by 2050 [5]. About 10% of individuals over 70 years have substantial memory loss, and in more than 50% of the cases AD is the cause. It is estimated that the median total cost of caring for a single patient with advanced AD is over \$50,000 (US) annually, not to mention the unmeasurable emotional toll for caregivers [8].

## **1.2. Clinical manifestations**

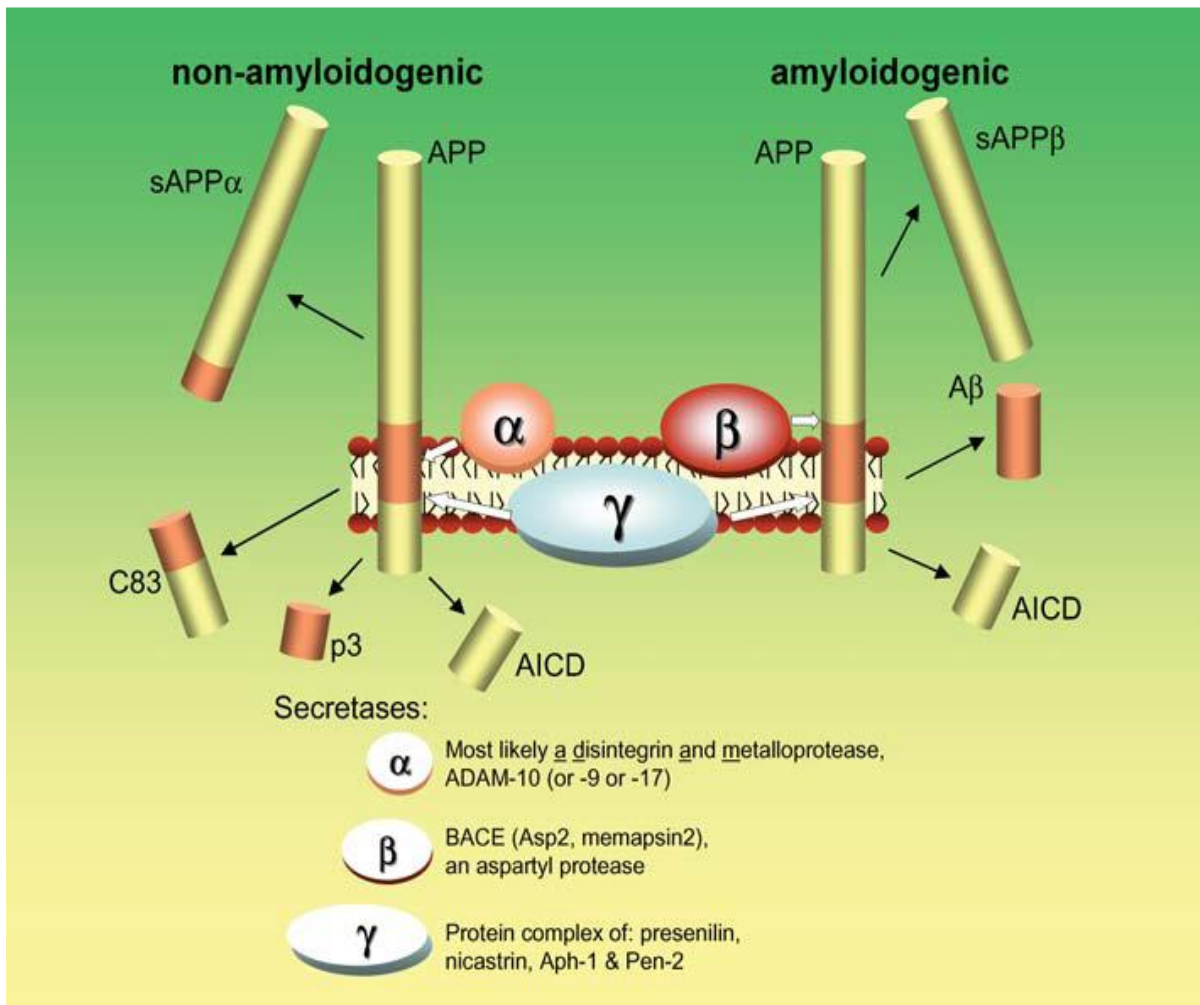
Cognitive changes in AD usually follow a characteristic pattern starting with memory impairment and development of language and visuospatial deficits. In the early stages of AD, memory loss may be undetectable, or be attributed to a benign forgetfulness associated with normal ageing. Once the memory loss becomes evident to patients and their family members together with the exhibition of falling one and a half standard deviations (SD) below normal on standardised memory tests, the term mild cognitive impairment (MCI) is applied. This pattern provides useful prognostic information, as about 50% of MCI patients will progress to AD in over four years [8,9]. Amnesic MCI, which usually leads to AD is gradually being replaced by the concept of “early symptomatic AD” in order to signify AD as the underlying disease. In the middle stages of AD, patients are unable to work, get lost easily and are confused. Therefore, they require daily supervision. They also develop language impairment (first naming, then comprehension and finally fluency). In the late stages of the disease, delusions are common including theft, disloyalty or misidentification, and patients often look parkinsonian. In the end stages, patients become rigid, mute, incontinent, and bedridden. Death often results from malnutrition, secondary infections, pulmonary emboli, cardiovascular diseases, or most commonly aspiration. The normal duration of AD is eight to 10 years, but the course varies from one to 25 years. For unknown reasons, some AD patients show a steady decline in function, while others have long term plateaus without substantial deterioration [8].

## **1.3. Pathogenesis**

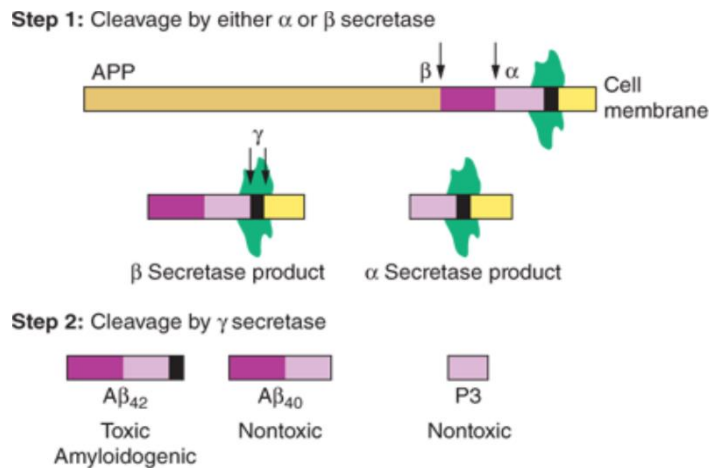
The pathogenesis of AD is complex; it involves excessive extracellular beta amyloid ( $A\beta$ ) deposition, intracellular tau hyperphosphorylation, neurofibrillary tangles (NFTs), cerebral amyloid angiopathy, widespread neuronal loss, oxidative stress, reactive glial and microglial changes as well as other pathological processes such as inflammation [8,10–12]. Although amyloid plaques consisting of deposited  $A\beta$  peptides are a neuropathological hallmark of AD, the soluble oligomers rather than the mature and insoluble amyloid fibrils most likely contribute to the  $A\beta$  toxicity and neurodegeneration [5,11,13].

$A\beta$  is a peptide consisting of 39-42 amino acids, that is derived from a larger transmembrane protein called amyloid precursor protein (APP). When APP is cleaved by  $\beta$  and  $\gamma$  secretases through the amyloidogenic pathway,  $A\beta$  is generated. The normal function of  $A\beta$  peptides still remains unclear, although they may have a role in controlling synaptic activity [8,12]. The most common form of  $A\beta$  is  $A\beta$  40. However,  $A\beta$  42, which is less common is the most pathogenic form of  $A\beta$  (Fig. 1 and 2) [5,8,12].

The core of the APP plaque is surrounded by a halo, containing dystrophic tau-immunoreactive neurites and deactivated microglia. NFTs are composed of abnormally phosphorylated tau protein [8]. Cholinergic neurons of the basal forebrain are some of the most severely affected neuronal populations. Cholinergic dysfunction has been correlated with synaptic loss and increased amyloid density in AD [6].



**Figure 1.** Proteolytic processing of APP via non-amyloidogenic and amyloidogenic cleavage [12]



**Figure 2.** A $\beta$  generation through the amyloid pathway in two steps [8]

It has been reported that A $\beta$  precipitation is caused by abnormal interactions with neocortical metal ions especially zinc, copper and iron. However, A $\beta$  may also participate in normal metal-ion homeostasis [14].

Early in the AD pathogenesis, an imbalance between the production of reactive oxygen species (ROS) such as metal ions and anti-oxidants (e.g. vitamin C and E) causes oxidative stress. Oxidative stress increases the levels of both A $\beta$  and APP. Consequently, by the overproduction of H<sub>2</sub>O<sub>2</sub>, the dysfunctional mitochondria lead to a series of reactions involving redox metals and oxidative stress response related elements [15]. Mitochondrial dysfunction and abnormal accumulation of A $\beta$  and tau can cause an imbalance between oxidants and antioxidants, which are responsible for oxidative damage in AD [16]. Another level of complexity is generating H<sub>2</sub>O<sub>2</sub> by A $\beta$  in the presence of redox-active ions, which is an additional threat to the neurons. Although it may be compensated by anti-oxidative responses; the extent to which the disease process is affected by oxidative stress, and the relationship between A $\beta$  and dysfunctional mitochondria requires further elucidation [15].

In addition, mounting evidence shows that various pro-inflammatory cytokines including interleukins, tumour necrosis factor (TNF)- $\alpha$ , transforming growth factor (TGF)- $\beta$  and interferons (INF) e.g. IFN- $\gamma$  all actively participate in the AD pathogenic process. Interleukin (IL)-1 $\beta$ , IL-6 and many other cytokines have also been found around amyloid plaques in brain autopsies [10,17,18]. C-reactive protein (CRP), a marker of chronic inflammation has also been detected in the senile (amyloid) plaques and NFTs in AD brains [19].

Glia maturation factor (GMF) is a brain-specific neuroinflammatory protein, which induces neurodegeneration, and has been previously found to be significantly upregulated in diverse regions of AD brains compared to non-AD control brains. Evidence regarding co-localization of GMF and apoE  $\epsilon$ 4 in AD brains has been provided for the first time. It has also been suggested that GMF and apo E  $\epsilon$ 4 are co-associated in amyloid plaques and in the reactive astrocytes surrounding amyloid plaques in AD [20]. The *APOE* gene on chromosome 19 contributes to the pathogenesis of AD. It has three common alleles:  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4. Its protein, apoE participates in cholesterol transport. It can be detected in neritic plaques, and may create neurofibrillary tangles by binding to tau [8].

There is also another theory which suggests that AD may be stimulated by infection with the obligate intracellular bacterium, Chlamydia pneumonia, which has been frequently found in glial cells in the pathogenic areas of AD brains. *In vivo* sites of chlamydial infection have shown chronic inflammation characterised by activated monocytes and macrophages. Immunopathogenesis resulting from inflammation is the hallmark of chlamydia-induced disease in which the presence of heat shock proteins (HSP) such as HSP 60, proinflammatory cytokines including IL-1 $\beta$ , IL-6 and TNF- $\alpha$  as well as TH1-related cytokines such as IFN- $\gamma$  and IL-12 have been detected at sites of chlamydial infection [21].

### **1.3.1. The mechanism of inflammation**

The brain has an atypical inflammatory process; because it does not have pain neuron fibres, the classic signs of inflammation including redness, swelling, heat, and pain are not usually detected in the central nervous system (CNS) [17]. Neuroinflammatory processes are a prominent feature of AD for which microglia are hyperactivated, and result in increased production of proinflammatory cytokines (Fig. 3) [22]. The activated microglia can be detected by various antibodies and lectins including macrophage receptor (Mac)-1/classification determinant (CD) 11b, human leukocyte antigen (HLA)-DR, CD45, RCA-1, and F4/80. Then, the microglia phagocytose foreign materials, and release pro-inflammatory molecules such as cytokines which further activate other inflammatory responses and eventually potentiate the inflammatory cycle [17].

Microglial M1/M2 polarisation of macrophage has an important role in controlling the balance between promotion and suppression of inflammation. M1 polarised microglia may produce pro-inflammatory cytokines, reactive oxygen species and nitric oxide, contributing to CNS

dysfunction. In contrast, M2 polarised microglia express cytokines and receptors, which are implicated in inhibiting inflammation and restoring homeostasis [23].

Moreover, deficiencies in the antibody inflammatory system can also contribute to neuroinflammation [22]. The role of IL-1 in AD, which is considered to be more important than other cytokines is further discussed below [17].

### **1.3.1.1. IL-1**

IL-1 was first defined in 1972 as a lymphocyte activating factor, and later it was shown to have a variety of effects inducing inflammation, production of acute phase proteins and prostaglandins, increasing body temperature, proliferating B and T cells and regulating haematopoiesis [17].

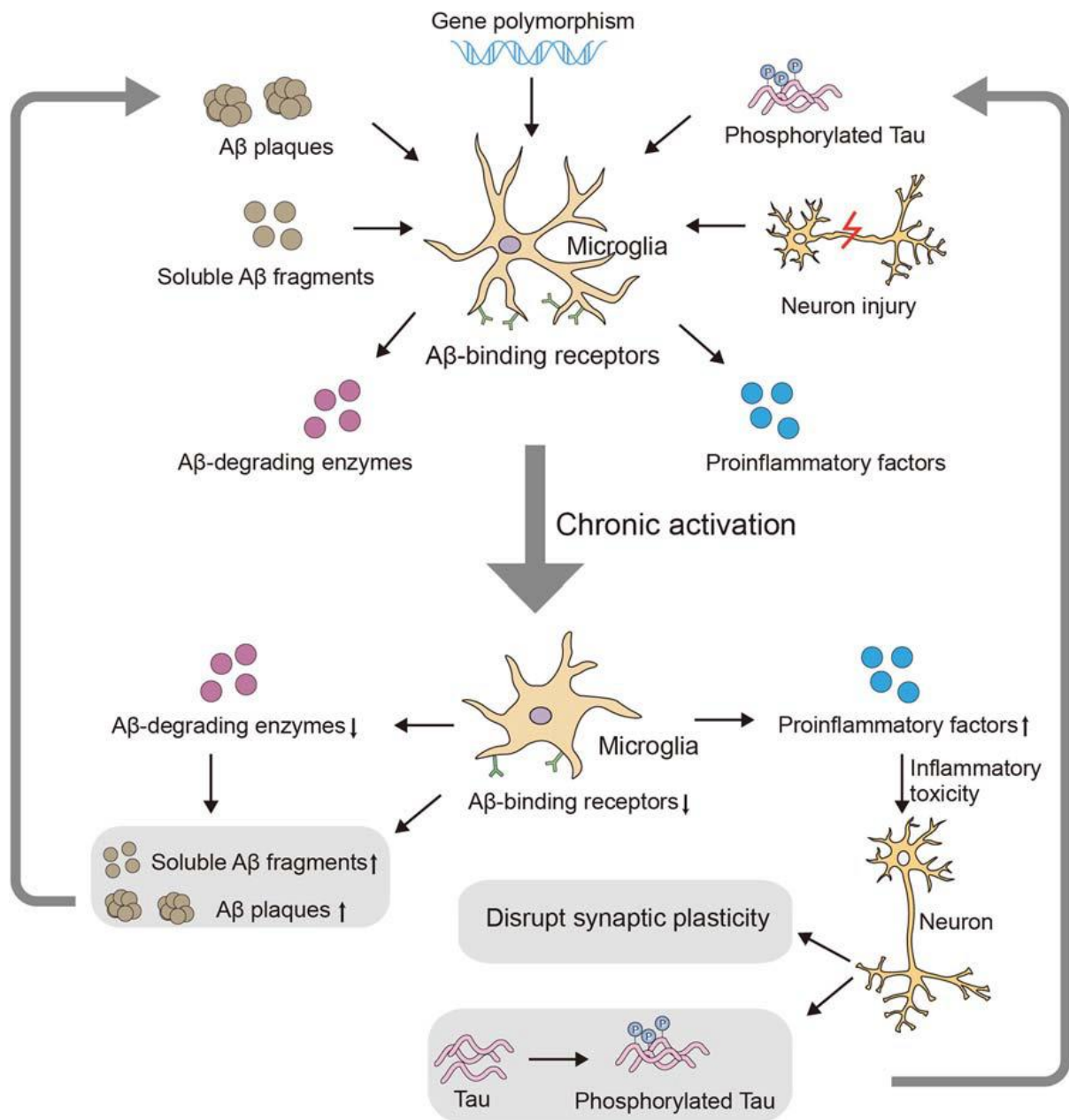
IL-1 has two isoforms,  $\alpha$  and  $\beta$ , which are encoded by separate genes, *IL-1A* and *IL-1B*, located on the long arm of chromosome two in a cluster with the gene for the IL-1 receptor antagonist. Both isoforms are synthesized as 33 kDa precursors that are cleaved to yield 17 kDa products. The secreted 17 kDa cleavage product is the only biologically active  $\beta$  isoform, whereas for the  $\alpha$  isoform both the non-secreted 33 kDa and the secreted 17 kDa molecules are biologically active [24].

The microglia are the principal source of both IL-1 $\alpha$  and IL-1 $\beta$  in the CNS, although they are also produced to a lesser extent by astrocytes and neurons [24]. The mechanism of IL-1 $\beta$  secretion has been found to be ambiguous, as it does not follow the typical endoplasmic reticulum-Golgi route of discharge [25].

IL-1 levels are increased in the AD brain, and its overexpression is related to A $\beta$  load [26]. It interacts with the gene products of several other known or possible genetic risk factors of AD including *APOE*  $\epsilon$ 4,  $\alpha$ <sub>1</sub>-antichymotrypsin and  $\alpha$ <sub>2</sub>-macroglobulin. The overexpression of IL-1 is also linked to environmental risk factors of AD such as ageing and head trauma. IL-1 stimulates both neuronal synthesis and processing of APP with potential contributions to neurotoxicity related to either discharged APP fragments or possibly with only A $\beta$ . In contrast,  $\alpha$  fragments of secreted APP promote microglial activation and overexpression of IL-1, and this latter effect is modified in an isoform-specific manner by *APOE*, wherein *APOE*  $\epsilon$ 3 blocks this effect, while *APOE*  $\epsilon$ 4 is ineffective [24]. Cleavage of APP by  $\alpha$ -secretase produces an extracellularly released fragment termed secreted APP-alpha (APP $\alpha$ ). APP $\alpha$  is neurogenic, neurotrophic,



neuroprotective, a stimulator of protein synthesis, and gene expression. It also improves long-term potentiation and memory [27].



**Figure 3. Neuroinflammation:** Chronic activation of microglia and involvement of proinflammatory factors on the disruption of synaptic plasticity and inhibition of A $\beta$  clearance [22]

#### **1.4. Risk factors**

The major genetic risk factor of AD is genetics. However, longevity plays an important contributing role to increasing risk of AD. Therefore, the higher life expectancy in women may partly explain their higher incidence of AD [1,8,28]. Another important risk factor for AD is type II diabetes mellitus (T2DM), which can increase the risk of AD three-fold. High blood levels of homocysteine and cholesterol, hypertension, folic acid deficiency, insufficient exercise, and smoking as well as low consumption of fruits and vegetables may all be potential risk factors for AD [2,8].

##### **1.4.1. Hypercholesteremia and AD**

Hypercholesteremia is a risk factor for AD. This hypothesis was established by discovering AD associated gene clusters involved in lipid binding and metabolism in the brain including the gene *APOE*  $\epsilon 4$  allele for which its protein, apoE is the major carrier of cholesterol in the CNS. Other implicated genes include the single-nucleotide polymorphism for clusterin, adenosine triphosphate (ATP) binding cassette subfamily A, member seven (*ABCA7*) and phosphatidylinositol binding clathrin assembly protein (*PICALM*) [29,30]. However, epidemiological evidence is limited due to the lack of data on the relationship between cholesterol and cognitive outcomes in general, and more specifically a lack of data compatible for pooling due to different methods of classifying serum cholesterol measures [29].

##### **1.4.2. Cholesterol subfractions**

Conventional cholesterol measurements may not reflect an accurate clinical outlook [31]. It has been shown that lower levels of the large high density lipoprotein (HDL) subfraction, higher levels of the small HDL subfraction and low density lipoprotein (LDL) subfractions have been identified in patients with coronary artery disease. This association is also linked to AD, where subfractions of cholesterol should also be considered besides cholesterol as AD risk factors [32].

#### **1.5. Genetic considerations**

Several genes are involved in the pathogenesis of AD, one of which is the *APP* gene located on chromosome 21, and two others which are presenilins (*PS*). Presenilin-1 (*PS-1*), located on chromosome 14 encodes a seven-transmembrane-spanning protein called S182, to which APP specifically binds. Mutations in this gene, which are more common than those in presenilin-2 (*PS-2*) cause an early-onset (familial autosomal dominant) AD, often before the age of 50 (mean: 45) [1,8,33,34]. Mutations of *PS-2*, which is on chromosome 1 and encodes another

seven-transmembrane-spanning protein (STM) 2, which is also bound to the APP, cause a later onset of the disease (average 53) with longer and less progressive course (mean duration:11 years compared with six to seven years for PS-1) [8,33,34]. Even though some carriers of PS-2 mutations, become afflicted with AD after the age of 70, PS mutations rarely lead to late-onset AD. Of note, patients with mutations in the presenilin genes have high plasma A $\beta$  42 levels [8].

The *APOE*  $\epsilon$  4 may also increase the risk of AD in the general population including sporadic and late-onset familial forms. Among AD patients, 40 to 65% have at least one  $\epsilon$  4 allele, which is significantly higher than controls [35]. However, many patients with AD are not carriers of the  $\epsilon$  4 allele, and many  $\epsilon$  4 carriers may never develop the disease. Therefore,  $\epsilon$ 4 is neither necessary nor sufficient to cause AD. Nevertheless, the *APOE*  $\epsilon$ 4 allele remains the most important genetic risk factor for AD. On the other hand, some evidence indicates that  $\epsilon$ 2 allele may reduce the risk of AD. However, *APOE* testing cannot be used as a diagnostic tool in predicting and diagnosing AD [8].

The translocase of outer mitochondrial membrane homolog (*TOMM*)40 gene next to the *APOE* gene, provides highly increased precision in the estimation of late onset AD for *APOE*  $\epsilon$ 3 carriers. *APOE* and *TOMM*40 interrupt mitochondrial functions with neurons [36–39].

## 1.6. Diagnosis

The gold standard test in evaluating cognitive performance is the Alzheimer's disease assessment scale-cognitive scale (ADAS-Cog) compared to other psychometric tests, which are considered screening measures for only general cognition, and cannot provide information for staging the dementia severity. This test usually requires 30 to 45 min to complete, and it is one of the most common cognitive tests conducted for the prescription of anti-dementia medications [40].

Diagnostic tests for patients with MCI should consist of tests to exclude reversible causes of cognitive impairment. Therefore, for most cases the evaluation should proceed similarly to the assessment of a patient with dementia. Nevertheless, neuropsychological tests may still have greater use in diagnosing MCI than dementia [41].

A clinical diagnosis of AD is confirmed by autopsy, 90% sensitivity and 80% specificity with misdiagnosed cases relating to other dementing disorders [42]. Cerebrospinal fluid (CSF) to measure the reduced level of A $\beta$  42 and elevated tau is not routine [5,8]. Magnetic resonance

imaging (MRI) and computerised tomography (CT) scan have been available for many years to help with the diagnosis of AD, and they can be used to measure the brain shrinkage to exclude other diseases [5]. However, they do not show a single specific pattern with AD [8]. A positron emission tomography (PET) scan using a chemical tracer,  $^{18}\text{F}$ -fluorodeoxyglucose (FDG) indicates brain regional glucose usage, which is low in certain areas of the brain in AD allows for early diagnosis of clinical AD [5]. PET amyloid scans, which have more recently become available are highly specific for identifying preclinical AD at least two decades before the onset of clinical symptoms. Several amyloid imaging radiotracers are available including Pittsburgh compound B ( $^{11}\text{C}$ PiB),  $^{18}\text{F}$ -Florbetaben (FBB) and  $^{18}\text{F}$ -Florbetapir, which play an important role in identifying high risk individuals for clinical trials [5,43].

Recently, studies have indicated that the retina, the internal posterior layer of the eye globe might serve as a reliable source for the diagnosis of AD based on  $\text{A}\beta$  deposits on the lens and the thickness of the retinal nerve fibre layer in patients within the primary stages of the disease [5].

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family is secreted by neurons, and is an important part of synaptic plasticity [44]. Reduction in BDNF levels has been shown to cause neurodegeneration in diabetic brains. Its low level is also broadly recognised as a pathogenic factor in numerous neurodegenerative brain diseases such as dementia (e.g. AD), autism spectrum disorders, schizophrenia, bipolar disorders, and major depression [45,46]. Circulating BDNF can be measured in serum, plasma or whole blood, although its level in serum is the most accurate measure [45]. However, it is unclear what is the normal range of BDNF levels, and if its blood levels reflect brain levels [44].

Another new diagnostic method is measuring plasma  $\text{A}\beta$  and its biomarkers including but not limited to  $\text{APP}_{669-711}/\text{A}\beta_{1-42}$  and  $\text{A}\beta_{1-40/1-42}$ , which has been measured by immunoprecipitation linked with mass spectrometry. [47,48].

Martins et al. reported that the levels of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase were both approximately two-fold higher in the brains of AD patients, which led to the concept that the AD brain is under oxidative stress [49].

## **1.7. Treatment**

The main strategy for managing AD is focusing on the long-term amelioration of related behavioural and neurological problems as well as providing caregiving support [8].

Some therapies for patients with AD have concentrated on reducing the A $\beta$  accumulation by antagonizing  $\beta$  or  $\gamma$  secretases or clearing A $\beta$  by using specific antibodies [8]. Aducanumab, which is a human monoclonal antibody (Ab) that selectively targets A $\beta$  has shown positive results as a potential treatment for AD. Additionally, BAN2401, which selectively targets soluble A $\beta$  protofibrils and currently in phase 2b, has shown a favourable safety profile in previous studies [50,51]. However, since 1998, more than 100 anti-amyloid therapeutics such as Tarenflurbil (Flurizan) and Bapineuzumab have failed to improve cognitive function in spite of reducing A $\beta$  [5]. Vaccination against A $\beta$  has been proved highly effective in AD mouse models facilitating brain amyloid clearance and preventing further amyloid accumulation. However, in human trials, this approach led to life-threatening complications including meningoencephalitis in rare cases [8,52]. Another approach to target A $\beta$  was using  $\gamma$  and  $\beta$  secretase inhibitors to reduce the production of A $\beta$ . However, the first two placebo-controlled trials of  $\gamma$  secretase inhibitors, tarenflurbil and semagacestat were unsuccessful [8]. Moreover, semagacestat may have enhanced cognitive decline compared with the placebo. On the other hand, passive immunisation with monoclonal antibodies against A $\beta$ , which has been used in mild to moderate AD had no positive outcomes. Therefore, new trials have been started among asymptomatic autosomal dominant forms and cognitively normal elderly individuals who are A $\beta$  positive with PET scan [8]. Tau antibodies, which modify tau phosphorylation and aggregation are also being investigated as a potential treatment for AD and non-AD tau-associated disorders [8].

The discovery of agents, which target soluble A $\beta$  oligomers is greatly desirable for more effective treatments. In this regard, a novel 15-amino acid peptide (15-mer) has been synthesised, which reduces the formation of A $\beta$  42 oligomers [13].

Acetylcholinesterase inhibitors (AChEI) such as Rivastigmine (Exenol), Galantamine (Razadyne), and Donepezil (Aricept) as well as an N-methyl-D-aspartate (NMDA) glutamate receptors antagonist called Memantine have been approved by the US food and drug administration (FDA) and European Medicines Agency (EMA) [5,8]. These medications target neurotransmitter deficits that occur in the later stages of AD. Notably, they do not suppress the disease progression, and only stabilise the cognitive function of AD patients for a maximum of one year by counteracting the functional consequences of the lost cholinergic neurons [5].

The relationship between T2DM and AD has been recently highlighted, and insulin regulating studies have been conducted as a potential treatment [5,8], because *APOE*  $\epsilon$ 4 and T2DM seem

to act together to cause cognitive dysfunction and increase the risk of AD. *APOE*  $\epsilon$ 4 has also been associated with decreased glucose metabolism, suggesting that hypometabolism of glucose in the brain could be a main contributing factor in both MCI and AD. As a result, the brains of patients with MCI and AD are functionally insulin resistant. Additionally, deposition of both A $\beta$  and hyperphosphorylated tau can also be detected in autopsies from pancreatic tissue in T2DM patients [49]. The insulin-degrading enzyme (IDE), which degrades insulin, glucagon and A $\beta$  peptides has a pathophysiological connection between T2DM and AD. Therefore, IDE modulators including IDE inhibitors and IDE activators are potential medications for the treatment of T2DM and AD, respectively [53].

Low levels of testosterone have been reported in AD and T2DM [54,55]. Testosterone therapy has been trialled to prevent cognitive decline and the progression of AD. Although clinical trials have revealed the improvement of some cognitive capacities in men with hypotestosteronemia, clinical efficacy has shown contradictory results [56].

In a cohort study, oestrogen therapy appeared to be protective about 50% against the development of AD in females. Contrary to these findings, a case-controlled study showed that a combination of oestrogen-progesterone therapy increased the prevalence of dementia [8].

There are some medical foods including Caprylidene (trade name: Axona), Souvenaid and Carefolin NAC that claim symptomatic benefits for mild to moderate AD. Axona, which consists of medium chain triglycerides (MCT) provides ketone bodies as an alternative source of energy for neurons. Souvenaid supplies precursors to enhance synaptic function. Carefolin NA, which is composed of methylfolate methylcobalamin (Vitamin B<sub>12</sub>) and N-acetylcysteine, prevents oxidative stress related to memory loss [57,58].

Common spices have been proposed as potential preventive and therapeutic agents for AD [59]. The extract of Ginkgo biloba leaves, as an approved Chinese medicine may reduce neurotoxicity of the A $\beta$  42 oligomer by increasing HSP70, and a controlled trial found a modest improvement in cognitive function of patients with AD and vascular dementia [8,16]. However, a six-year prevention study using ginkgo found no decrease in the progression rate of AD in the treated group [8]. Saffron has also been found to be as effective as Donepezil in the treatment of mild to moderate AD after five and a half months. In addition, it caused a significant improvement in cognitive function than the placebo in patients with mild to moderate AD after four months supplementation [60]. Another spice, curcumin (the bioactive compound of the spice turmeric), can be considered as the most probable inhibitor of APP [59].

The interaction between heavy metals such as cadmium and curcumin has been shown to prevent the neurotoxicity caused by these metals in AD. In addition, in traditional Middle Eastern medicine, black pepper has been used as a nerve tonic with anti-amyloid properties. Nevertheless, comprehensive clinical trials are required to test the medical use and potential side effects of these spices [59].

Several case-controlled studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, which targets cytokines are considered as potential goals in the treatment of AD, and also 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, known as statins may have a protective effect on AD, if used before the onset of the disease. However, they are not effective in symptomatic AD [8,10].

Danish researchers have reported that HDL, the principal lipoprotein in the human brain can reduce amyloid formation *in vitro*. Some studies have shown that A $\beta$  associates directly with HDL, while others have reported that HDL particles decrease the A $\beta$  neurotoxicity. Ultimately, HDL may intensively alter the structural and biochemical properties of A $\beta$  [61,62], because apo A-I, which includes apo E, apo J, transthyretin, and  $\alpha$ -antichymotrypsin as well as being the main lipoprotein in HDL and the major component of senile plaques in the brain of patients with AD binds to A $\beta$  [63,64].

Finally, an interesting approach to the treatment of AD is gene therapy via cell transfers such as stem cells or via genetic materials for example viral vectors particularly adeno-associated-viruses-2 (AAV2), DNA, RNA or antisense agents [65,66]. New developments in molecular medicine have made gene therapy in the nervous system a potentially practical method for the delivery of therapeutic molecules such as growth factors, which are neurotrophic factors [6].

In the 1980's, it was discovered that nerve growth factor (NGF) not only influences neuronal survival and axon growth during brain development, but it was also produced in the adult hippocampus and neocortex [67]. Subsequent studies showed that NGF infusion into the ventricles could prevent the lesions, which had induced degeneration of cholinergic neurons in the aged rats. However, other brain structures sensitive to NGF, which were exposed to the increased NGF led to adverse effects such as pain and weight loss. Therefore, gene therapy, which was explored in the late 1980's solved this problem by targeting a specific site and allowing more time in delivering the NGF without further manipulation. Despite long-term NGF infusions, which would require refilling of a pump reservoir, and would be subject to infection and malfunction, a single gene infusion can last for years. In *ex vivo* NGF gene

delivery, retroviral vectors specifically coded for NGF are used, and it is advised to go through a stereotaxic surgery to circumvent the obstacle presented by the BBB [6,66].

On the other hand, the disadvantages of this method include no conclusive evidence for clinical or biomarker benefits, no definitive statistical significance after two years and a lack of scientific validity for open-label comparisons with historical controls in clinical trials [65].

So far, currently approved medications have had little effect on dementia especially on AD, and they have not been able to suppress its progression. Therefore, identification and implementation of preventive strategies seem to be more practical and essential rather than only combating the disease once it has been developed [2].

Besides lifestyle factors such as sleep pattern, physical and mental activities, diet may also play a prominent role in preventing AD [2,8,68]. In this regard, special attention is given to the three types of nuts including hazelnut, walnut and almond, considered brain food in manuscripts of traditional Persian medicine as preventive agents against brain atrophy and memory loss. According to several studies, these nuts consist of unsaturated fatty acids, phenolic acids, and other nutrients that play an important role in amyloidogenic ( $\beta$ -secretase and  $\gamma$ -secretase), tau phosphorylation and cholinergic (cholineacetyl transferase, acetylcholine receptors and acetylcholinesterase) pathways. However, in some people these nuts may cause allergic reactions [7,69–71].

The Mediterranean diet seems to have many components reported as potentially beneficial for cognitive performance, and reduce the risk of AD. It features the high consumption of vegetables, legume, fruits, cereals, unsaturated fatty acids (mostly olive oil, but low intake of saturated fatty acids), moderate to high intake of fish, low to moderate use of dairy products (mainly cheese and yoghurt), low intake of red meat and poultry, and regular but moderate amount of alcohol especially red wine usually with meals. The Mediterranean diet may exert its effects by multiple potential anti-AD mechanisms including against oxidative stress and inflammation by having antioxidants such as complex phenols, vitamin C, vitamin E and carotenoids. However, Mediterranean diet scores did not differ between subjects who remained in the study and those who quit [72].

Furthermore, natural products and medical foods particularly with multi-target approaches are currently being investigated as novel interventions for AD and also as valuable sources of bioactive compounds in drug discovery [7].



## **2. Coconut oil in medicine**

In recent years, this oil has become popular in the healthy food world as a highly nutritious functional food, rich in dietary fibres, vitamins and minerals. Several health benefits have been attributed to coconut oil (virgin and extra virgin), coconut water and coconut cream including hair and skin care, anxiety relief, obesity treatment, cholesterol control, antimicrobial effects, cardiovascular uses by preventing elevation of blood pressure, insulin regulation and more recently in AD. Among the coconut products, its oil is of the highest interest to human health. Nevertheless, for several years, coconut oil was vilified for causing atherosclerosis due to having mainly saturated fatty acids (~92%). Opinions, however, are shifting, and recently its health benefits are increasingly being recognised [68,73].

Between the 1920s and 1930s, it was demonstrated that coconut oil was different from other fats and oils for which it was found to consist of 62-70% MCTs/ medium chain fatty acids (MCFAs). The predominant fatty acid in coconut oil is lauric acid (12 carbons) with a concentration of 48.5% [68,73].

The main difference between MCFAs and long chain fatty acids (LCFAs) is the length of the fatty acid carbon chain. MCFAs have a chain length of six to 12 carbons, whereas LCFAs contain 14 or more carbons. The length of the carbonic chain determines the physical and chemical properties of the fats as well as their metabolism [68,73].

MCFAs are unique as they are easily absorbed and quickly metabolised in the liver. They enter the mitochondria in the liver, and get metabolised into ketone bodies. These ketone bodies are transferred from the liver to the brain, heart and skeletal muscles to give rise to energy by converting to acetyl-CoA that enters the citric acid cycle (Krebs cycle) eventually giving rise to ATP (energy). In contrast, LCFAs primarily get packed in the chylomicrons to be transferred to the liver, and require the assistance of carnitine to pass through the mitochondrial membrane before it can be used for energy production. Further, MCFAs have been reported to increase energy expenditure more than LCFAs, most likely due to increased metabolic rates and thermogenesis [68,73].

### **2.1. Coconut oil in AD**

AD has also been described as type III diabetes mellitus (T3DM), wherein the brain is unable to metabolise and use glucose, which is its primary source of energy. Due to disturbances in insulin signalling, because of the expression of genes encoding insulin, insulin-like growth factor (IGF)-1 and IGF-II as well as the significant reduction of their receptors [74]. The

MCFAs in coconut oil provide the brain with an alternative source of energy as they can readily convert to the ketone bodies, naturally produced during fasting/starvation [73,75].

In the brain, the lipid macromolecule, cholesterol is utilised as an antioxidant for structural scaffolding of neural network as an electrical insulator in order to prevent ion leakage and as a functional component of the cell membrane. Cholesterol is used in wrapping and synaptic delivery of the neurotransmitters. It also has a major role in synaptic formation and functions in the brain. Several studies have demonstrated the deficiency of cholesterol in AD brains [73,76]. Moreover, a positive relation was found between high levels of cholesterol and longevity due to improving memory function and reducing dementia in a population above 85 years old [73,77].

*In vitro* studies on flavonoids have shown that hydroxyl groups in phenolic compounds can trap hydrogen bonds of A $\beta$ , and may reduce A $\beta$  accumulation. Moreover, phenolic acids and cytokinins (phytohormones) present in coconut oil and water, respectively, are recognised for their antioxidant and eventually anti-ageing and anti-inflammatory properties [68,78]. *p*-Coumaric acid, ferulic acid, catechin acid, and caffeic acid are the major phenolic acids found in coconut oil.

In an innovative recent animal study, potential neuroprotective effects of VCO on A $\beta$  toxicity was found in a high-fat diet fed rats in an AD model (receiving A $\beta$ ) for which VCO improved the function of hippocampus, memory and learning by inhibiting nucleotide-binding oligomerisation domain (NOD)-like receptor family pyrin domain containing 3 (NLRP) 3 inflammasome and reducing oxidative stress compared with the control group [79].

Dutch researchers have led a study on the efficacy of adjunctive extra VCO on moderate to severe AD in which 20 g of VCO oil was administered to the patients for six weeks. Subjects were on a normal diet, and continued with their prescribed medications for AD. The ADAS-Cog often considered the gold standard in evaluating cognitive results indicated a significant improvement in scores between weeks zero, two, four, and six [40].

## **2.2. MCTs and inflammation**

An animal study showed that a high fat diet can cause obesity, impaired glucose metabolism, insulin resistance, and inflammatory response in mice, whereas MCTs significantly prevent visceral fat obesity and improve glucose intolerance in high fat diet-fed mice. Moreover, MCTs may suppress upregulation of IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2

(COX-2), and downregulation of IL-10, while deactivate pro-inflammatory signal pathways including nuclear factor-kappa beta (NF- $\kappa$ B) and p38 mitogen-activated protein kinase (MAPK) pathways [80].

Nevertheless, a significant amount of data has shown that high fat diets (HFDs) such as coconut oil induce neuroinflammation and cognitive decline in normal mice. HFDs also increase AD inflammatory biomarkers, impair synaptic plasticity, and induce insulin resistance [18,81,82]. A long-term high cholesterol diet may change the cerebral vasculature, BBB integrity and the accumulation of A $\beta$  in the brain of transgenic mice, which increases tau hyperphosphorylation. Nevertheless, HFDs do not increase tau phosphorylation in an AD animal model [18,83].

### **2.3. Association between dyslipidaemia and inflammation**

In spite of evidence indicating a strong relationship between lipid disorders and inflammation in metabolic disorders such as obesity, impaired glucose metabolism, dyslipidaemia, and hypertension, a few studies have reported a connection between these disorders and AD from which the molecular mechanisms of this association require further clarification [18,80].

A relationship between cholesterol and inflammasome is a connection between lipid metabolism and inflammation, which are two important processes in AD [18].

This project will investigate the levels of cytokine including IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in pre-clinical AD in order to prevent and the management of this disease, which can be more beneficial and cost-effective than treatment after afflicting with the disease. It will also look into the plasma A $\beta$  to evaluate the A $\beta$  40/42 ratio as a potential diagnostic biomarker in preclinical AD. To date, no other studies have measured the plasma levels of cytokines and A $\beta$  in cognitively normal elderly individuals with high neocortical amyloid- $\beta$  load (NAL).

### **3. Aims**

The aims of this study are listed below.

1. To investigate the plasma levels of cytokines including IL-1  $\beta$ , IL-6 and TNF- $\alpha$  in high and low NAL (NAL<sup>+</sup> and NAL<sup>-</sup>) groups
2. To investigate the association between the cytokines and cognitive performance and between cytokines and AD risk factors including age, gender, *APOE*  $\epsilon$ 4 carrier status, lipid profiles, education, and BMI
3. To investigate the association between the cytokines and CRP
4. To investigate the levels of plasma A $\beta$  in high and low NAL groups
5. To investigate the association between plasma A $\beta$  and cognitive performance and between plasma A $\beta$  and AD risk factors including age, gender, *APOE*  $\epsilon$ 4 carrier status, lipid profiles, education, and BMI
6. To evaluate the A $\beta$  40/42 ratio as a potential diagnostic biomarker for preclinical AD

## Materials and Methods

Ninety-seven plasma samples (non-repeated freeze-thaw cycles) from the McCusker Kerr Anglican Retirement Village Initiative in Ageing Health (KARVIAH) cohort were used to measure IL-1 $\beta$  by immunosorbent assay (ELISA), which employs the quantitative sandwich enzyme immunoassay technique. Notably, three samples (two NAL<sup>+</sup> and one NAL<sup>-</sup>) were missing due to the shortage of plasma [84,85].

### 1. Participants

Study participants were recruited from Anglicare (formally known as Anglican retirement villages), New South Wales (NSW), Australia. The study was conducted from 2015 to 2016. Two hundred and six volunteers after giving their written informed consent were screened for the following inclusion and exclusion criteria [84,86–88].

#### **Inclusion criteria:**

1. Age 65-90 years
2. Good general health
3. Living in independent units or similar residence place with no or minimal impairment in their daily activities as determined by a clinical interview
4. Fluent in English
5. No known substantial cerebral vascular disease
6. Sufficient visual and auricular power to do the tests
7. No objective cognitive impairment using a Montreal cognitive assessment (MoCA) cut off score  $\geq 26$  (MoCA scores between 18 and 25 would be assessed individually by the study neuropsychologist following the stratification of MoCA score according to age and education) [89].
8. Memory complaint as determined by memory assessment clinic-questionnaire (MAC-Q), and preferably verified by an informant.
9. No objective memory impairment based on cognitive test scores

**Exclusion criteria:**

1. Being diagnosed for dementia based on other revised criteria from the National Institute on Ageing-Alzheimer's Association
2. Having acute functional psychiatric disorder including lifetime history of schizophrenia or bipolar disorder
3. History of stroke
4. Severe or extremely severe depression, based on the depression, anxiety, stress scales (DASS)
5. Uncontrolled high blood pressure (BP): Systolic BP > 170 mm Hg or diastolic BP > 100 mm Hg
6. History of alcohol or drug abuse/dependence within two years of screening
7. History of regular alcohol consumption exceeding seven standard units per week for women or 14 standard units per week for men (One standard unit is equal to five ounces of wine or 12 ounces of beer or one and a half ounces of hard liquor) within six months of screening
8. History of using anticoagulants such as warfarin within four weeks of screening
9. Obstruction of the biliary tract
10. Contradiction to MRI including but not limited to the individuals with a pacemaker, presence of metallic fragments near eyes or spinal cord or cochlear implant (Dental fillings are not considered as a risk for MRI.)
11. History of closed-angle glaucoma or related conditions, which are contraindicated for retinal scanning
12. Any significant systemic disease or unstable medical conditions that may lead to difficulty complying with the study protocol including a history of myocardial infarction in the past year or unstable/severe cardiovascular disease including angina or congestive heart disease, chronic renal failure, chronic hepatic disease, and severe pulmonary disease.

One hundred and five out of the 134 volunteers, who met the inclusion and exclusion criteria underwent neuropsychiatric evaluation, blood collection and neuroimaging. The remaining either declined neuroimaging or withdrew from the study. One hundred out of the remaining 105, who had MMSE scores equal or above 26 at baseline were included in the study. Sixty-

five participants were then categorised as low NAL (NAL<sup>-</sup>) and 35 as high NAL (NAL<sup>+</sup>), based on their neocortical A $\beta$  load measured via a PET scan using ligand <sup>18</sup>F-FBB, wherein a NAL standard uptake value ratio (SUVR) cut-off score of 1.35 was used. Subjects with scores equal or above 1.35 were classified as high NAL, and those with scores below 1.35 were classified as low NAL.

Participants were recruited with the intent to treat through the consumption of curcumin capsules for one year. For this substudy, only baseline plasma samples and data were assessed.

Baseline assessments included a comprehensive physical examination by measuring weight, height, girth, BMI, BP, pulse rate, respiratory rate, and temperature, chest and abdominal examination, followed by a full blood examination, clinical chemistry, and iron studies.

## **2. Neuropsychological assessments**

Participants underwent a battery of neuropsychological test including but not limited to mini mental state examination (MMSE), MoCA and MAC-Q.

**MMSE:** This test is probably the most common cognitive screening test to evaluate orientation, attention, memory, language, and praxis. However, it does not include all the important cognitive abilities [90]. MMSE scores range from 0 to 30, and indicate severe to no cognitive impairment [87].

**MoCA:** It is a 30-item screening instrument of global cognition, which was designed to detect MCI. It includes measurements of visuospatial/executive, naming, attention, language, abstraction, and orientation functions [89,91].

**MAC-Q:** It is a self-reported measure of age-related memory weakness, which was used to identify subject memory complainers (SMCs). It includes six items, one of which is a general question to check global memory, and five others which determine specific memory failures. Participants with a total score <25 will be classified as normal memory cognition [91,92].

## **3. Neuroimaging**

Participants underwent MRI brain scan and two PET brain scans at Macquarie medical imaging centre in Sydney. They were administered an intravenous bolus of FBB slowly over 30 seconds in the supine position. Images were obtained over a 20 min scan, in five min expositions, which started 50 min after injection. NAL was calculated as the mean SUVR of the frontal, superior parietal, lateral temporal, lateral occipital, anterior and posterior cingulate regions normalised

with that of the cerebellum using an image processing software, computerised analysis of PET (Cap) AIBL [84,86].

#### **4. Blood collection and storage**

Eighty millilitres of blood was collected from the participants in 2015 after an overnight fast for a minimum of 10 h. The blood collection was performed according to standard serological techniques. Blood samples were collected in heparin ethylenediamine tetraacetic acid (EDTA) coated tubes. All tubes were kept on rockers at ambient temperature for at least 20 min. Blood processing started about 20 min after collection to allow for the gel tube to clot. Approximately 53 mL of each blood sample, was fractionated and stored at -80 °C within 3.5 h of the collection for research. The remaining 27 mL was used for clinical pathology testing in a private laboratory [87].

#### **5. APOE genotyping**

From the blood collected, 0.5 mL of whole blood was used to extract purified genomic DNA for APOE genotyping to determine the alleles including  $\epsilon$  2,  $\epsilon$  3 and  $\epsilon$  4 according to TaqMan single nucleotide polymorphism (SNP) genotyping assays for rs7412 (C 904973) and rs429358 (C 3084793) as per the manufacturer's instructions (AB Applied Biosystems by Life Technologies, Scoresby, VIC, Australia). Five percent of the samples were genotyped in duplicate, and demonstrated 100% inter- and intra-assay concordance [87,88]. This test was carried out at Macquarie University, NSW.

#### **6. CRP measurement**

CRP concentrations were measured in serum samples using COBAS Tina-quant immunoturbidimetric high sensitivity (hs) CRP assay and AVIDA wide range (wr) CRP assay in a private laboratory [87].

#### **7. Measuring plasma A $\beta$**

The plasma A $\beta$  measurements were carried out by our collaborators at the University of Gothenburg, Mölndal, Sweden in 2017 using the ultra-sensitive single molecule array (SIMOA) platform. The SIMOA A $\beta$  40 and A $\beta$  42 assays both use the same capture Ab, which targets the nitrogen (N)-terminal of A $\beta$ , whereas different biotinylated and specific antibodies for A $\beta$  40 and A $\beta$  42 detect carbon (C) terminal. Calibrators or diluted samples with four-fold dilution for A $\beta$  42 and eight-fold for A $\beta$  40 are mixed with the capture beads and biotinylated



detection Ab. After washing away any non-specifically bound proteins, incubation and adding a substrate solution, detection is performed on a SIMOA disk [93,94].

## 8. Cytokine assays

### 8.1. ELISA method to measure IL-1 $\beta$

This assay was done by me.

The ELISA kits were stored at 4 °C, and was brought to room temperature before the experiment (Fig. 4).



**Figure 4.** IL-1 $\beta$  ELISA kit

Relevant materials in the ELISA kit: **biotechne<sup>®</sup>** (2018 R&D Systems, Inc).

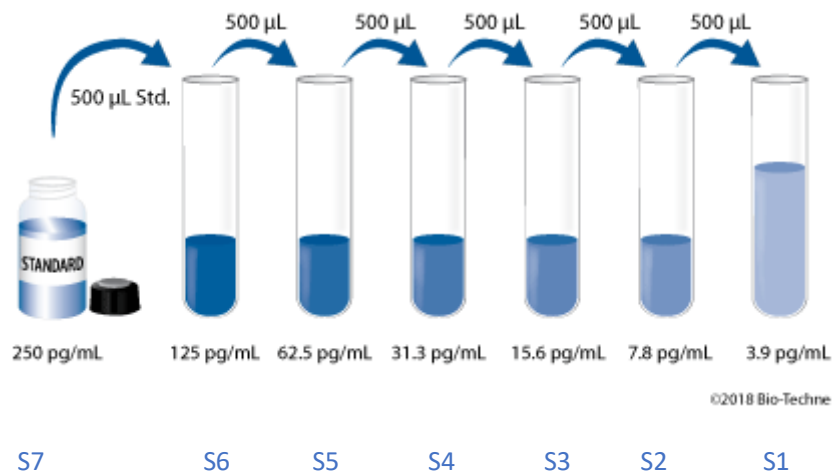
- Human IL-1 $\beta$  microplate:  
96 well polystyrene microplate (12 strips of eight wells) coated with a monoclonal Ab specific for human IL-1 $\beta$
- Human IL-1 $\beta$  standard
- Human IL-1  $\beta$  conjugate

- Assay diluent RD1-83
- Calibrator diluent RD6C
- Wash buffer concentrate
- Colour reagents A and B
- Stop solution
- Plate sealers (adhesive strips)

As IL-1  $\beta$  is detectable in saliva and sweat, precautionary measures were taken to prevent contamination of the kit reagents while running the assay.

### 8.1.1. Assay procedure

1. Reagents were brought to room temperature before use. Samples were thawed on the same day that the assay was performed.
2. The wash buffer was mixed gently until any crystals were completely dissolved, and 20 mL of wash buffer was added to 480 mL of deionised water in order to prepare 500 mL of wash buffer.
3. Human IL-1 $\beta$  standard was reconstituted with 5 mL calibrator diluent RD6C according to the vial label for reconstitution volume. This reconstitution produced a stock solution of 250 pg/mL. The standard sat at least 15 min with gentle agitation before any dilutions were made.
4. Five-hundred microlitres of calibrator diluent RD6C was pipetted into each tube. Then, the stock solution was used to produce a dilution series from standard (S) one to seven as follows (See Fig.5).



**Figure 5.** Dilution process of the standard stock solution (Reference: Quantikine® ELISA manual)

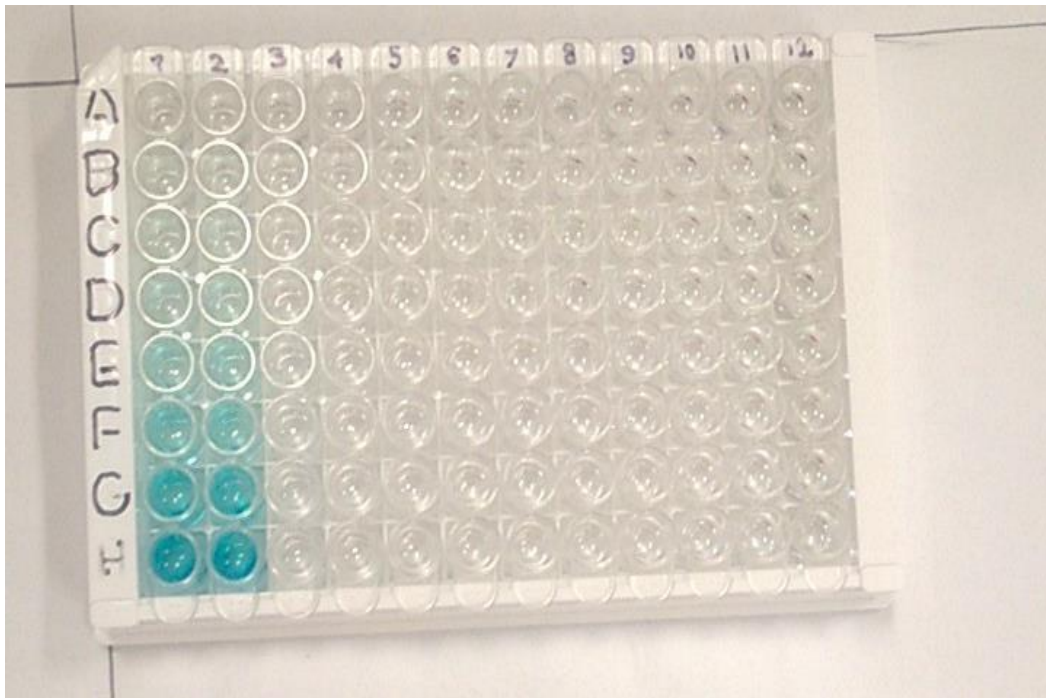
Each tube was thoroughly mixed before the next transfer. The undiluted human IL-1 $\beta$  (250 pg/mL) served as the high standard, and the calibrator diluent served as the zero standard (0 pg/mL).

5. Fifty microlitres of the assay diluent RD1-83 was added to each well, and it was mixed well before and during use.
6. Two-hundred microlitres of standard, blank or sample was added to each well. The microplate was covered with the adhesive strip, and incubated for 2 h at room temperature.
7. The liquids in the ELISA plate were evacuated. All wells were washed with 400  $\mu$ L of wash buffer using a 300  $\mu$ L multi-channel pipette by filling each well with 200  $\mu$ L of wash buffer twice. The washing process was repeated twice. After each wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels until the last paper towel was completely dry.
8. Two hundred microlitres of the human IL-1 $\beta$  conjugate was added to each well. The microplate was covered with another adhesive strip, and incubated for 2 h at room temperature.
9. The washing process was repeated three times as in step seven.
10. Within 15 min of use, reagents A and B were mixed in equal volumes to make the substrate solution from which 200  $\mu$ L was added to each well. The plate was then incubated in the dark for 20 min at room temperature. The substrate solution changed from colourless to gradations of blue.
11. Finally, 50  $\mu$ L of the stop solution was added to each well. The colour in the wells changed from blue to yellow (Fig. 6 and 7). If the colour in the wells was green, or it did not change homogeneously, the plate was gently tapped to ensure thorough mixing.
12. The optical density (OD) of each well was determined within 30 min using a microplate reader. The OD at 450 nm, 540 nm and 570 nm were read to check the consistency of the results between 540 nm and 570 nm. Then, the reading at 570 nm was subtracted from the reading at 450 nm.

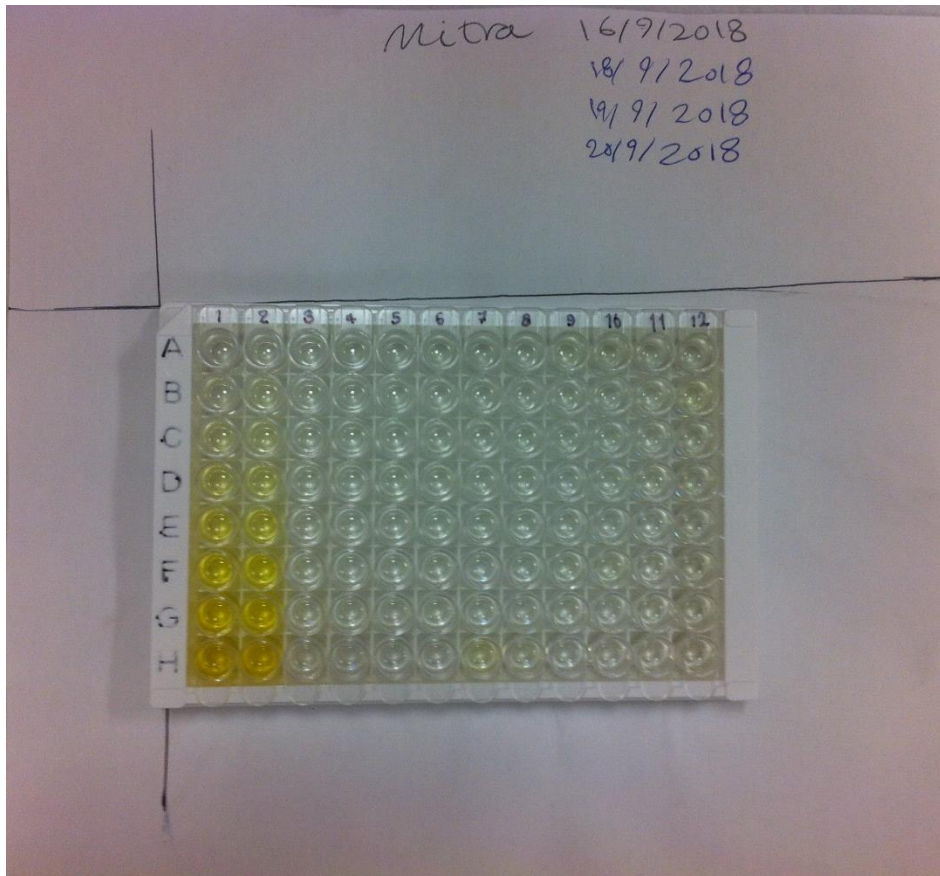
Online software ([www.elisaanalysis.com](http://www.elisaanalysis.com) and [www.mycurvefit.com](http://www.mycurvefit.com)) was used to calculate the concentrations and create standard curves based on a four-parameter logistic curve fit.

The average of the duplicate readings for each standard and sample was calculated. Then, the average zero standard OD was subtracted. The formula to calculate the mean concentration of duplicate samples can be found below.

Regression formula:  $y = d + \frac{a-d}{1+(x/c)^b}$       Inverse Formula:  $X = c \left( \frac{a-d}{y-d} - 1 \right)^{1/b}$   
where:  $y$ =response value e.g. OD,  $x$ =dose value e.g. concentration



**Figure 6.** Image of the microplate after the third incubation: Standard series are duplicated horizontally in column one and two.



**Figure 7.** Image of the microplate after adding the stop solution

## 8.2. ELISA method to measure IL-6

This assay was done by another student in our research team in order to share the data. An additional 100 plasma samples were taken from the same cohort. The method is very similar to the previous one to measure IL-1 $\beta$  with the following modifications.

1. Four and a half millilitres of RD6C was added to the human IL-6 standard
2. Six-hundred and sixty-seven microlitres of RD6F was added into each tube.
3. Three-hundred and thirty-three microlitres of standard stock solution was added into the eight tubes to produce a dilution series as follows.

S7= 300 pg/mL, S6=100 pg/mL, S5=50 pg/mL, S4=25 pg/mL, S3=12.5 pg/mL,  
S2=6.25 pg/mL, S1= 3.13 pg/mL and S0 (RD6F): 0 pg/mL

4. One hundred microlitres of standard or samples was added to each well.
5. All wells were washed four times.
6. The OD was measured at 450 nm and 540 nm.

### 8.3. ELISA method to measure TNF- $\alpha$

This assay was also done by another student with 100 extra plasma samples from the same cohort, and it was slightly different from the assays for interleukins. The different processes are as follows.

1. One millilitre of calibrator diluent RD6-12 was added to the human TNF- $\alpha$  standard for the standard reconstitution.
2. Four-hundred and fifty microlitres of calibrator diluent RD6-12 was added into each tube.
3. Fifty microlitres of the stock solution was added into the eight tubes to produce a dilution series as follows.  
S7= 1000 pg/mL, S6=500 pg/mL, S5=250 pg/mL, S4=125 pg/mL, S3=62.5 pg/mL, S2=31.3 pg/mL, S1=15.6 pg/mL and finally RD6-12 served as S0=0 pg/mL
4. Fifty microlitres of standard or samples was added to each well.
5. Four times washing with the wash buffer was done during steps seven and nine (See IL-1 $\beta$  procedure).
6. Opposed to the ILs, the incubation was done on a horizontal orbital microplate shaker (0.12" orbit), set at 500 $\pm$ 50 rpm.
7. Similar to IL-6, the OD was read at 450 nm and 540 nm.

### 9. Statistical Analyses

Statistical analyses were performed using the IBM statistics package for social sciences (SPSS) statistics 19 software package (version 25). The data of cytokines were not normally distributed, and log transfer was not possible due to having some undetectable (zero) values for the cytokines. Therefore, general linear models, under one-way analysis of variance (ANOVA) were used to compare continuous variables, and chi-square ( $\chi^2$ ) tests were used to compare categorical variables between low and high NAL groups. *P* values were calculated with and without adjusting for covariates such as age, gender, APOE  $\epsilon$ 4 carrier status and years of education. *P* value <0.05 was considered as significant. Logistic regression was used to create receiver operating characteristic (ROC) curves to evaluate predictive models.

## Results

### 1. Baseline data

The descriptive characteristics of the KARVAIAH cohort participants are presented in Table 1. As the colours show, there are two significant results from AD risk factors between the two groups, which are the percentage of *APOE*  $\epsilon 4$  carriers and total cholesterol. However, total cholesterol is slightly higher in the NAL- group.

Table 1. Characteristics of the study participants

	Total N	Low NAL	High NAL	<i>P</i> Value
Gender (M: F)	32:68	19:46	13:22	0.419
Age (years)	78.18±5.52	77.62±5.56	79.23±5.38	0.165
BMI	27.62±4.56	27.38±4.47	28.05±4.74	0.486
Education (years)	14.43±3.26	14.85±3.37	13.64±2.92	0.078
n <i>APOE</i> $\epsilon 4$ carriers (%)	21 (17.4)	5 (7.7)	16 (45.7)	<0.001
Global cognition score	-0.0002±0.55	0.06±0.49	-0.13±0.63	0.08
MMSE	28.61±1.15	28.50±1.16	28.80±1.11	0.225
MoCA	26.97±1.77	27.14±1.73	26.66±1.81	0.299
MAC-Q	26.08±2.70	26.02±2.55	26.20±3.00	0.746
n SMC (%)	76 (76)	51(78.5)	25 (71.4)	0.432
<sup>18</sup> F-FBB-PET SUVR	1.35±0.31	1.16±0.09	1.71±0.26	-
Total cholesterol (mmol/L)	4.73±0.98	4.82±0.98	4.35±1.19	0.045
Triglycerides (mmol/L)	1.27±0.62	1.34±0.70	1.1±0.42	0.071
HDL cholesterol (mmol/L)	1.66±0.45	1.64±0.55	1.52±0.48	0.292
LDL cholesterol (mmol/L)	2.46±0.78	2.39±0.85	2.34±0.88	0.794
*hs-CRP (mg/L)	2.49±4.42	2.16±2.23	3.09±6.84	0.317
*wr-CRP (mg/L)	3.10±6.09	2.68±3.18	3.88±9.37	0.349

N: number ~ 100, M: male, F: female, BMI: body mass index, MMSE: mini mental state examination, MoCA: Montreal cognitive assessment, MAC-Q: memory assessment clinic-questionnaire, SMC: subjective memory complainer, FBB-PET SUVR: Florbetaben positron emission tomography, standard uptake value ratio, HDL: high density lipoprotein, LDL: low density lipoprotein, hs-CRP: high sensitivity-C-reactive protein, wr-CRP: wide range-CRP. Data are presented as mean ± SD unless otherwise specified. \*There are two types of assays for measuring CRP in blood, which are wide-range and high sensitivity [95].

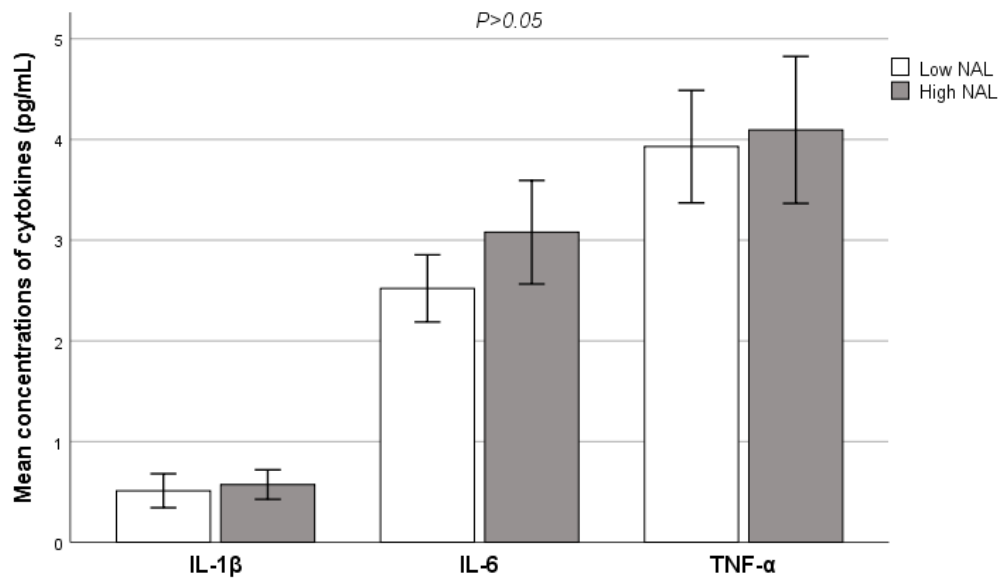
## 2. Plasma cytokines in NAL groups

The mean concentrations of cytokines in NAL<sup>+</sup> versus NAL<sup>-</sup> with or without adjusting the *p* value for covariates including age, gender, *APOE*  $\epsilon$ 4 carrier status, and years of education are presented in Table 2. The *p* values show that the mean concentrations of the cytokines are not statistically significant between NAL<sup>-</sup> and NAL<sup>+</sup> groups. However, as it is shown in figure 8, there is a mild trend of increased levels in the high NAL group.

Table 2. Plasma cytokine concentrations (pg/mL) in NAL<sup>-</sup> and NAL<sup>+</sup>

	Total N	NAL <sup>-</sup>	NAL <sup>+</sup>	<i>P</i> Value	<i>P</i> Value <sup>a</sup>
IL-1 $\beta$	0.53 $\pm$ 1.2	0.51 $\pm$ 1.35	0.57 $\pm$ 0.83	0.902	0.859
IL-6	2.71 $\pm$ 2.82	2.52 $\pm$ 2.69	3.08 $\pm$ 3.04	0.347	0.850
TNF- $\alpha$	3.99 $\pm$ 4.42	3.93 $\pm$ 4.50	4.09 $\pm$ 4.32	0.859	0.760

N: number= 97 for IL-1 $\beta$ , 100 for IL-6 and TNF- $\alpha$ , IL-1 $\beta$ : interleukin-1 $\beta$ , IL-6: interleukin-6, TNF- $\alpha$ : tumour necrosis factor- $\alpha$ . Data are presented as mean $\pm$ SD unless otherwise specifies. The general linear model was used to analyse the data. *P* value<sup>a</sup> was adjusted for age, gender, *APOE*  $\epsilon$ 4 carrier status, and years of education.



**Figure 8.** The bar graphs illustrate the mean concentrations of cytokines in low and high NAL groups. *P* values are not significant. Error bars represent SE $\pm$ 1 not adjusted for the covariates.



## 2.1. Association between cytokines and cognition as well as AD risk factors

The association between the cytokines and cognitive performance as well as between cytokines and AD risk factors including age, gender, *APOE*  $\epsilon_4$  carrier status, lipid profiles, education, and BMI of the study participants was investigated, and significant correlations are presented in the table below.

Table 3. Cytokines, cognitive performance and AD risk factors

Correlation Bivariate	<i>r</i>	<i>p</i> value
TNF- $\alpha$ and Global cognition score	-0.253*	0.011
TNF- $\alpha$ and MMSE	-0.218*	0.029
IL-6 and Age	0.218*	0.029
TNF- $\alpha$ and Age	0.368**	<0.001
TNF- $\alpha$ and Triglycerides	0.216*	0.031

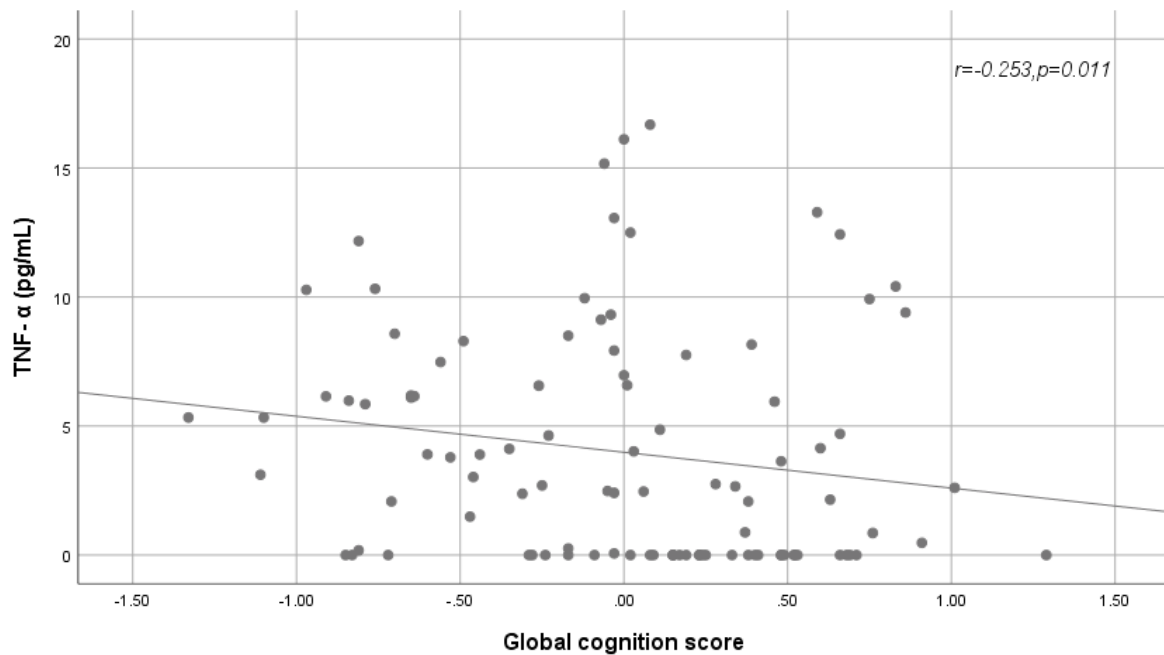
\**r* = Correlation coefficient is significant at the 0.05 level (two-tailed).

\*\*Correlation is significant at the 0.01 level (two-tailed).

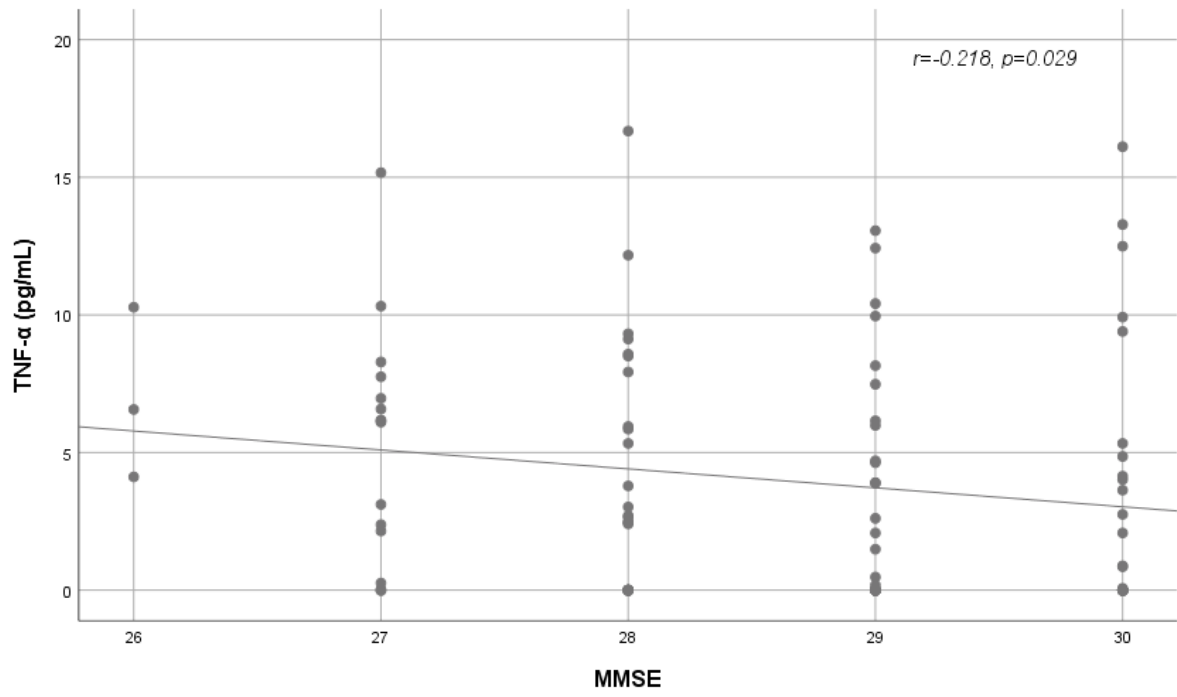
Inverse correlations are negative values.

AD: Alzheimer's disease, IL-6: interleukin-6, TNF- $\alpha$ : tumour necrosis factor- $\alpha$ , MMSE: mini mental state examination. Spearman's rho test was used to calculate the correlation coefficient.

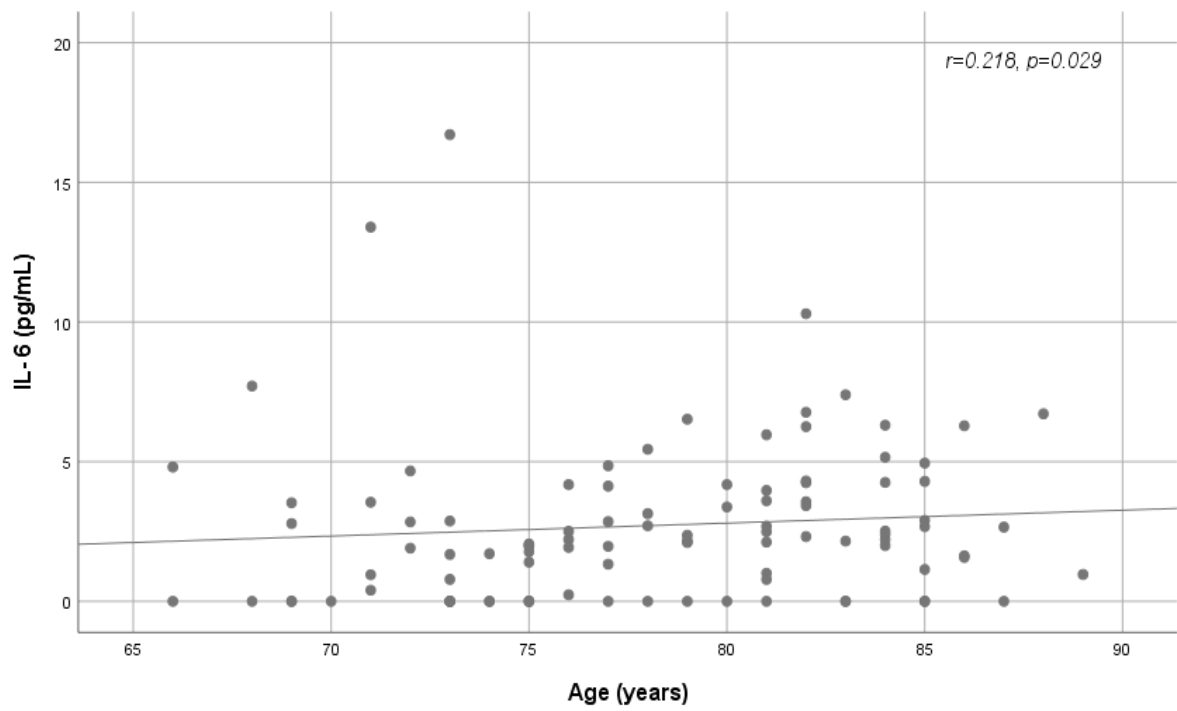
Graphs for the correlations shown in Table 3 are presented in figure 9 to figure 13 as follows.



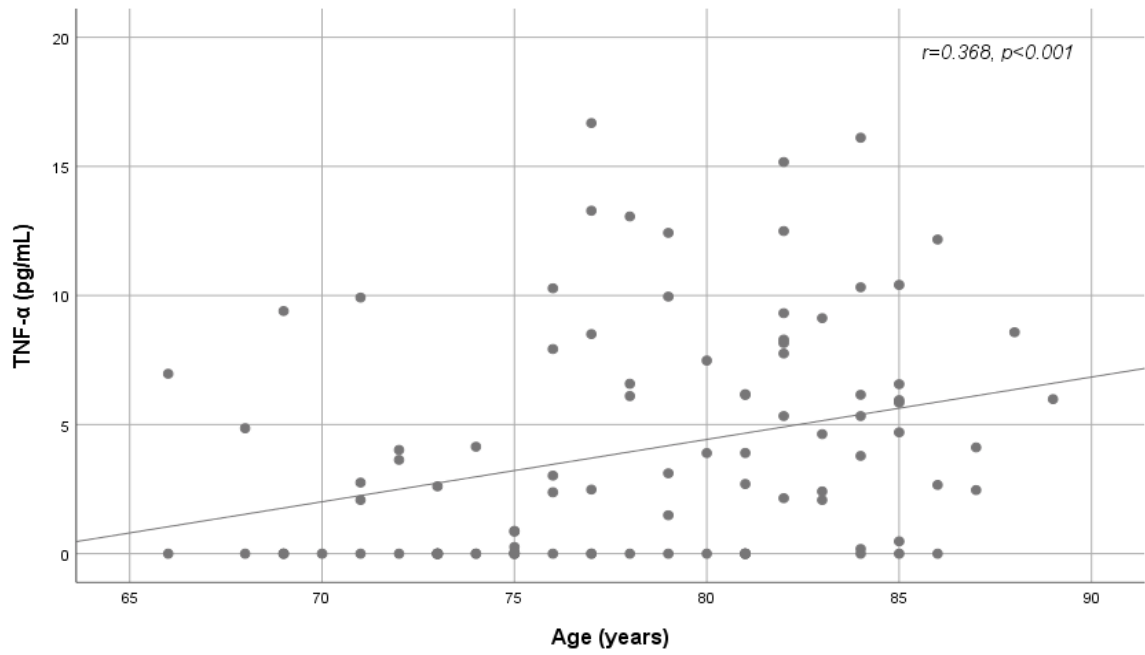
**Figure 9.** A significant correlation was observed between TNF- $\alpha$  and global cognition score using Spearman's rho test to calculate the correlation coefficient.



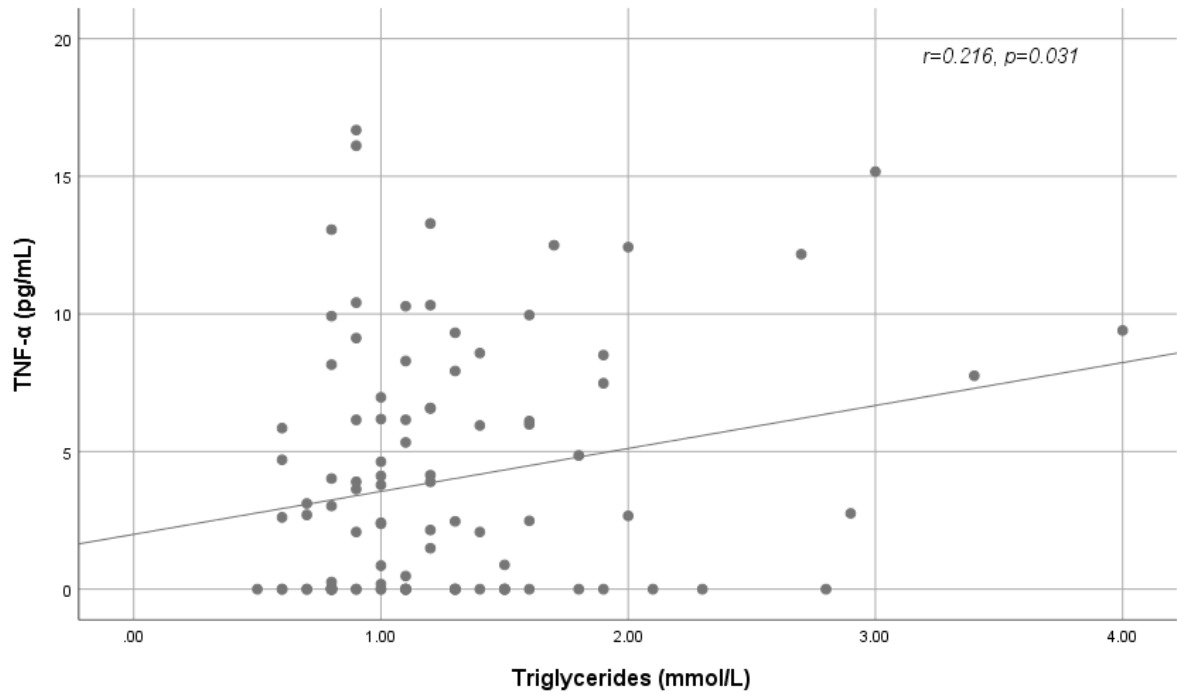
**Figure 10.** A significant correlation was observed between TNF- $\alpha$  and MMSE score using Spearman's rho test to calculate the correlation coefficient.



**Figure 11.** A significant correlation was observed between plasma IL-6 and age using Spearman's rho test to calculate the correlation coefficient.



**Figure 12.** A significant correlation was observed between TNF- $\alpha$  and age using Spearman's rho test to calculate the correlation coefficient.



**Figure 13.** A significant correlation was observed between TNF- $\alpha$  and triglycerides using Spearman's rho test to calculate the correlation coefficient.

### Non-parametric tests to investigate the association between the cytokines and *APOE* $\epsilon_4$ :

The distributions of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were similar across *APOE*  $\epsilon_4$  genotype categories based on the statistical tests presented in the table below.

Table 4. Hypothesis test summary based

Null Hypothesis	Test	<i>P</i> value	Decision
The distribution of IL-1 $\beta$ is the same across categories of <i>APOE</i> $\epsilon_4$ genotype.	Independent-Samples Kruskal-Wallis Test	.657	Retain the null hypothesis.
The distribution of IL-6 is the same across categories of <i>APOE</i> $\epsilon_4$ genotype.	Independent-Samples Kruskal-Wallis Test	0.771	Retain the null hypothesis.
The distribution of TNF- $\alpha$ is the same across categories of <i>APOE</i> $\epsilon_4$ genotype.	Independent-Samples Kruskal-Wallis Test	0.309	Retain the null hypothesis.

IL-1 $\beta$ : interleukin-1 $\beta$ , IL-6: interleukin-6 and TNF- $\alpha$ : tumour necrosis factor- $\alpha$ . Asymptomatic significances are displayed. The significance level is 0.05.

### 2.2. Association between cytokines and CRP

The association between the cytokines with CRP, the other pro-inflammatory biomarker was also investigated. Surprisingly, there was no significant correlation between them. CRPs including hs-CRP and wr-CRP as well as the cytokines were not normally distributed (See the correlations in Table 5).

Table 5. Cytokines and CRP

Correlation Bivariate	<i>r</i>	<i>p</i>
IL-1 $\beta$ and hs-CRP	-0.49	0.633
IL-1 $\beta$ and wr-CRP	-0.056	0.588
IL-6 and hs-CRP	0.144	0.153
IL-6 and wr-CRP	0.120	0.236
TNF- $\alpha$ and hs-CRP	0.032	0.752
TNF- $\alpha$ and wr-CRP	0.026	0.795

hs-CRP: high sensitivity- C-reactive protein, wr-CRP: wide rang- CRP, IL-1 $\beta$ : interleukin-1 $\beta$ , IL-6: interleukin-6 and TNF- $\alpha$ : Tumour necrosis factor- $\alpha$ . Spearman's rho test was used to calculate the correlation coefficient.

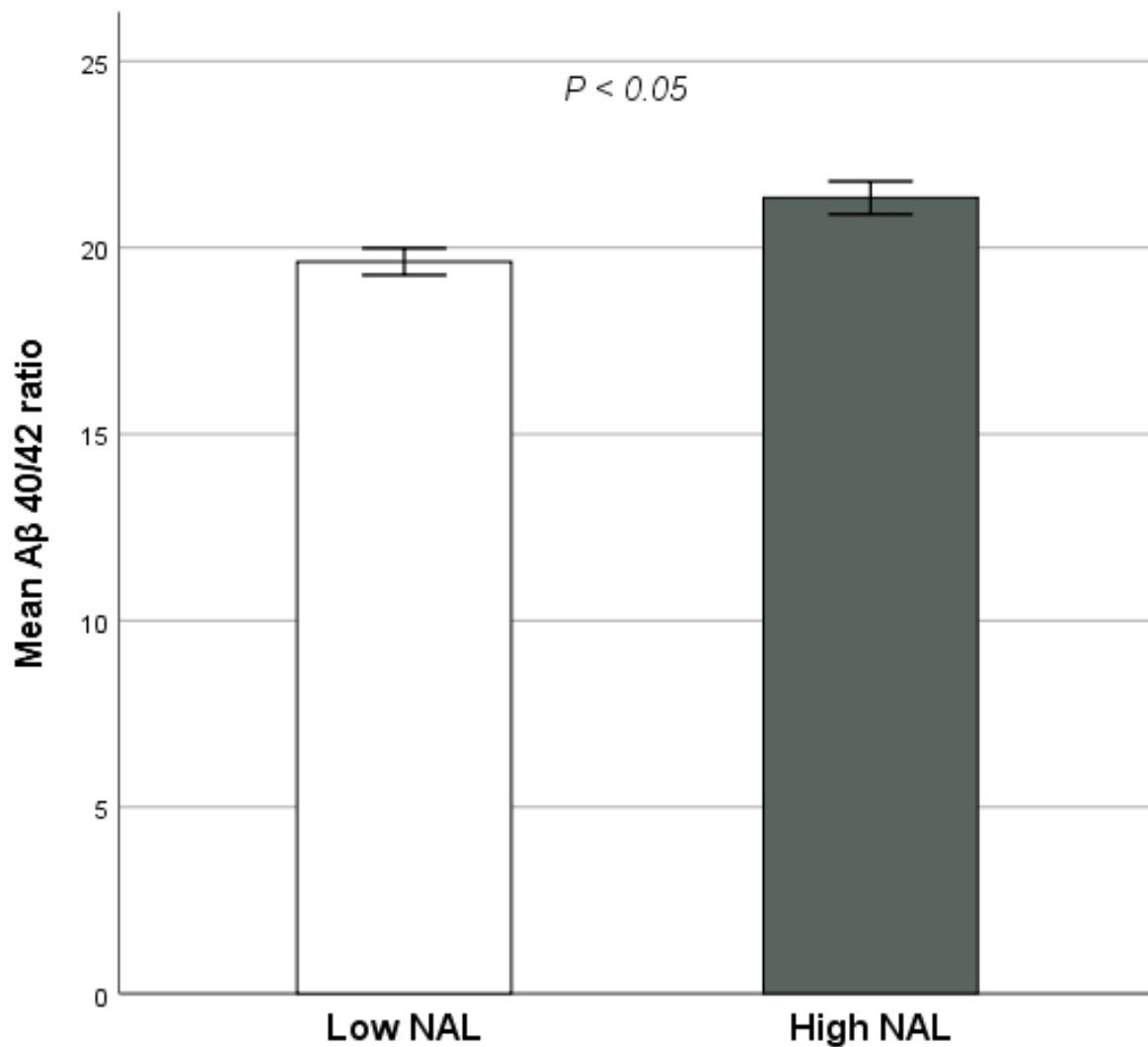
### 3. Plasma A $\beta$ 40 and A $\beta$ 42 in NAL groups

I investigated the plasma A $\beta$  40 and A $\beta$  42 from the baseline dataset. Then, I calculated the ratio of A $\beta$  40/42, and compared them between low and high NAL groups. There were no significant differences in the mean concentrations of A $\beta$  40 and A $\beta$  42 between NAL<sup>-</sup> and NAL<sup>+</sup> groups with or without adjusting the *p* value for the covariates (Table 6). However, the A $\beta$  40/42 ratio was significantly higher in the NAL<sup>+</sup> group compared with the NAL<sup>-</sup>. The mean A $\beta$  40/42 ratio between NAL<sup>-</sup> and NAL<sup>+</sup> is presented in figure 14.

Table 6. Plasma A $\beta$  40, 42 (pg/mL) and A $\beta$  40/42 in NAL<sup>-</sup> and NAL<sup>+</sup> subjects

	Total N	NAL-	NAL+	<i>P</i> Value	<i>P</i> Value <sup>a</sup>
A $\beta$ 40	315.95 $\pm$ 61.67	307.44 $\pm$ 54.16	331.27 $\pm$ 71.59	0.067	0.105
A $\beta$ 42	15.89 $\pm$ 3.62	15.97 $\pm$ 3.71	15.71 $\pm$ 3.48	0.744	0.488
A $\beta$ 40/42	20.2 $\pm$ 2.84	19.62 $\pm$ 2.84	21.34 $\pm$ 2.5	0.03	0.008

N: number~100, A $\beta$ : amyloid- $\beta$ . Data are presented as mean  $\pm$  SD unless otherwise specified. The general linear model was used to analyse the data. *P* value<sup>a</sup> was adjusted for age, gender, *APOE*  $\epsilon$ 4 status, and years of education.



**Figure 14.** The bar graphs illustrate the mean Aβ 40/42 ratio in low and high NAL groups. The  $p$  value = 0.03 is significant. Error bars represent  $SE \pm 1$  not adjusted for the covariates.

### 3.1. Association between plasma Aβ and cognitive performance as well as AD risk factors

The association between the plasma Aβ 40 and Aβ 42 and cognitive performance as well as between the and plasma Aβ 40 and Aβ 42 and AD risk factors such as age, gender, *APOE* ε4 carrier status, lipid profiles, education, and BMI of the study participants was investigated, and significant correlations are presented in Table 7 on the next page.

Table 7. Association between A $\beta$ , cognitive performance and AD risk factors

Correlation Bivariate	<i>r</i>	<i>p</i>
A $\beta$ 40 and Global cognition score	-0.293	0.003
A $\beta$ 40 and working memory and executive function composite z-score	-0.231*	0.022
A $\beta$ 42 and composite verbal and visual episodic memory	-0.212*	0.037
A $\beta$ 40 and composite verbal and visual episodic memory	-0.269**	0.007
A $\beta$ 40 and Age	0.259*	0.01

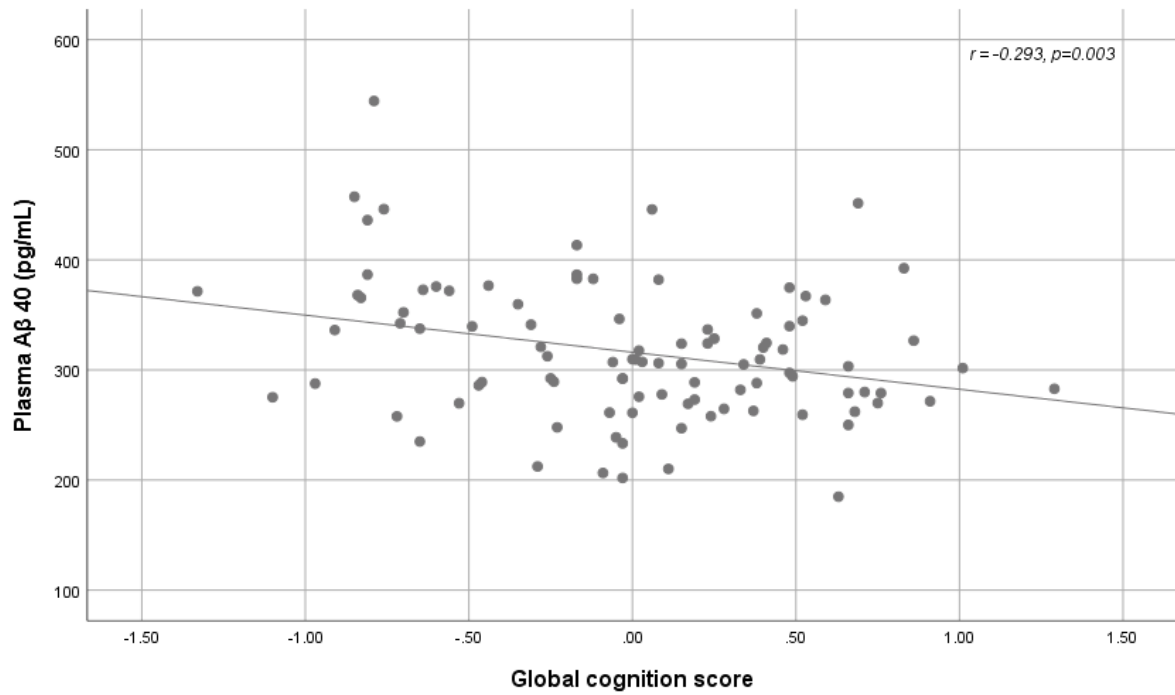
\**r* = Correlation coefficient is significant at the 0.05 level (two-tailed).

\*\*Correlation is significant at the 0.01 level (two-tailed).

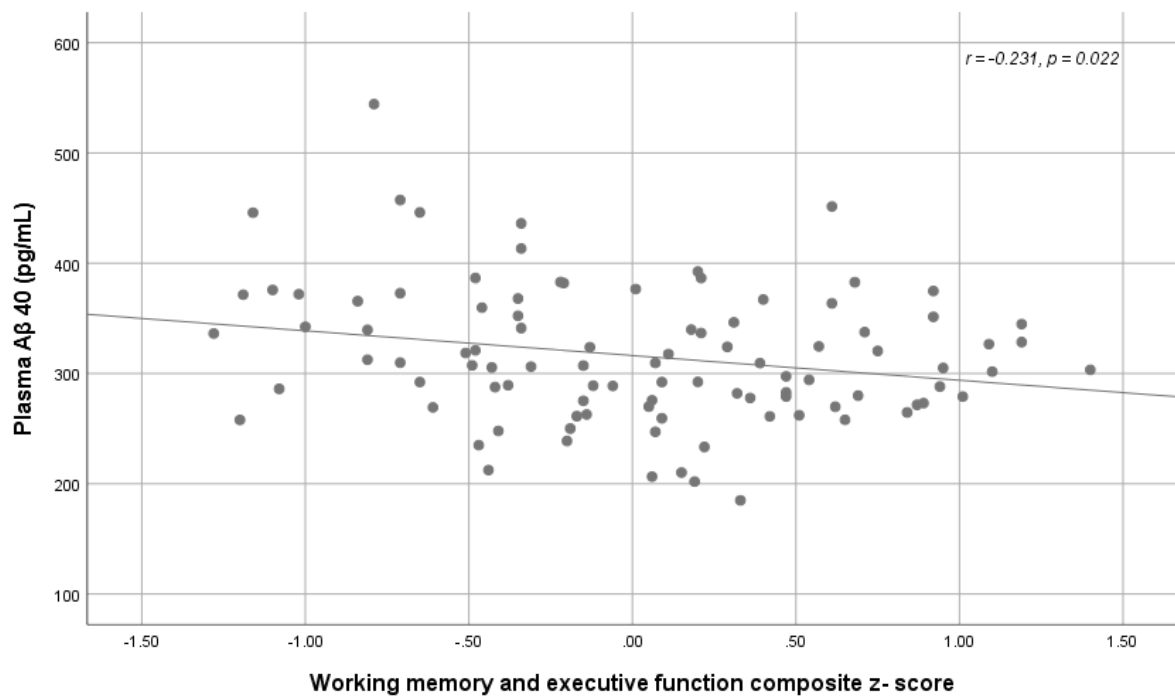
Inverse correlations are negative values. Pearson correlation was used to calculate the correlation coefficient, as data were normally distributed.

AD: Alzheimer's disease and A $\beta$ : amyloid- $\beta$

Graphs for the correlations shown in Table 7 have been presented in figure 15 to figure 19 (See the next page).

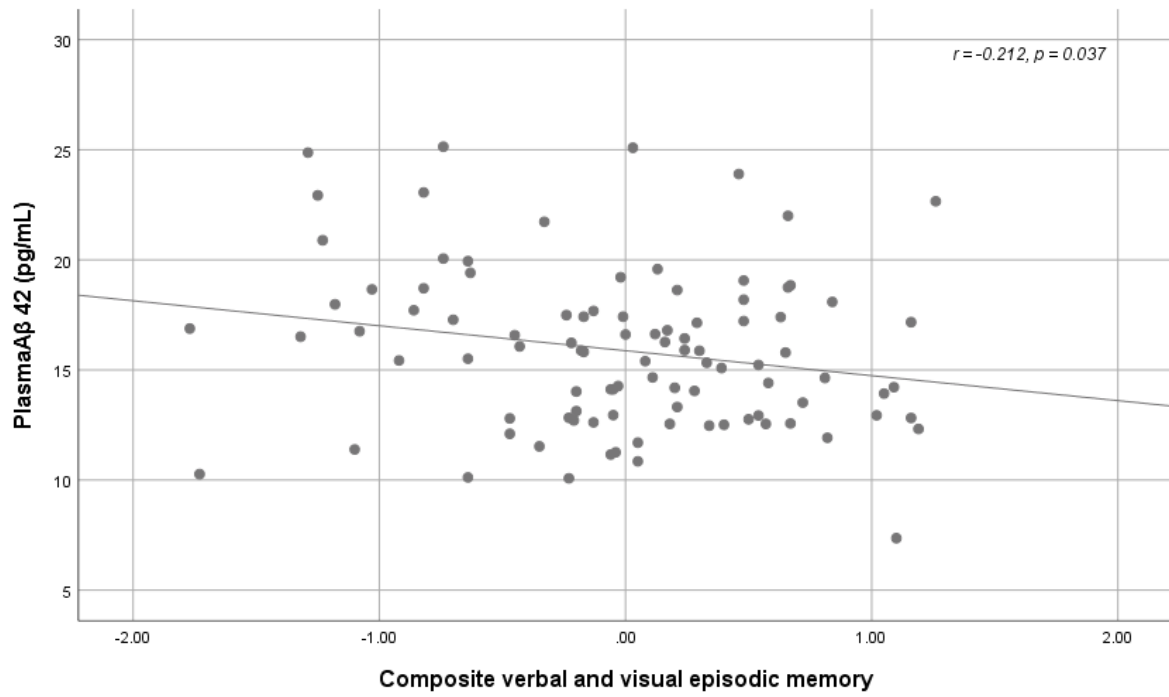


**Figure 15.** A significant correlation was observed between plasma Aβ 40 and global cognition score using Pearson test.

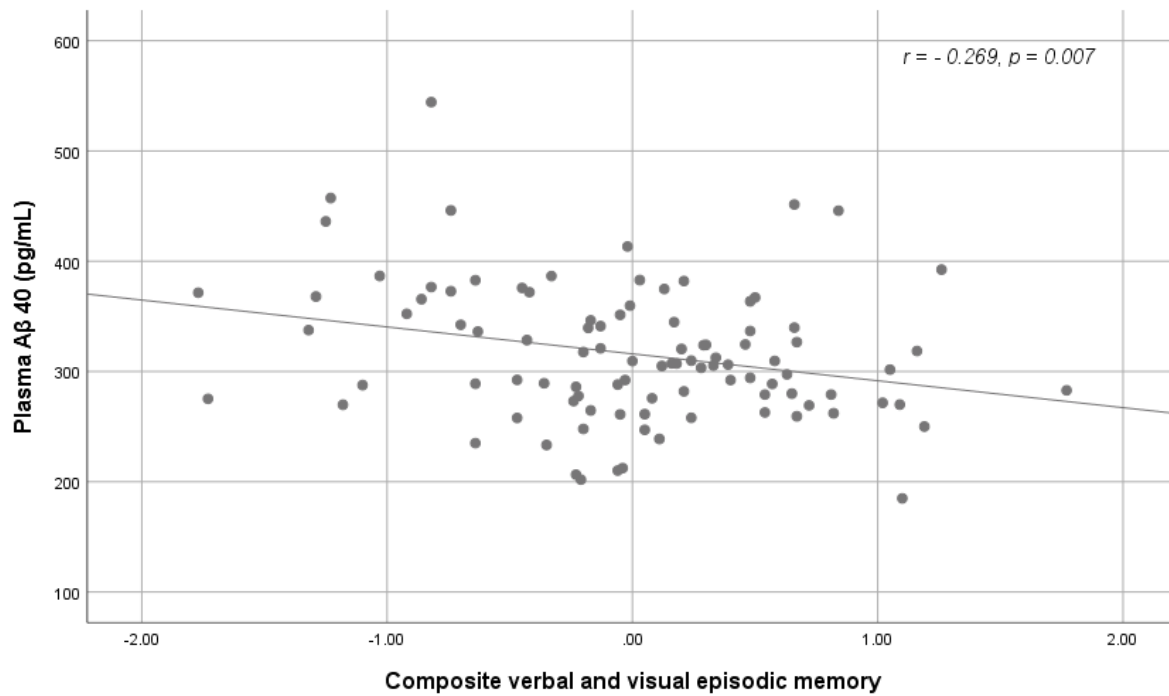


**Figure 16.** A significant correlation was observed between plasma Aβ 40 and working memory and executive function composite z-score using Pearson test.

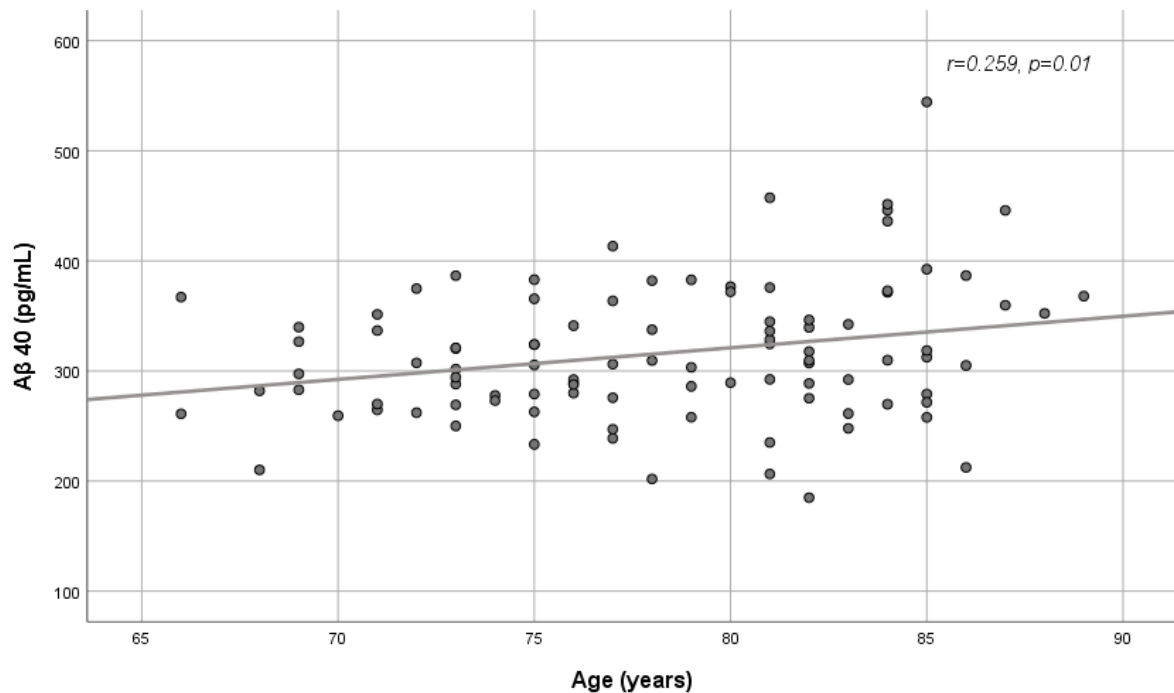




**Figure 17.** A significant correlation was observed between plasma Aβ 42 and composite verbal and visual episodic memory using Pearson test.



**Figure 18.** A significant correlation was observed between plasma Aβ 40 and composite verbal and visual episodic memory using Pearson test.

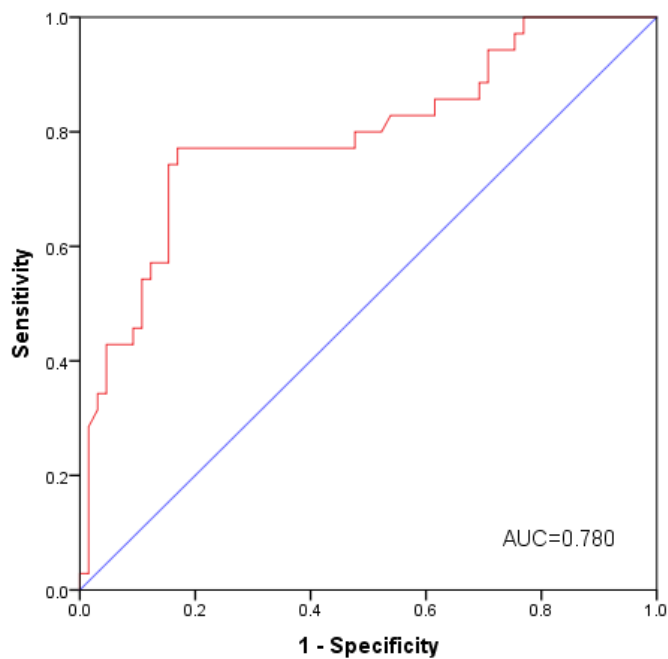


**Figure 19.** A significant correlation was found between plasma Aβ 40 and age using Pearson test.

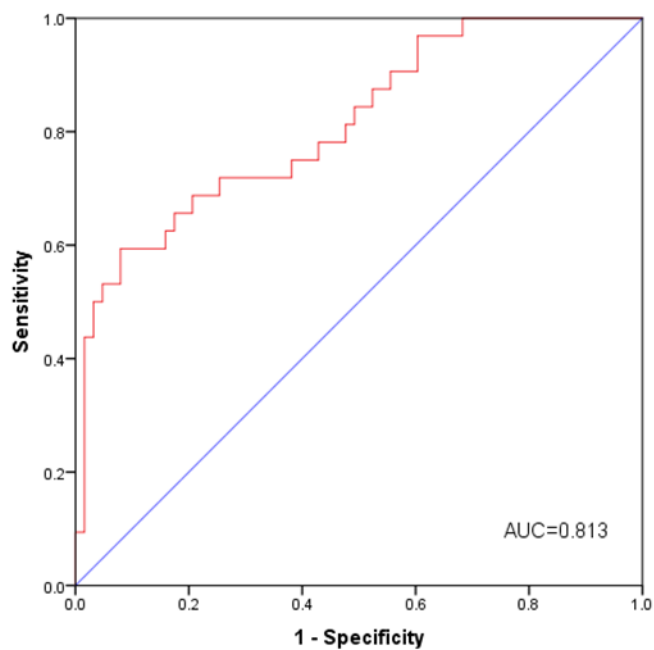
### 3.2. Aβ 40/42 ratio as a potential diagnostic biomarker

Finally, given that significant difference was observed in Aβ 40/42 ratio between NAL<sup>-</sup> and NAL<sup>+</sup> groups, I also evaluated this ratio as a potential diagnostic biomarker for NAL<sup>+</sup> by creating a base model comprising major AD risk factors such as age, gender, *APOE* ε4 carrier status and the years of education. Then, it was compared with the “base model + Aβ 40/42 ratio”, wherein the ratio of Aβ 40/42 was added to the base model. The ROC curves for these models are presented in Fig. 20 (A and B). The area under the curve (AUC) of the “base + Aβ 40/42 ratio” (AUC = 0.813, 80% sensitivity and ~ 52% specificity) slightly outperformed the base model (AUC= 0.780, 80% sensitivity and ~ 47% specificity) in predicting high and low NAL.

A) Base model



B) Base model + A $\beta$  40/42



**Figure 20.** ROC curves for the prediction of high NAL were created using logistic regression that show the specificity of the “base” model (A) comprising major AD risk factors including age, gender, *APOE*  $\epsilon 4$  carrier status, and education was enhanced by adding the A $\beta$  40/42 ratio i.e. “base+A $\beta$  40/42” model (B). ROC: receiver operating characteristic, NAL: neocortical amyloid load, AD: Alzheimer’s disease, A $\beta$ : amyloid- $\beta$  and AUC: area under curve.

## Discussion

Brain inflammation is a major feature of the AD brain, and several studies have shown that inflammatory factors particularly proinflammatory cytokines are elevated in the blood of individuals with clinical symptoms of AD. However, there are no definitive studies on the levels of proinflammatory cytokines in high risk individuals who are positive for cerebral A $\beta$ , which is a major objective of my thesis together with determining plasma A $\beta$  levels. Evidence in support of the latter has recently been shown to demonstrate an accuracy of 90% when plasma A $\beta$  was compared with brain A $\beta$  as reported by Nakamura et al [47,96]. While these recent findings are exciting, and hold great promise for A $\beta$  to be an important blood biomarker for AD diagnosis, the published method employed is not conducive for use in clinical practice as the procedure is complex involving immunoprecipitation followed by mass spectrometry. My approach is attractive as it utilises an ultrasensitive assay for measuring A $\beta$  thus obviating the need for the prior immunoprecipitation step as reported by Nakamura et al [47].

This study focused on the analysis of plasma cytokines (IL1- $\beta$ , IL-6, and TNF- $\alpha$ ) to determine whether their levels were higher in patients with preclinical AD. Although they were associated with several risk factors for AD, these cytokines alone are not reliable markers for preclinical AD. Thus, further investigation was pursued to evaluate whether the ratio of A $\beta$  40/42 would provide an indicator of early preclinical AD. A $\beta$  40/42 ratio was indeed significantly higher in the high NAL group, and was also correlated with some AD risk factors. The relevance of the findings presented in this thesis is discussed below.

### 1. Cytokines

The release of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), IL-8, and macrophage inflammatory protein (MIP)-1 $\alpha$ , as well as anti-inflammatory cytokines such as IL-10, sTNF-RII and IL-1 receptor antagonist (Ra) have been reported to change with ageing based on the acute, chronic or recovery phase of inflammatory processes. For example, the concentrations of these cytokines in cultured supernatants from blood monocytes in acute phase of inflammation in old subjects with pneumonia are lower than young patients with the same disease. These results with the impaired production of cytokines in elderly patients may partially explain the characteristic features of host defence mechanisms in ageing. However, the outcomes of different studies using ELISA methods are inconsistent [97,98]. In the present

study, plasma IL-6 and TNF- $\alpha$  were observed to correlate with age. However, IL-1 $\beta$  plasma concentrations in this cohort were not found to correlate with age. According to the Invecchiare in Chianti, ageing in the Chianti area (InCHIANTI) study, a reason for these inconsistencies, maybe due to the use of a different ELISA protocol for the dosage of cytokines including but not limited to IL-1 $\beta$ , IL-6 and TNF- $\alpha$  has been discussed [99]. In another study, Fagiolo et al. did not find differences in IL-6, TNF- $\alpha$  and IL-1 $\beta$  concentrations in unstimulated cultures from young and old individuals, but substantial differences were observed in mitogen-stimulated cultures, occurring at 24-72h after stimulation. This may suggest that the capacity of cytokine production may not be reflected by serum concentration [100]. IL-1 $\beta$  serum concentrations might represent only an indirect biomarker for chronic inflammation and a proportion of the actual generation capacity of activated monocytes/macrophages. Since the InCHIANTI study had large and representative samples (1292 participants), reflecting sufficient statistical power, the lack of correlation between age and IL-1 $\beta$  concentrations corroborates the study findings of Di Iorio et al [99]. Hence these findings are in line with observations within the current study.

In this study, there was a non-significant association between IL-1 $\beta$  and LDL. Similarly, according to the InCHIANTI study, patients afflicted with dyslipidaemia and deep venous thrombosis had a non-significant trend to higher levels of IL-1 $\beta$ . No significant differences in IL-1 $\beta$  levels were found in the other studied clinical conditions [99].

According to a systematic review and meta-analysis of 175 studies, higher levels of peripheral inflammatory factors such as IL-1 $\beta$ , IL-6, TNF- $\alpha$  converting enzyme, soluble TNF receptors 1 and 2, hs-CRP,  $\alpha$ 1-antichymotrypsin, IL-2, IL-18, INF- $\gamma$ , homocysteine, C-X-C motif chemokine-10, epidermal growth factor, vascular cell adhesion molecule-1, leptin, and decreased IL-1Ra have been found in AD patients compared with controls [101]. In contrast, plasma cytokine concentrations in the current study were not significantly elevated in cognitively normal elderly adults with high NAL (preclinical AD) versus low NAL (those individuals at no apparent risk of AD). Interestingly, these findings are similar to other studies showing no significant difference in plasma cytokines namely, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in AD patients, wherein normal or undetectable concentrations of the cytokines were identified [100-104].

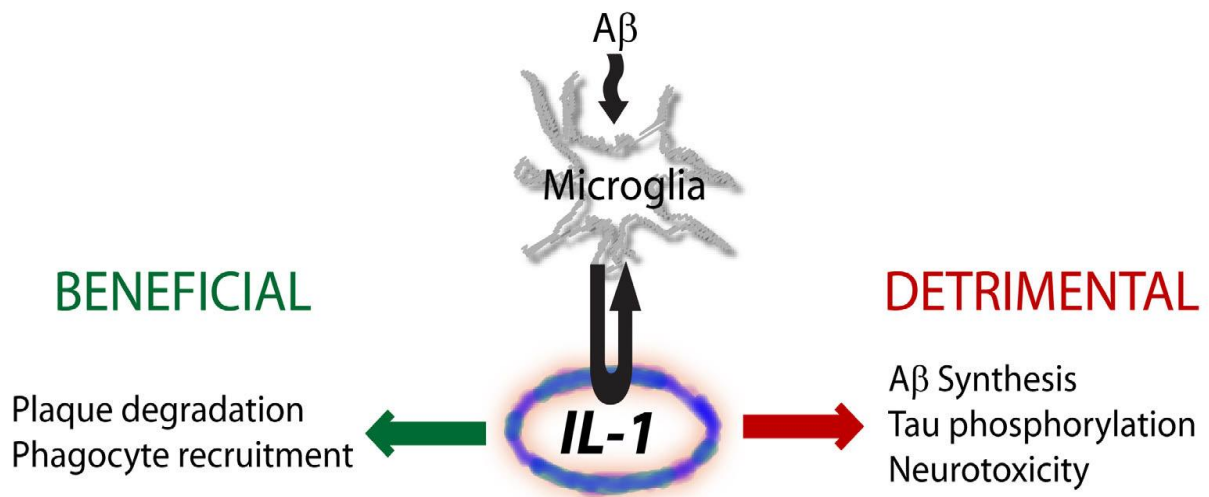
In a German study, where conventional ELISA was employed, it was found that the levels of cytokines in the CSF and serum were decreased or under the detection range. The

concentrations of IL-1 $\beta$ , IL-2, IL-6, and TNF- $\alpha$  in CSF and serum of 20 AD patients and 21 controls were measured. Additionally, other pro-inflammatory biomarkers such as IL-12, IFN- $\gamma$  and IL-13 were investigated in stimulated blood cell cultures from 27 AD patients and 25 controls. The aforementioned study suggested a general decline of the immune response, which served as a pathogenetically relevant factor in AD. Considering the putative role of ageing in AD, a premature immunosenescence attributing to the AD pathogenesis was assumed [105]. However, the clinical significance of the latter finding is questionable as the sample size of 20 is low [107].

Given that preclinical AD individuals as defined by their cerebral amyloid positivity do not show symptoms, and that they represent at least 30% of the population over 60 years old it is not surprising that mixed findings have been reported for cytokine levels in previous studies. Most previous studies did not conduct brain amyloid imaging on participants thus were unable to identify controls that were amyloid negative. Therefore, the contamination of cerebral A $\beta$  positive individuals in the control groups of these studies would be variable thereby contributing to the difference in findings between different studies [108].

In the current study, the plasma levels of the two cytokines, IL-6 and TNF- $\alpha$  were observed to correlate with age, some neuropsychometric parameters and lipoproteins. Therefore, although according to the findings in my study the plasma cytokines lack biomarker value for preclinical AD, they have the potential to act as a prodromal marker of neurodegeneration, because they exhibited a trend towards increased levels in NAL<sup>+</sup> subjects [86]. Future studies using ultrasensitive SIMOA based assays should determine the significance of this notion [109]. Correlations are not always clear between increased cytokine concentrations and cognitive scores, which may be attributed to heterogeneous mental capacity in healthy elderly individuals [110].

The rise in IL-1 levels, associated with inflammation has been correlated to the pathogenesis of AD. However, a clear connection between IL-1 genetic loci and AD has not been reported, and a beneficial role of inflammation for AD cannot be ruled out. Thus, new avenues for AD therapy may evolve from this understanding of the proposed beneficial role of inflammation in AD (See Fig. 21 on the next page) [110].



**Figure 21.** A schematic depiction of potential roles of IL-1 in AD [110]

As mentioned previously, surprisingly, no statistically significant correlations were found between the cytokines and CRP in this study. However, it could be argued that given that the preclinical AD subjects were early stage, about two decades before the onset of symptoms, the levels of plasma cytokines were too low in my study to be measured accurately by conventional ELISA that I employed. A definite answer to this question may be obtained using a much more sensitive assay such as SIMOA, which is about 1000 times more sensitive than conventional ELISA [108]. According to an American study, which was done on 192 AD patients and 174 cognitively healthy controls, the average of CRP concentrations was observed to be significantly decreased in AD compared to controls. In modified models, the increased CRP levels substantially prognosed lesser clinical dementia rating scale sum of boxes (CDR SB) scores in patients with AD, whereas in the control group, CRP had an inverse correlation with MMSE scores and positively correlated with CDR SB scores. O’Bryant et al. have reported that their findings connected with previously published outcomes have been concordant with the primary hypothesis, which presumes that CRP rise in middle age is related to a high risk of AD progression though increased CRP concentrations are not valuable for prognosis in the instant prodrome years before appearing clinical AD. However, for a subclass of AD patients, raised CRP remains to prognose the incremental severity of the disease, suggesting a probable proinflammatory endophenotype in AD [111]. Additionally, a case-control general population study of patients diagnosed with AD found that CRP levels were not significantly different between individuals who remained cognitively healthy for ten years, and those who developed AD

within the same duration. However, surprisingly, the CRP concentrations in the AD cases had a substantial descending slope nearer the diagnosis time, whereas the control group showed an ascending curve to a limited extent towards the same time-point similar to the sigmoidal trajectories detected by Jack et al. in association with other biomarkers especially A $\beta$  and tau [112]. The aforementioned study had limitations such as assessing a single inflammatory biomarker and the older age of the population (>65 years old) [113].

Di Iorio et al. found no correlation between CRP and erythrocyte sedimentation rate (ESR), a marker of inflammation probably, due to the low sensitivity of their method (the name of the method has not been mentioned in their article) [97]. On the other hand, according to an Israeli study, former less sensitive assays were not able to measure serum CRP levels lower than 5 mg/L down to 0.16 mg/L. The innovation of high-sensitivity assays such as the Dade-Behring assay has provided novel current approaches that can be employed in clinical practice. Additionally, the newly introduced Bayer wr-CRP is able to measure a broad range of CRP concentrations including values as low as 0.12 mg/L. In fact, a significant correlation has been found between the results of these two assays. Therefore, low cost and on-line (real time) availability of the wr-CRP assay can be striking for clinicians [95].

The value of forthcoming epidemiological studies on inflammatory biomarkers in the preclinical stage is highly dependent on their ability to test a broader span of biomarkers during this period particularly from middle age, when exposure to numerous major inflammatory diseases is also implicated in the risk of AD including obesity, T2DM and cerebrovascular accident (CVA) that are currently hypothesised to have a major effect [114].

In this study, the years of education was not significantly different between high and low NAL groups. This area has been systematically reviewed by Sharp and Gatz, who reported that lower education was related to a higher risk for AD in many but not all studies, whereas it was varied by study population characteristics such as age, gender and race/ethnicity. Higher levels of education did not homogeneously reduce the risk of dementia either. They have described a greater association with AD appears when the level of education highlights cognitive capacity. Nonetheless, education alone cannot provide a simple indicator for the risk of AD. It may be used to aid risk calculation in context together with other considerations, such as the specific demands of a person's environment



and as a representative point for the course of life events, commencing prior to formal education and spanning events that take place throughout the lifespan. Taken together, the outcomes of the aforementioned review article have suggested that the education-AD correlation may be more multifaceted than formerly proposed in the literature. No clear evidence was found to indicate that prevalence studies have supported stronger or more coherent substantial effects of education on AD risk in comparison to incidence studies. Future work with the cohort in my study will address the role of lifestyle factors and their interplay with education on influencing NAL positivity [115].

## **2. Plasma A $\beta$**

Despite extensive research worldwide, there has not been a definite plasma or blood biomarker, which can indicate a high or low risk of AD except for TOMM 40, which was previously described as one of the main players in the regulations of CSF biomarkers has an important role in AD pathogenesis [114]. The levels of A $\beta$  in blood have been extensively investigated as a potential biomarker for AD. However, contradicting results have been obtained using different molecular detection methods or research designs, which show the lack of availability of ultra-sensitive assays [116]. Measuring serum/plasma A $\beta$  and tau with ELISA platforms have resulted in contradictory findings with levels either unchanged, decreased or increased from controls [94].

The concentrations of A $\beta$  species and tau in plasma and serum are much lower than their levels in CSF, because these molecules are predominantly synthesised in the brain, and do not directly enter the blood circulation due to the presence of BBB. Some A $\beta$  molecules are cleared at the BBB through receptor-mediated mechanisms, while others are removed from the CSF through the lymphatic drainage system [94].

According to a Swedish study, plasma concentrations of A $\beta$  40 and A $\beta$  42 were reduced in AD compared with all other testing groups, which indicate the significant changes in A $\beta$  metabolism occur later in the periphery compared to the brain. However, during the preclinical or prodromal stages of AD (i.e. in NAL<sup>+</sup> groups, subjective cognitive decline and MCI) plasma concentration of A $\beta$  42 was just moderately decreased, whereas A $\beta$  40 levels were unaffected [92].

According to the Alzheimer's disease neuroimaging initiative (ADNI) study, plasma A $\beta$  failed to differentiate AD patients from controls and also NAL<sup>+</sup> from NAL<sup>-</sup> individuals,

although a positive correlation between plasma A $\beta$  40/42 ratio and A $\beta$  ligand retention on PET was found in *APOE*  $\epsilon$ 4-negative subjects only [92].

The Australian imaging biomarkers and lifestyle (AIBL) research team have reported that plasma levels of either A $\beta$  40 or A $\beta$  42, are not related to AD or NAL. However, the A $\beta$  42/A $\beta$  40 ratio (inverse ratio as compared to the ADNI study findings) was slightly decreased in AD patients, and it had an inverse correlation with A $\beta$  load as determined by the amyloid PET. They found that plasma levels of A $\beta$  42 and A $\beta$  40 were reduced in AD, and plasma A $\beta$  42/40 ratio was decreased in MCI and even more in AD [116]. Although most of the previous studies showed no differences in plasma A $\beta$  42 between AD patients and cognitively healthy controls, data from the Swedish study agreed with some studies showing low plasma A $\beta$  42 and/or low plasma A $\beta$  42/A $\beta$  40 ratio in AD. Furthermore, their results of decreased plasma A $\beta$  42 in *APOE*  $\epsilon$ 4 carriers compared with non-carriers were also consistent with former reports. Finally, according to the aforementioned study, although low plasma A $\beta$  42 and A $\beta$  42/40 ratio were associated with A $\beta$  deposition in the brain, these markers did not show diagnostic value in AD [93].

According to a study by Fendos et al., a lower plasma A $\beta$  42/40 ratio is related to a higher risk of dementia, and a greater cognitive decline in healthy control subjects at follow-up. However, some studies have reported weak or no relation between A $\beta$  42/40 plasma ratio with AD. Some of these inconsistent results are likely related to the complexity of measuring plasma A $\beta$ , preanalytical and analytical differences between quantitative methods, differences in study populations in terms of age, stage of the disease and confounding factors [48].

The results of this study regarding the plasma A $\beta$  40/42 ratio is consistent with the main literature review, although no significant differences were found in plasma A $\beta$ 40 and A $\beta$  42 between NAL<sup>-</sup> and NAL<sup>+</sup> groups. More sophisticated approaches for measuring A $\beta$  in the blood have recently been undertaken, and cohorts such as AIBL, which did not show plasma A $\beta$  to be a strong biomarker are now demonstrating that this peptide is a very strong biomarker [40]. Therefore, as it was mentioned in the results section, A $\beta$  40/42 may serve as a potential diagnostic biomarker for early diagnosis in preclinical AD, and the strength of this biomarker is likely to increase with the development of more sensitive and specific assays.

According to a review article by Toledo et al. most studies have shown a strong correlation between older age and higher levels of plasma A $\beta$  [117]. In this study, a correlation was found between plasma A $\beta$  40 and age as well, which was one of my aims.

### **3. Limitations**

This study has strengths such as a characterised, cognitively normal cohort with a representative percentage of NAL<sup>+</sup> participants in an aged population. However, it also has limitations which include a relatively modest sample size for NAL<sup>+</sup> individuals (N=35) though it should be noted that the relative proportion of NAL<sup>+</sup> to NAL<sup>-</sup> participants is consistent with major studies including AIBL and ADNI. Unlike the two latter studies, the findings presented in my thesis is cross-sectional only which is another limitation. The plasma samples in my study were collected in 2015 and thus three years since collection though all samples were stored frozen at minus 80 °C. It has been reported that in samples stored for more than two years old, the levels of cytokines are reduced when measured by multiplex immunoassays. Long term storage (greater than 4 years) results in cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-8, IL-10, and IL-15 to be degraded up to 75% compared to values measured fresh following collection [119,120]. Moreover, we also measured the levels of cytokines in 20 extra samples from the interventional phase of the KARVIAH cohort to not waste the third kit for the three cytokines, and these samples were found to have slightly higher cytokine levels, which could be attributed to the one year lesser storage duration compared to the samples of the current study. The long term storage of the samples used in my study may thus have resulted in degradation of the cytokines that I measured reducing the significance of my findings. Another limitation of this study was the assessment of only three cytokines in the baseline phase.

## Conclusions/Future Directions

The cytokine results while not significant in my study did exhibit a trend towards increased levels in NAL<sup>+</sup> subjects. Future studies using samples stored for less than 12 months and an ultrasensitive assay such as SIMOA should provide more definitive answers as to their significance whether A $\beta$  build up in the brain precedes, and leads to the elevation of cytokines. Longitudinal studies will provide insight into the role of cytokines not only as biomarkers but also in the pathogenesis of AD. Plasma A $\beta$  is a promising biomarker for detection of preclinical AD, which may contribute to reflecting A $\beta$  build up in the brain either alone or more likely as a panel of biomarkers. My finding with the ultrasensitive SIMOA assay shows that plasma A $\beta$  can be measured directly, which would facilitate its use in the clinic. Using higher affinity A $\beta$  antibodies may allow even greater sensitivity and accuracy increasing its value as a blood biomarker.

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## Appendix

### Final ethics approval letter

Office of the Deputy Vice( Research)

-Chancellor



Research Services  
Research Hub, Building C5C East  
Macquarie University  
NSW 2109 Australia T: +61 (2) 9850 4459  
<http://www.research.mq.edu.au/>  
CRICOS Provider No 00002J

30 November 2017

Dear Professor Martins

**Reference No:** 5201701078

**Title:** *Investigating blood and brain biomarkers for preclinical Alzheimer's disease in the McCusker KARVIAH (Kerr Anglican Retirement Village Initiative in Ageing Health) cohort*

Thank you for submitting the above application for ethical and scientific review. Your application was considered by the Macquarie University Human Research Ethics Committee (HREC (Medical Sciences)) at its meeting on 23 November 2017.

The Executive noted the prior approval of the research from the Bellberry Human Research Ethics Committee.

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated May 2015) (the *National Statement*).

This letter constitutes ethical and scientific approval only.



NB. Approval is for the use of deidentified data only.

**Standard Conditions of Approval:**

1. Approval is contingent on continuing compliance with the requirements of the *National Statement*, which is available at the following website:

<http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research>

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. Proposed changes to the protocol and associated documents must be submitted to the Committee for approval before implementation.

It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email [ethics.secretariat@mq.edu.au](mailto:ethics.secretariat@mq.edu.au)

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

[http://www.research.mq.edu.au/for/researchers/how\\_to\\_obtain\\_ethics\\_approval/human\\_research\\_ethics](http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics)

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely



**Professor Tony Eyers**

Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

**Details of this approval are as follows:**

**Approval Date:** 23 November 2017

The following documentation has been reviewed and approved by the HREC (Human Sciences & Humanities):

Documents reviewed	Version no.	Date
Macquarie University HREC Prior Review Form	1*	Received 08 Nov 2017
McCusker_KARVIAH_MK002 Protocol	6	15 Jul 2016

Documents Noted	Version no.	Date
Letter from Prof Martins re Notification of further data analyses to Bellberry HREC	N/A	06 Nov 2017
Bellberry HREC Trial Completion Acknowledgement Letter	N/A	21 Sep 2017
Bellberry HREC Approval Letter (Appl 2012-0901086)	N/A	02 Apr 2013
Bellberry HREC Application Form		Submitted 05 Oct 2012
McCusker_KARVIAH_MK002 Research Foundation Participant Information and Consent Form	3	12 Apr 2013

## Amendment

Macquarie University Student Email and Calendar Mail - HREC...

<https://mail.google.com/mail/u/0?ik=6838318ae7&view=pt&sea...>



Mitra Elmi <mitra.elmi@students.mq.edu.au>

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### HREC Application - Amendment Approved - 5201826144153 - Martins

1 message

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donotreply@infonetica.net <donotreply@infonetica.net> Mon, Sep 3, 2018 at 12:57 PM To: ralph.martins@mq.edu.au  
Cc: ralph.martins@mq.edu.au, mitra.elmi@hdr.mq.edu.au, kathryn.goozee@mq.edu.au, tejal.shah@mq.edu.au,  
pratishtha.chatterjee@mq.edu.au, danit.saks@hdr.mq.edu.au, cintia.botelhodias@mq.edu.au, hamid.sohrabi@mq.edu.au,  
maryam.mohammadi@hdr.mq.edu.au

Dear Professor Martins

RE: 5201826144153 - Investigating blood and brain biomarkers for preclinical Alzheimer's disease in the McCusker KARVIAH (Kerr Anglican Retirement Village Initiative in Ageing Health) cohort

Your amendment request has been approved.

You may access the application by logging into the Human Research Ethics Management System.

Kind regards,

Ethics Secretariat

Research Services Level 3, 17 Wally's Walk  
Macquarie University, NSW 2109, Australia

T: +61 2 9850 4459 (Administration)

T: +61 2 9850 7850 (HREC: Humanities and Social Sciences)

T: +61 2 9850 4194 (HREC: Medical Sciences)

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#### 1.1.4 Macquarie University Research Team Members

Search for additional personnel by name.

Title	First Name	Surname
Dr	Mitra	Elmi
Department		
Faculty	Faculty of Medicine and Health Sciences	
Staff OneID	45042802	
AOU Code	5031	
Email	mitra.elmi@hdr.mq.edu.au	

##### 1.1.4.1. Detail the expertise, qualifications and competence held by this member of the research team.

##### 1.1.4.2. If this member of the research team is a student, please provide a response to the following:

###### 1.1.4.2.1. Identify the student's educational program.

Masters

###### 1.1.4.2.2. Detail the supervisory arrangements, support, and training provided to the student researcher.

Prof Ralph Martins, Dr Hamid Sohrabi, Dr Pratihtha Chatterjee, Dr Tejal Shah and Dr Cintia Dias will be providing supervisory support and training.

##### 1.1.4.3. What position does this research team member have on the project?

Associate Investigator

##### 1.1.4.4. Describe the research activities this research team member will be responsible for.

Investigate lipoprotein fractions and inflammatory marker levels in blood samples from the KARVIAH cohort, that have already been collected and stored frozen for research purposes, in order to investigate whether lipoprotein fractions and inflammatory markers in the blood can serve as blood biomarkers for preclinical Alzheimer's disease, using enzyme linked immunosorbent assays.

Search for additional personnel by name.