Investigating carotenoid and α-tocopherol levels in preclinical Alzheimer's disease

Danit Gila Saks

BSc. (Neuroscience), MBMSc

Student number: 45331138

Department of Biomedical Sciences

Faculty of Medicine and Health Science

Macquarie University

NSW Australia

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Summary

Background: Oxidative stress, a well-documented contributor to neurodegeneration, has been shown to be an early feature of Alzheimer's disease (AD) and may have preclinical significance. Carotenoids and vitamins have been proposed to have antioxidant, anti-inflammatory and anti-amyloidogenic potentials, which interrupts the oxidative stress pathway, protecting against neurodegeneration. The protective effect of these biomolecules has been reported in cognitive decline and AD progression, however, it has not yet been investigated in cognitively normal individuals at higher risk for AD, in relation to amyloid-β. This study investigates whether there is an association between plasma carotenoids and α-tocopherol levels, and neocortical amyloid-β load (NAL) prior to the onset of clinical AD.

Methods: Cognitively normal participants (n=99, aged 65-90 years), recruited from the Kerr Anglican Retirement Village, were characterised as being preclinical AD (the high NAL group (n=35)) or at no apparent risk of AD (low NAL group (n=65)) determined using positron emission tomography. Carotenoid and α-tocopherol levels were analysed using high-performance liquid chromatography (HPLC) and correlated with plasma amyloid-β (Aβ) levels determined using single-molecule array (SIMOA) assays.

Results: Plasma carotenoid and α-tocopherol levels were not significantly associated with NAL. After controlling for clinical characteristics, and dietary and supplement intake, higher α-carotene was significantly associated with high NAL (p=0.033), and after adjusting for diet and supplements, α-tocopherol levels were significantly lower in the high NAL group (p=0.044). Lutein was significantly inversely associated with plasma $A\beta_{40}$ and $A\beta_{42}$ (p=0.025 and 0.045, respectively), β-carotene was significantly inversely associated with plasma $A\beta_{40}$ (p=0.030).

Conclusion: The current study reveals minimal association between plasma carotenoids and α-tocopherol, and brain Aβ levels. The significant inverse association of lutein and β-carotene to plasma Aβ, but not cerebral Aβ, suggests a potential interaction of these carotenoids with Aβ peptides. The nature of this association should be investigated by future research as it may help determine the potential therapeutic benefit of increased dietary intake of carotenoids in preclinical AD.

Declaration

This thesis entitled 'Investigating carotenoid and α-tocopherol levels in preclinical Alzheimer's disease' is original research and has been written by me. Any assistance I received in the thesis preparation and any collaborations have been acknowledged. All information sources used in the making of this thesis have been cited in the text.

This work has been created for the 10-month Master of Research degree and has not been previously submitted for a degree to this or any other university or institution.

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

Danit Saks

Student number: 45331138

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Abbreviations

Abbreviation	Full Term
AD	Alzheimer's disease
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid-beta
BMI	Body mass index
BOLD	Blood-oxygen-level-dependent
CCV	Cancer Council of Victoria
COWAT	Controlled Oral Word Association Test
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DASS	Depression, Anxiety, Stress Scales
D-KEFS	Delis-Kaplan Executive Function System
DSST	Digit Symbol Substitution Test
ELISA	Enzyme-linked immunosorbent assay
^{18}F-FBB	^{18}F-Florbetaben
FDG	Fluoro-2-deoxy-D-glucose
FFQ	Food frequency questionnaire
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
KARVIAH	Kerr Anglican Retirement Village Initiative in Ageing Health
MCI	Mild cognitive impairment
MMSE	Mini Mental State Examination
MoCA	Montreal Cognitive Assessment
MRI	Magnetic resonance imaging
NAL	Neocortical amyloid load
NFT	Neurofibrillary tangles
NMDA	N-Methyl-D-aspartate
PET	Positron emission tomography
PiB	Pittsburgh compound-B
PSEN	Presenilin
RAVLT	Rey Auditory Verbal Learning Test
RCFT	Rey Complex Figure Test
ROS	Reactive oxygen species
SIMOA	Single-molecule array
SR-B1	Scavenger receptor class B type 1
SUVR	Standardized uptake value ratio
TNF-α	Tumour necrosis factor- alpha
WTAR	Wechsler Test of Adult Reading

Chapter One

1. Introduction

Over 400 000 people are currently living with dementia in Australia, of which Alzheimer's disease (AD) is the most common cause [1]. With the ageing population, the frequency of AD cases worldwide is increasing and expected to surpass 150 million by 2050 [2]. Thus, the associated care and financial burden will continue to greatly impact economies and societies worldwide [1]. AD involves complex, progressive neurodegeneration which currently cannot be effectively treated. AD-related pathology begins to develop up to 20 years prior to the onset of clinical symptoms and the condition has a typical clinical duration of 8-10 years [3]. To date, effective and safe treatment methods have not been developed [4, 5]. This may be because by the time symptoms have appeared, extensive synaptic loss and neuron death has already occurred. Treatment research is now focusing on treating the condition in the long preclinical phase, to slow or prevent further pathological damage and thus delay or prevent the emergence of dementia symptoms. To this end, accurate preclinical diagnostic markers are needed. Therefore, AD research has extensively investigated early, clinically-relevant biomarkers, that are detectable before the occurrence of cognitive decline and irreversible neurological damage. These investigations have included biomarkers in the brain, blood, cerebrospinal fluid (CSF), and the retina.

Of the etiological explanations for AD, oxidative stress and inflammation are increasingly gaining support [6, 7]. The brain has high metabolic activity, yet low levels of endogenous antioxidants, and so it is vulnerable to oxidative injury [8]. This antioxidant deficit leads to gradual damage which, over time, could explain the progressive neurodegeneration in AD. Nutrition has been shown to greatly influence AD [9, 10], yet the full impact of dietary antioxidants in cognition and disease progression remains unclear [11-15]. Fruits and vegetables are rich in nutritional factors known as carotenoids and vitamins. These factors may influence oxidative stress-induced cognitive decline, as they have been proposed to have antioxidative, anti-inflammatory and anti-amyloidogenic potential [16-19]. Numerous studies have explored antioxidants with regard to general health, as well as cognitive performance and AD, however, the discrepancies in controlling for confounders, lack of pathological AD

outcomes such as beta-amyloid (Aβ) deposits, and the absence of studies in the preclinical AD phase have led to limited and inconsistent results.

This thesis seeks to address some of these limitations. The literature review explores the current understanding of AD as well as the currently-known links between common dietary antioxidants (five major carotenoids and α-tocopherol) and cognitive performance. The study then explores the association between levels of these antioxidants and a preclinical measure of increased AD risk, relative to potential confounds.

1.1 Pathology

The hallmark features of AD are senile plaques comprising mostly aggregated Aβ [20], neurofibrillary tangles (NFTs) composed of hyperphosphorylated aggregated tau protein [21], as well as cerebral amyloid angiopathy [22] and widespread neuronal atrophy [23]. AD characteristics were first recognised by Dr. Alois Alzheimer in 1906 when he noted psychological worsening in one of his patients and *post-mortem*, revealed cerebral atrophy and unusual deposits around nerve cells [24].

1.1.1 Beta-Amyloid

Dr. Alzheimer's finding of abnormal extracellular cerebral deposits have since been identified as the accumulation of the Aβ protein. These Aβ deposits (Figure 1, [25]) are arguably the key component of AD pathology, thought to begin accumulating decades prior to the onset of clinical symptoms [26]. The Aβ peptide is a normal physiological product from the enzymatic breakdown of the amyloid precursor protein (APP). The APP amino (N)-terminal lies within the extracellular space and the carboxyl (C)-terminal within the cytosol. APP cleavage by enzymes known as secretases is achieved by two pathways: non-amyloidogenic and

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Figure 1. Examples of amyloid-β plaques, antibody stained in brain sections. Image obtained from Koronyo et al. [25].

amyloidogenic (Figure 2). In the former, α-secretase cleaves APP within the Aβ sequence, generating a truncated APP C-terminal fragment, lacking the Aβ N-terminal, thereby precluding

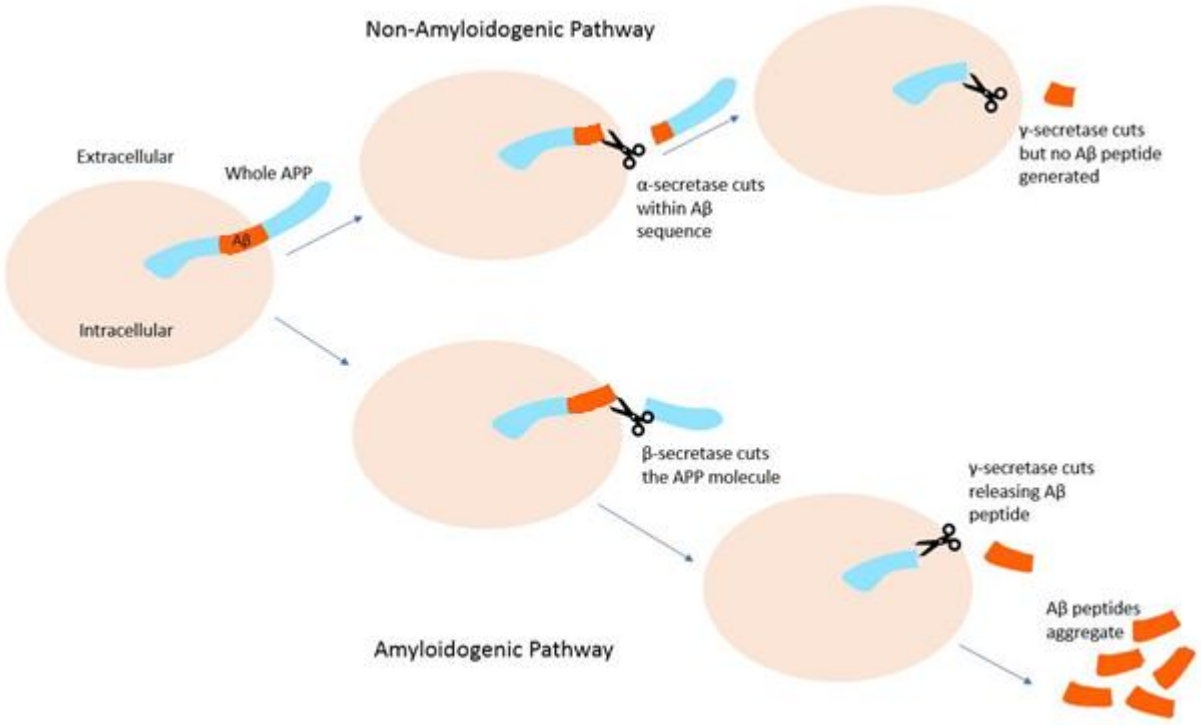


Figure 2. Amyloidogenic (producing Aβ) and non-amyloidogenic (preventing Aβ-production) pathways of APP processing. Abbreviations: APP, amyloid precursor protein; Aβ, amyloid-beta.

the generation of Aβ. In the latter, β-secretase initially cleaves APP, generating an APP C-terminal fragment, which is further cleaved by γ-secretase to release the Aβ peptide [27]. Cleavage site variability causes the generated Aβ peptides to vary in length. The longer Aβ peptides, particularly $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (often abbreviated to $A\beta_{40}$ and $A\beta_{42}$, respectively) are most associated with AD pathology [28]. Though levels of $A\beta_{40}$ are usually much higher than levels of the $A\beta_{42}$ peptides, the $A\beta_{42}$ peptides are more likely to aggregate, initially as small soluble oligomers, then as larger insoluble fibrils, finally to be deposited in the brain as AD-typical plaques. This toxic accumulation of Aβ can occur due to overproduction and/or reduced clearance of the peptide [28]. The role of Aβ throughout AD pathogenesis is still not fully understood. When observed longitudinally, Aβ deposition rates have been shown to plateau at higher Aβ burden and with declining cognition, approximately 25-30 years after Aβ accumulation begins [26].

It should be noted that although Aβ is a recognised hallmark of AD, the exact contribution of Aβ to AD development is controversial. The most extensive AD research has surrounded the amyloid cascade hypothesis, in which Aβ accumulation triggers a cascade of events including neuronal loss, inflammation and dementia [29]. Several less-recognised theories have also been postulated to explain the disease course, including the tau, cholinergic, inflammatory and oxidative stress hypotheses [29]. However, particularly in the case of inflammation and

oxidative stress, these hypotheses overlap, as there is much evidence suggesting that high Aβ or Aβ oligomer levels cause inflammation and oxidative stress, and conversely, chronic inflammation and oxidative stress have been shown to cause an increase in Aβ levels [30-33]. These links are discussed in greater detail later.

1.1.2 Tau

Tau proteins, key protein filaments in the neuronal cytoskeleton, assist in stabilizing microtubule formation, thus enabling neuronal transport. Ordinarily this tau protein is phosphorylated, however, under disease conditions it can become hyperphosphorylated, preventing it from functioning normally, resulting in its aggregation into filaments. These filaments bundle together in a helical manner and hence are referred to as tangles (Figure 3) [34]. In AD, tau proteins in the brain are three to four times more phosphorylated [35], leading to extensive NFTs.

Figure 3. Brain section from Alzheimer's disease patients. Red arrow heads have been used to identify intraneuronal tau protein tangles labelled with antibody. Adapted from Bennett et al. [34].

1.1.3 Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy is an important contributor to AD pathogenesis, and is recognised by Aβ-deposition in and around the cerebral blood vessels (Figure 4) [36]. There are various methods by which cerebral amyloid angiopathy has been proposed to cause neurodegeneration,

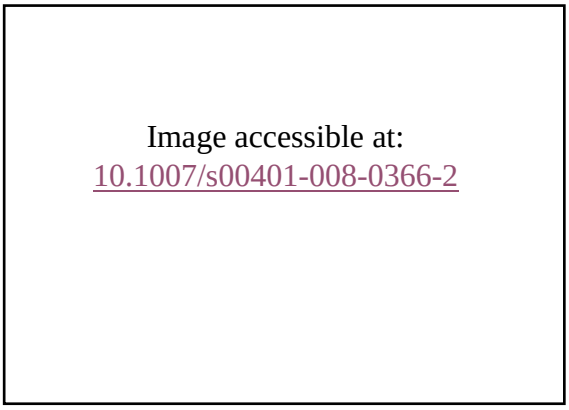


Figure 4. Cerebrovascular amyloid-β deposits in the vessel wall in moderate cerebral amyloid angiopathy. Amyloid-β is indicated by brown staining. Image obtained from Thal et al. [36].

including disruption of vascular functions and limiting blood supply, therefore resulting in oxygen deprivation and cell toxicity [22]. The distribution of Aβ peptide type in cerebral amyloid angiopathy varies such that parenchymal deposits and those in the capillary network tend to be of $A\beta_{42}$, while vascular deposits in leptomeningeal vessels are primarily $A\beta_{40}$, and a mixture of both Aβ forms is found in arterioles and small cortical arteries [22].

1.2 Types of AD

There are two main forms of AD: early onset AD, which occurs in individuals younger than 65 years, and late onset AD, the more common form of AD which occurs after age 65. The term 'early onset' AD encompasses both familial autosomal dominant and sporadic cases. Familial autosomal dominant cases account for approximately 1% of total AD cases [37] and can occur in people as young as 30 years of age. Currently, the only identified deterministic factors of AD, are relevant to the autosomal dominant type and involve mutations in one of three genes: amyloid precursor protein *(APP)*, presenilin 1 *(PSEN1)* and presenilin 2 *(PSEN2). APP* mutations affect the secretase cleavage sites, and interfere with the normal production of Aβ from APP. The presenilins are components of the γ-secretase cleavage enzyme, and thus interfere with γ-secretase function and activity [38]. Overall, most of these mutations increase the amount of $A\beta_{42}$ produced. Mutations in *APP* and *PSEN1* genes are fully penetrant and predictive of the age of symptom onset, based on the parent's age of symptom onset and mutation type [37]. The biological offspring of affected parents who inherit the mutation, develop AD at a predictable, often early age. Unlike *PSEN1* and *APP* mutations, *PSEN2* mutations are far less predictive of the age of symptom onset (from 40 to 80 years old), have variable penetrance, are rare, and are involved in other dementia-associated disorders [39].

Additionally, while most AD cases are sporadic and typically present later in life, early onset sporadic cases have also been reported. There is no deterministic factor for sporadic AD, however it is associated with various genetic and environmental risk factors.

1.3 Signs and symptoms

AD is characterized by a gradual decline in cognitive and neuropsychiatric functions to an extent that interferes with normal daily functioning. AD typically presents with memory disturbance, including learning and recall, yet can also be initially non-amnestic, with affected domains including executive function, personality and behaviour [40]. A rare version of AD presents with prominent visual disturbances, which can precede memory loss and cognitive dysfunction [41].

1.4 Risk factors

Though age is the strongest risk factor for AD, with incidence doubling every five years over 65 years [42], genetic and modifiable risk factors are thought to play a major role.

1.4.1 Genetic risk factors

The risk of developing sporadic AD is significantly increased with a family history of the disease, particularly maternal history [43]. AD is also genetically associated with the apolipoprotein E (*APOE*) gene. *APOE* is involved in the transport and clearance of brain-Aβ into cells and the bloodstream [38]. The *APOE* gene has three alleles: ε2, ε3 and ε4. Approximately 25% of the population carries either one or two ε4 alleles, yet 50% of AD patients are ε4 allele carriers; indicating carriage of ε4 alleles is associated with an increased risk of AD development [38, 44]. However, this denotes susceptibility and is not deterministic. In contrast, carrying the ε2 allele seems to have a protective effect, associated with lower AD risk [44] relative to the most common ε3 allele [38]. Previously, it was believed that female carriers of the ε4 allele were at a greater risk of developing AD than their male counterparts, however, a recent meta-analysis of 58 000 participants, between the ages of 55-85 years, revealed no overall sex-difference in developing mild cognitive impairment (MCI) and AD [44]. The study did show an age-related risk whereby the odds of developing AD were higher in women than men between 65 and 75 years, and 55-70 years for developing MCI.

Research has also established a genetic association between Down syndrome and AD. Down syndrome is a genetic disorder, characterised by developmental and cognitive impairment. Individuals with Down syndrome are at a high risk of AD because of a direct genetic link. Down syndrome is caused by trisomy 21, given that the APP gene is found on chromosome 21, the result is a triplicate of the APP gene in Down syndrome individuals [45].

1.4.2 Cardiovascular risk factors

Various cardiovascular diseases and associated risk factors, such as obesity, diabetes, hypertension, high cholesterol and smoking, have been linked to AD. For example, the direct impact of stroke causes neuronal loss, inflammation and increased Aβ deposition [46]. Diabetes mellitus is an established risk factor for vascular dementia and AD. Diabetic older adults have been associated with a 65% higher risk of developing AD over a 5.5 years longitudinal follow-up, than non-diabetic older adults [47]. The metabolic dysregulation associated with diabetes may explain the pathogenic impact in AD, such that soluble Aβ oligomers cause the loss of neuronal insulin receptors and inhibit the neuronal response to insulin [48]. Type-2 diabetes is

linked to altered cerebral insulin processing, glucose metabolism, mitochondrial function and lipid metabolism, all of which are thought to occur in the early stages of AD [49].

1.4.3 Modifiable factors

Physical activity influences the risk of AD development, such that higher physical activity levels have been associated with lower insulin and plasma $A\beta_{42/40}$ ratio [50], and light and vigorous physical activity during mid-life, and light and moderate physical activity in late-life reduced the risk of MCI [51]. Low physical activity was associated with higher brain-Aβ load in *APOE* ε4 carriers but not in non-carriers of the allele [50]. Additionally, sedentary behaviour is a risk for reduced brain health. Indeed, in cognitively normal individuals, greater sedentary behaviour, but not physical activity levels, was associated with low medial temporal lobe thickness, a region commonly atrophied in AD [52].

Diet can be associated with increased AD risk, as seen with the Western diet and heavy alcohol consumption, or it can have a protective effect, as seen with the Mediterranean diet [53, 54]. The Mediterranean diet has also been associated with various brain changes. A 3-year observational study in cognitively normal individuals revealed that compliance to the Mediterranean diet was associated with lower rates of cerebral glucose hypometabolism and brain Aβ deposition, yet no structural MRI differences were observed [55]. While a separate study showed that cognitively normal individuals with low adherence to a Mediterranean diet showed greater localised cortical thinning, in areas common to AD, when compared with higher adherence participants [56]. Diets high in fruit and vegetables have been positively associated with cognitive function [9], with particular benefit from increased intake of green leafy and cruciferous vegetables [10]. This relationship will be explored in more detail throughout this thesis.

Disruptions to the circadian system are associated with aging and chronic diseases, and have been demonstrated to cause neuroinflammation, oxidative stress and neuronal damage [57]. Widely reported in symptomatic AD, there is also evidence that sleep-wake cycle dysfunction arises prior to the onset of cognitive decline. For example, in preclinical AD participants, brain Aβ load and increased phosphorylated tau/$A\beta_{42}$ ratios were associated with greater circadian fragmentation [57].

Depression is regarded as a prodromal risk for AD as well as a comorbid condition [58]. In a 44-year longitudinal study in women, the timing of depression was found to influence the incidence of AD, such that early-life, early midlife and late-life depression increased the risk of AD [59].

Higher education levels have a protective effect on the risk of developing AD [60]. Similarly, cognitive engagement has been shown to improve cognitive scores and to associate with greater grey matter volumes in typical AD-affected areas [61]. Higher cognitive activity in early and midlife has been associated with low brain Aβ deposition independent of educational attainment [62].

Social engagement has a protective effect against cognitive decline. In a 12-year longitudinal follow-up study in older adults, frequent social activity was associated with a 70% reduction in the rate of global cognitive decline, compared to infrequent social activity [63].

The concepts of brain reserve and cognitive reserve should be noted regarding susceptibility for developing AD. Brain reserve reflects the quantity of neuronal resources, while cognitive reserve involves functioning efficiency through existing or alternate neural networks, often associated with education level [64]. Greater reserve indicates greater propensity to cope with neuropathological changes, such that equal amounts of AD-pathology may manifest differently between individuals, thus may not correlate with symptom severity.

1.5 Diagnosis

Currently, a definitive diagnosis of AD involves *post-mortem* examination of the Aβ plaques and NFTs in the brain. *Pre-mortem* diagnosis is limited to 'probable' or 'possible' AD, determined by clinically assessing cognitive and behavioural functioning using neuropsychological tests often coupled with pathophysiological examination, including neuroimaging and CSF examination [40]. Due to the similarity of AD symptoms with those seen in other neurological conditions, a clinical diagnostic error rate for AD has been reported at approximately 10-15% [65]. This diagnosis has limitations in that it is reliant on clinical symptoms which arise after irreversible neurological damage has occurred.

More recent research has developed new diagnostic tools. Although expensive, sometimes relatively invasive and not applicable to the whole population, these are the current gold standards for AD diagnosis. These include CSF examination for $A\beta_{42}$, total tau and phosphorylated tau concentrations and positron emission tomography (PET) using Aβ-specific tracers to assess brain Aβ deposition levels [66, 67]. These are used in clinical research for diagnosis including preclinical diagnosis, along with magnetic resonance imaging (MRI) to evaluate structural changes [23]. Recently, new developments in plasma Aβ measurement and the measurement of many other inflammation and lipid metabolism-related markers in AD suggest that a blood biomarker panel may have sensitivity as a future diagnostic tool [68].

1.5.1 Cerebrospinal fluid

Elevated CSF tau and low CSF $A\beta_{42}$ levels are strongly associated with AD and have been shown to distinguish AD patients from controls with a sensitivity and specificity of 92% and 89%, respectively [67]. CSF is relatively accessible, via lumbar puncture, however, this is invasive, uncomfortable and can be expensive. Consequently, a more viable biomarker is needed.

1.5.2 Positron emission tomography

PET imaging is a form of nuclear imaging technology. Radioligands, which are radioactive compounds having strong affinity to the biomolecule of interest, are administered intravenously and then detected and quantified by PET technology. The most commonly used radioligand to assess brain Aβ deposition is carbon-11 Pittsburgh compound-B (^{11}C-PiB), due to its high binding capability with Aβ, enabling quantification of AD pathology in the brain [69]. PET has been used to identify individuals likely to develop AD as characterised by high PiB retention, indicating high brain Aβ levels, in cognitively normal elderly individuals [66]. Similarly, ^{18}F-florbetaben (^{18}F-FBB) has been used to distinguish AD patients from cognitively normal individuals [70]. Compared with ^{11}C-PiB, ^{18}F-FBB is more practical as it has a longer half-life (110min compared with 20min for ^{11}C-PiB [70]), thus it was chosen for use in the current study.

^{18}F-Fluoro-2-deoxy-D-glucose (^{18}F-FDG)-PET is also often used in AD. The ^{18}F-FDG radiotracer indicates cerebral glucose metabolic activity, which represents neuronal and synaptic function. Hypometabolism is common in neurodegeneration, with certain patterns of cerebral hypometabolism specific to AD. AD-like changes in cerebral hypometabolism have been associated with cognitively normal individuals at high genetic or familial risk for AD, MCI patients and global cognitive decline [71], suggesting a prognostic use of FDG-PET in AD diagnosis.

However, as with CSF investigations, PET is not without its limitations. It is expensive, involves radiation which limits regular use, and though has been highly successful and productive in research, the method is not clinically accessible to meet the high demand of individuals with, or developing, AD.

1.5.3 Magnetic Resonance Imaging

MRI scans are used clinically to visualise the location and extent of cortical atrophy to aid in the diagnosis of conditions including AD. Hippocampal atrophy and global atrophy are typically regarded as markers of AD [72]. Combining measures of anatomical MRI markers, including cortical thickness, grey matter density, subcortical volumes, and hippocampal shape may improve the accuracy of the clinical diagnosis of AD [23]. Decreases in hippocampal and

amygdala volumes have been shown to predict development of dementia and AD during a 6-year follow-up, independent of subjective memory complaints or poor cognitive performance at baseline [73]. However, although useful in conjunction with clinical assessment, MRI markers can be confounded by age, and other neurodegenerative diseases and so cannot provide a definitive diagnosis of AD, as yet.

1.5.4 Blood biomarkers

Recently, a new technique was developed for measuring peripheral Aβ in plasma, which is more sensitive and has the potential to be more successful than traditional Enzyme-linked Immunosorbent Assay (ELISA) techniques [74]. This new method involves the use of immunoprecipitation and mass spectrometry, and a large two-site study using this method found that blood Aβ levels predicted brain Aβ with an accuracy of 90% [68]. While this is an exciting prospect, as it is only minimally-invasive, inexpensive and scalable, further investigation is needed to confirm these results, before this could become a commercially available blood test.

1.6 Treatments

AD treatments that have been trialled have limited efficacy, possibly due to the already extensive neurological damage at the time of intervention and high diagnostic uncertainty. The available drugs provide temporary symptomatic relief to only some people diagnosed with AD, improving the quality of life and functioning of this subset of people with AD. However, clinical benefits are seen for a relatively short period and they do not stop disease progression. There are two main types of established AD treatments: cholinesterase inhibitors and N-Methyl-D-aspartate (NMDA) receptor antagonists. The cholinergic hypothesis of AD posits that disruption to the acetylcholine processes in the basal forebrain results in cognitive decline. Cholinesterase inhibitors block the normal breakdown of acetylcholine in the synaptic cleft, enhancing neuronal activity [75]. Current licensed cholinesterase inhibitors for AD treatment are: Donepezil (for mild-moderate and moderate-severe AD) [76], Rivastigmine (for mild-moderate and severe AD) [77] and Galantamine (for mild-moderate AD) [78]. Side effects of cholinesterase inhibitors range from mild discomfort to severe enough to require hospitalisation, and tend to be gastrointestinal, yet can also include weight loss, cardiovascular changes and neuropsychiatric symptoms [79]. NMDA receptor antagonists act by binding to glutamate receptors, regulating excessive glutamate activity which could otherwise cause excitotoxicity. Memantine [76] is effective in moderate-severe AD and has been associated with mild adverse effects, most commonly dizziness, headache, somnolence and hypertension [80]. Additionally, antipsychotics and antidepressants are often used to treat mood and behavioural disturbances common in AD [81].

A summary of the current treatments, as well as the aforementioned pathology, signs and symptoms, genetics, risk factors, protective factors, and diagnostic techniques, is represented in Figure 5.

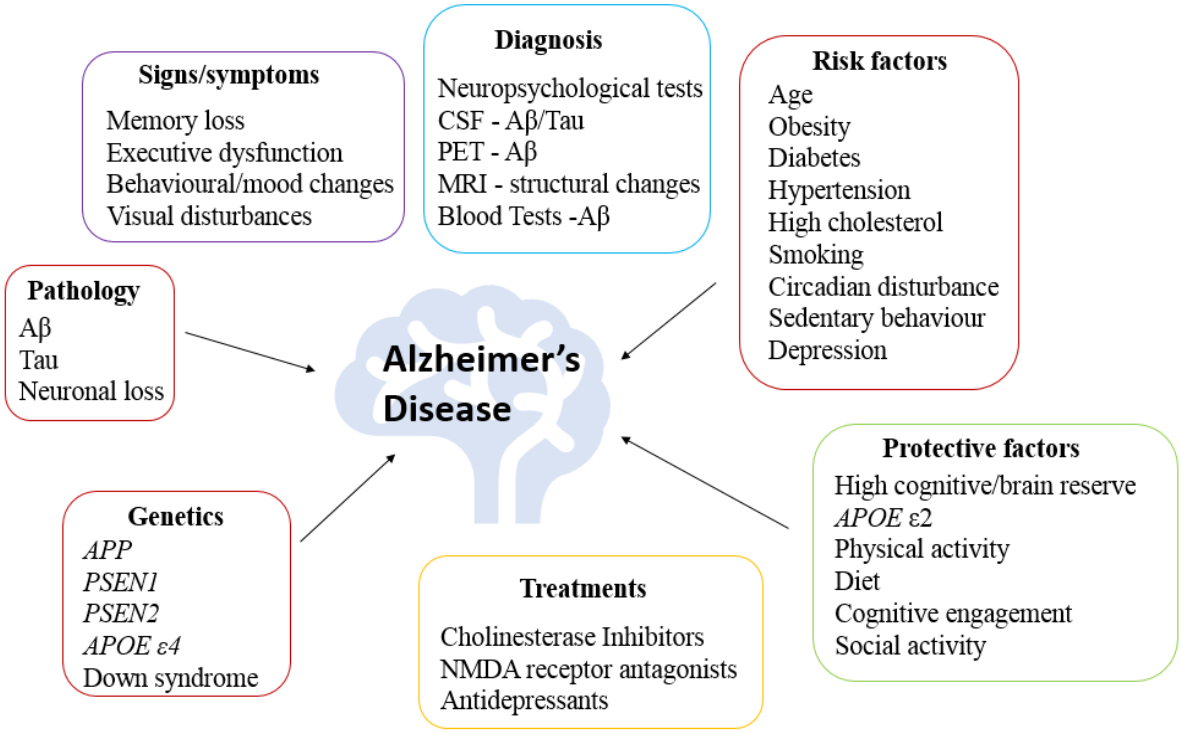


Figure 5. Summary of contributors, diagnostic techniques, risk factors, protective factors and treatments of Alzheimer's Disease. Abbreviations: CSF, Cerebrospinal Fluid; PET, Positron Emission Tomography; MRI, magnetic resonance imaging; NMDA, N-Methyl-D-aspartate; *APOE*, Apolipoprotein; *APP*, Amyloid Precursor Protein; Aβ, amyloid-beta; *PSEN*, Presenilin.

1.7 Oxidative stress and inflammation

As mentioned earlier, oxidative stress and chronic inflammation are believed to be early and/or accelerating factors in the pathogenesis of AD [6, 7]. Oxidative stress involves an imbalance between the production of reactive oxygen species (ROS), free radicals, and antioxidant processes, favouring the oxidants, causing tissue damage [82]. ROS are mainly released from the inner membrane of mitochondria. They are necessary regulators of biological processes including metabolism and cell signalling, and they have a role in the body's immune response [30]. Given that in moderation, ROS are physiologically vital but when in excess they have harmful consequence, it is imperative that the body maintains the balance of this system. There are two categories of antioxidants which are tasked with regulating this system and preventing oxidative stress, enzymatic endogenous antioxidants (naturally produced within the body) and non-enzymatic exogenous (consumed via diet) antioxidants [83].

Oxidative stress is recognised in the pathology of AD, yet whether it arises prior to, or as a consequence of, pathological changes in the brain remains in question. There is evidence that oxidative stress is present during preclinical AD [84] and MCI [31], that is, prior to the development of clinical AD. Yet Aβ may also promote excess ROS generation, inducing oxidative stress, causing dysfunction and degeneration of synapses [30, 32].

While oxidative stress has been directly related to AD, it has also been associated with conditions that are known to increase AD risk, such as cardiovascular disease, diabetes, obesity and insulin resistance [6]. Ageing has been associated with increased ROS and oxidative damage [83], thus, in order to compensate for this potential source of increased AD risk, it may be beneficial to increase the intake of exogenous antioxidants in the diet, such as foods containing antioxidant vitamins and carotenoids.

Similarly, inflammation has direct and indirect associations with AD. Inflammation is the body's normal response to pathogens. However, chronic inflammation is also common in obesity, type 2 diabetes and cardiovascular disease; and as with oxidative stress, chronic inflammation can result in damage to neurons [85]. Neuroinflammation and microglial responses can be detected as a result of the presence of Aβ oligomers and NFTs [7, 33]. Initially arising as a neuroprotective response, degrading and removing neurotoxic Aβ and NFTs, the binding of microglia to Aβ oligomers and fibrils and APP can result in overproduction of cytokines and proinflammatory mediators such as interleukins, tumour necrosis factor-α, chemokines, prostaglandins and C-reactive protein [33]. When prolonged and uncontrolled, this production leads to (further) chronic inflammation, stimulating disease progression.

1.8 Carotenoids

As previously mentioned, AD pathogenesis is greatly influenced by the amount of oxidative stress and inflammation. A nutritional impact on these processes has been proposed, yet the specific influence of dietary antioxidants remains in debate. Carotenoids and vitamins have been researched due to their antioxidant and anti-inflammatory properties [8, 16], which may be mechanistic in reducing oxidative stress and inflammation. Carotenoids are naturally occurring phytochemicals, present in many fruits and vegetables, as well as in some dairy products and eggs (Table 1), which cause these foods to have their yellow, red, and orange colours [86].

Carotenoids can be characterised by polarity, that is: xanthophylls (polar molecules) including lutein, zeaxanthin and β-cryptoxanthin or carotenes (non-polar) including α-carotene, β-carotene, and lycopene. The chemical structure of these biomolecules is shown in Figure 6.

Carotenoids with at least one β-type ring have the potential to be retinol, or vitamin A, precursors [87]. Common carotenoids with at least one β-type ring include α-carotene, β-carotene and β-cryptoxanthin. Unlike these other carotenoids, β-carotene has two β-rings enabling its conversion to two molecules of vitamin A [87].

Table 1. Content of major carotenoids in selected commonly consumed foods

Carotenoid	Food Source	Reference
Lutein	Fruits/vegetables: Corn, avocado, broccoli, banana, kiwi fruit, spinach, zucchini, pumpkin, squash, spinach, cucumber, pea, capsicum, butternut, honeydew, celery, tomato juice, brussels sprouts, scallions, green beans, apple, green lettuce **Animal products**: Egg yolk, egg, butter	[88-91]
Zeaxanthin	Capsicum, egg yolk, corn, grapes, spinach, orange, honeydew, mango	[89, 91]
β-cryptoxanthin	Apricots, avocado, basil, dill, guava, mango, corn, mandarin, nectarine, orange, papaya, peach, capsicum, pineapple, plum, pumpkin, sage, watermelon	[88, 90, 91]
α-carotene	Carrots, banana, capsicum, apricot, avocado, yellow squash, celery, green lettuce, peach	[89, 90]
β-carotene	Fruits/vegetables: Carrots, apricot, broccoli, red grapes, pumpkin, spinach, capsicum, mango, sweet potato, orange, honeydew, brussels sprouts, orange, apple, tomato, peach, nectarine, cantaloupe **Animal products**: Butter, cheese, milk	[89, 91]
Lycopene	Tomato, tomato juice, apricots, orange, guava, grapefruit, capsicum, watermelon, papaya	[88, 89, 91]

Table 1 shows the main dietary sources of common carotenoids: lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. These carotenoids are mostly found in fruits and vegetables however, lutein and β-carotene can also be found in animal products such as dairy and eggs.

Carotenoids can be detected throughout the body, including the central nervous system, i.e. the brain and the retina, and these levels are strongly correlated with serum/plasma levels as well as dietary intake [11, 92, 93]. Carotenoid distribution varies in the different areas of the body. For example, xanthophylls account for 66–77% of the total carotenoids in the human brain [94]; lutein and zeaxanthin are also highly concentrated in the macula and retina where they protect against damage by blue light, improve visual acuity and may reduce the risk of age-related macular degeneration and cataracts [95].

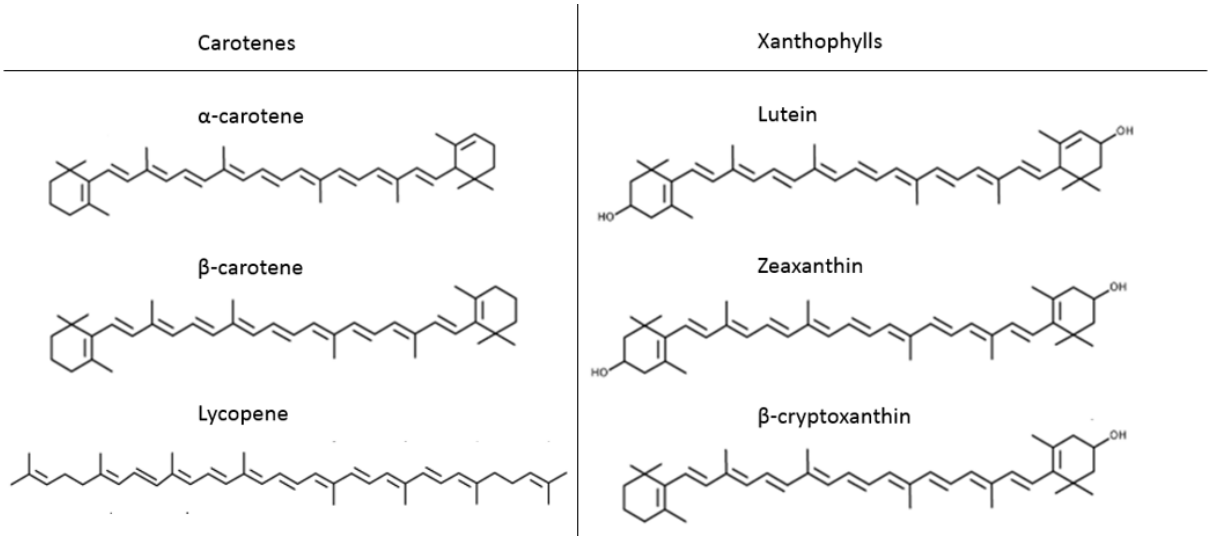


Figure 6. Carotenoid structures (six most commonly analysed). Carotenes and xanthophylls share a basic hydrocarbon structure, xanthophylls have the addition of an oxygenated functional group.

1.9 Vitamin E

One of the more researched antioxidant vitamins is vitamin E. Vitamin E refers to a group of isoforms comprising four structurally different tocotrienols and four tocopherols. The α-tocopherol form is the most abundant and is the only compound of vitamin E to be maintained by the body [96]. Vitamin E is most commonly found in vegetable oils (Table 2), as well as in nuts and leafy greens.

Table 2. Structure and source of α-tocopherol

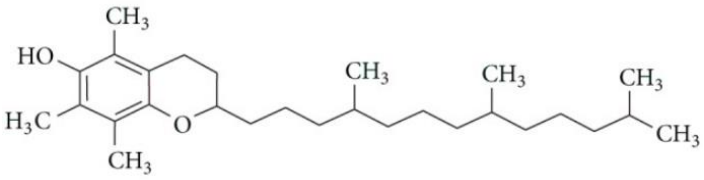
Compound	Structure	Food source	Reference
α-tocopherol		Almonds, spinach, **Oils**: canola, sunflower, soybean, corn, wheat germ, rice bran, palm, olive	[96-98]

Table 2 shows the structure and dietary sources of vitamin E (α-tocopherol).

1.10 Bioactivities

Carotenoids and vitamin E have been shown to have protective effects in general health and neurodegeneration, although, the exact mechanism(s) by which these effects occur is not fully understood. The specific structures of carotenoids and vitamins, specifically their binding capabilities, give these molecules antioxidant, anti-inflammatory and anti-amyloidogenic potential (Figure 7), further explained below.

1.10.1 Antioxidant potential

Increasing the dietary intake of carotenoids and vitamin E has been proposed as a mechanism to reduce the effect of oxidative stress. This is because these compounds are known to have vast antioxidant potential [17], quenching singlet oxygen ($^1O_2$), and neutralising ROS and free radicals [99]. Not all carotenoids have the same bioactivities, for example, in one study only α-carotene and β-carotene (of the major carotenoids) were found to correlate with antioxidant capacity [16]. In another study which investigated the ability of carotenoids to scavenge a radical cation, the major carotenoids demonstrated relative antioxidant activity in the order of lycopene > β-cryptoxanthin/β-carotene > lutein/zeaxanthin > α-carotene [100]. Notably, vitamin E has been shown to prevent the increased protein oxidation, ROS formation and neurotoxicity induced by Aβ, proposed to be reflective of vitamin E scavenging free radicals [101].

1.10.2 Anti-inflammatory potential

Though most known for their antioxidant capacities, carotenoids and vitamin E also possess anti-inflammatory properties [16, 18]. α-tocopherol induces its anti-inflammatory effect mainly through inhibition of cell signalling, seemingly distinct from its antioxidant behaviours [18]. The anti-inflammatory impact of carotenoids is commonly reported particularly with respect to cardiovascular health [102]. The anti-inflammatory activity has been demonstrated by the inverse association of carotenoid (α-carotene, β-carotene, zeaxanthin/lutein, and β-cryptoxanthin) concentrations with markers of inflammation (leukocyte count, C-reactive protein and fibrinogen) and oxidative stress [103]. Yet a separate study found that while β-cryptoxanthin and lycopene levels were inversely associated with inflammatory markers, α-carotene was positively associated with TNF-α [16]. As β-carotene has been shown to increase the secretion of TNF-α from monocytes and macrophages, it has been proposed that α-carotene, due to its structural similarities with β-carotene, may similarly stimulate TNF-α secretion [16]. Furthermore, TNF-α at levels below 20pg/mL, as was reported, have been proposed to have neuromodulatory and neuroprotective functions [104, 105], thus the positive association of TNF-α and α-carotene may reflect this normal activity. The study also showed no significant associations between lutein and zeaxanthin and markers of oxidative stress [16]. Similarly, no interaction between lutein and markers of oxidative stress has been shown in a supplementation study of daily lutein (15mg) for three months [106]. This study also showed no significant interactions with oxidative stress, and lycopene or β-carotene supplementation. However, it should be noted that participants were limited to healthy males, which may not reflect the broader population. Since there have been varying reports on the association of carotenoids and

inflammatory markers, future studies are necessary to validate the results, and to determine the association with regard to AD and preclinical AD.

1.10.3 Anti-amyloidogenic potential

Another mechanism by which carotenoids may have therapeutic benefit in preventing AD is by their ability to inhibit cerebral Aβ aggregation [19]. This is achieved by varying mechanisms dependent on the carotenoid structure, for example, the hydroxyl groups in zeaxanthin have been shown to interact with amino acids in Aβ, likely leading to disintegration of β-fibrils [19]. β-carotene has also been shown to have anti-amyloidogenic properties by inhibiting the

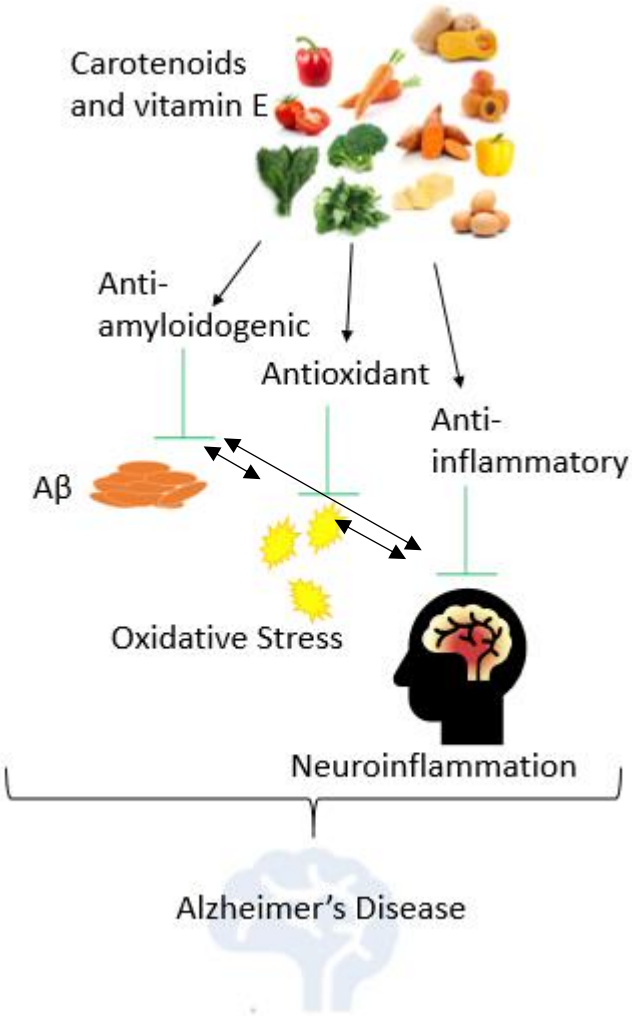


Figure 7. Carotenoid action against amyloid-beta (Aβ), oxidative stress and neuroinflammation, protecting against neurodegeneration in Alzheimer's disease.

formation of fibrillar Aβ and destabilizing fibrillar Aβ [107]. This anti-amyloidogenic property can be attributed to the hydrophobic nature of β-carotene along with its rod-like structure, that enables binding to both non-fibrillar Aβ and fibrillar-Aβ which in turn initiates anti-amyloidogenic activity. However, these are at present molecular modelling or *in vitro* studies, it is not known if these compounds influence fibrillisation *in vivo.*

1.11 Antioxidants and cognition

There is an understanding of the levels of antioxidants and anti-inflammatory compounds needed in the body to have a protective effect for general physiological health [108]. However, the evaluation of which carotenoids and vitamins may have a similar protective effect, with respect to cognition is still ongoing. Commonly, the bioactive effect is determined by the association of plasma or serum carotenoids and/or vitamins with cognitive outcomes or other clinical measures of AD progression. Some studies report on the effect of antioxidants using dietary intake as the primary outcome [12, 109], however, this method is more subjective, and may result in over or under-reporting the antioxidant's actual intake. The association of self-reported vitamin supplement use and the risk of dementia and AD, and cognitive decline, has been investigated, with one study reporting reduced risk of dementia and AD with vitamin E and/or C supplements [110]. Interestingly, while another study observed no effect on dementia risk, after five-years follow up combined vitamin E and C, or multivitamin consumption at baseline, was associated with a protective effect on cognitive performance [111]. However, since neither study reported on dietary intake of vitamins, or controlled for incorrect self-reporting of supplements, by plasma analysis, these results require further investigation. Serum/plasma analysis reflects the concentration of carotenoids and α-tocopherol actually absorbed from the diet and is therefore more reliable. Table 3 reviews a selection of previous human studies which exemplify the varying effects of carotenoid and vitamin E on cognition.

Notably, some studies reported associations between cognitive function and individual (but not all) plasma carotenoids [11-13]. Furthermore, lower carotenoid concentrations have consistently been shown in AD participants compared with cognitively intact individuals [8, 112-114] yet often with conflicting results concerning certain specific carotenoids [112, 113]. In addition, serum lutein and zeaxanthin have been reported to be inversely correlated with functional MRI blood-oxygen-level-dependent (BOLD) [115] signalling across multiple brain regions during encoding and recall tasks, suggesting an influence on neural efficiency [115].

Prospective and longitudinal studies have revealed heterogeneous results. Some have found no interaction between plasma levels of carotenoids or tocopherols with cognitive function [14], whereas other studies reported a reduced risk of dementia associated with individual plasma and/or dietary carotenoids and vitamin levels [109, 116, 117]. One study showed that in combination, low levels of carotenes, 25(OH)D and polyunsaturated fatty acids/saturated fatty acids ratio is strongly associated, while higher levels of α-tocopherol were mildly associated, with a higher risk of dementia after approximately 10 years follow-up [118]. Similarly, a recent study revealed that a combined intervention of xanthophylls and fish oil was more effective

than xanthophylls alone in improving AD outcomes such as mood and memory, and in lessening the progression of AD [119].

Plasma carotenoid levels have also been shown to correlate with AD severity, whereby lower levels of lutein and β-carotene were found in moderately severe AD patients compared to mild stage AD patients and controls [120]. Higher serum lycopene and lutein/zeaxanthin levels were associated with lower risk of AD mortality however, these associations were not found for α-carotene, β-carotene or β-cryptoxanthin [121]. Whether there is a significant difference in carotenoid levels in other types of dementia (such as vascular dementia and Parkinson's disease) also remains unclear, given that observations of no significant difference [112] and minimal differences within individual carotenoid levels [122] have both been reported. Interestingly, MCI participants had lower plasma concentrations of lutein, zeaxanthin and α-carotene than controls, yet higher vitamin A, zeaxanthin, β-cryptoxanthin and lycopene concentrations when compared with AD patients [114].

Table 3. Key findings from select studies regarding the effect of carotenoids and vitamin E on cognition and Alzheimer's disease

Study	Participants	Antioxidants investigated	Key Findings
Akbaraly et al. [13]	589 older adults (age=73.55, range= 68-79)	Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-, β-carotene	Probability of having low cognitive function was most associated with low levels of plasma lycopene and zeaxanthin
Amadieu et al. [118]	666 older adults (age=73.3)	Lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-, β-carotene, α-, γ-tocopherol, retinol, 25(OH)D and 12 fatty acids	Combined pattern of low levels of carotenes, 25(OH)D and polyunsaturated fatty acids/saturated fatty acids ratio is strongly associated with higher risk of dementia after approximately 10 years follow-up. Higher levels of α-tocopherol were mildly associated with higher risk of dementia at follow-up.
Devore et al. [116]	5 395 older adults (age=67.7, range=55+)	Vitamins E and C, β-carotene, and flavonoids	After up-to-9.6 years follow-up, higher dietary vitamin E was associated with lower risk of dementia and AD; no associations found between intake of vitamin C, β-carotene or flavonoids and dementia or AD risk.
Feart et al. [117]	1 092 older adults (age=74.4)	α-, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin	In this prospective study, after up-to-10 years follow-up, higher levels of lutein, but no other carotenoid, were associated with decreased risk of all-cause dementia and AD.
Foy et al. [122]	79 AD 37 VaD 18 PDem 58 controls (overall age median=73, range=62-85)	α-, β-carotene, lycopene, and vitamins A, C and E	Vitamins A, C and E were lower in AD than controls; β-carotene was lower in VaD than AD; lycopene was lower in PDem than AD and VaD

Jiménez-Jiménez et al. [113]	38 AD 42 controls (age=70.95)	α-, β-carotene and retinol	AD patients showed lower levels of serum β-carotene and vitamin A than controls, but no difference in α-carotene levels
Johnson et al. [11]	78 octogenarians (age=84.2, range=80-89), 220 centenarians (age=100.4, range=98+)	Lutein, zeaxanthin, cryptoxanthin, α-, β-carotene, lycopene, α-tocopherol and retinol	Various interactions found including: higher serum lutein, zeaxanthin and β-carotene concentrations were most associated with better cognitive performance; and α-tocopherol was inversely associated with dementia severity
Kang et al. [14]	858 older women (age=65)	Lutein/zeaxanthin, β-cryptoxanthin, α-, β-carotene, retinol, lycopene, α-, γ-tocopherol	After a 10-year follow-up, plasma carotenoids and tocopherols were not associated with rate of cognitive decline
Lindbergh et al. [115]	43 older adults (age=72, range=65-86)	Lutein and zeaxanthin	Serum lutein and zeaxanthin were inversely correlated with BOLD signalling on fMRI across multiple brain regions during encoding and recall tasks
Min et al. [121]	6 958 older adults (25% aged 50-59, 32% aged 60-69, 25% aged 70-79, 17% 80-89, 2% ≥90)	Lutein/zeaxanthin, β-cryptoxanthin, α-, β-carotene, and lycopene	Compared with those without AD mortality, participants with AD mortality (n=75) had lower serum lycopene and lutein/zeaxanthin levels, but no difference in α-carotene, β-carotene or β-cryptoxanthin
Mullan et al. [8]	251 AD 308 controls (age=78.1, range=65+)	Lutein, zeaxanthin, β-cryptoxanthin, α-, β-carotene, retinol, lycopene, α-, γ-tocopherol	AD participants had lower serum α- tocopherol, retinol, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene levels; and higher γ-tocopherol levels than controls.

Polidori et al. [112]	63 AD 23 VaD 55 controls (age=77)	Vitamins A, C and E, uric acid, lutein, zeaxanthin, lycopene, β-cryptoxanthin, α-, β-carotene	Compared with controls, AD and VaD patients showed lower levels of plasma vitamins C, E and A, uric acid, lutein, zeaxanthin, β-cryptoxanthin, lycopene and α-carotene
Rinaldi et al. [123]	25 MCI 63 AD 56 controls (age=76)	Vitamins A, C, and E, uric acid, lutein, zeaxanthin, lycopene, β-cryptoxanthin, α-, β-carotene	Compared with controls, MCI and AD patients showed similarly lower levels of plasma vitamins C, E and A, uric acid, lutein, zeaxanthin and α-carotene (AD also had reduced β-cryptoxanthin). Yet MCI patients had higher vitamin A, zeaxanthin, β-cryptoxanthin and lycopene, than AD patients
Schmidt et al. [15]	1769 middle-aged and older adults (age= 62, range=50-75)	Lutein/zeaxanthin, cryptoxanthin, canthaxanthin, lycopene, α-, β-carotene, retinol, α-, γ-tocopherol, and ascorbate	Low cognitive performance was associated with lower levels of plasma β-carotene and α-tocopherol. Yet, only α-tocopherol remained significant after controlling for confounding clinical factors
Wang et al. [120]	13 mild AD 13 moderate AD 10 severe AD 10 controls (age=74.25)	Lutein, zeaxanthin, β-cryptoxanthin, α-, β-carotene, and lycopene	Moderate AD patients had lower plasma lutein and β-carotene than mild AD patients. No between-groups difference in zeaxanthin, β-cryptoxanthin, α-carotene, and lycopene. Poorer cognitive function was associated with lower lutein and β-carotene.

Table 3 shows the main publications which investigated the association between carotenoids and vitamin E with cognition. These results demonstrate that cognition is inconclusively associated with dietary carotenoids and vitamin E, however the extent of this association and which antioxidant is most effective in this relationship remains unclear. Studies obtained via database searches on PubMed, Macquarie University Library and Google Scholar, using the key words 'antioxidant', 'carotenoids', 'vitamin E', 'cognition', 'Alzheimer's disease' and 'dementia'. Abbreviations: AD, Alzheimer's disease; VaD, Vascular dementia; PDem, Parkinson's disease and dementia; BOLD, Blood-oxygen-level-dependent; fMRI, Functional magnetic resonance imaging. Age given as mean, unless otherwise specified. Range given if provided in original article.

Though considerable research has been undertaken on the effect of carotenoids and vitamin E on cognition in healthy older adults, AD with some reports on the early stage of MCI, as per the author's knowledge, there are no studies directly analysing the impact of carotenoids and vitamin E in regard to cerebral Aβ load in preclinical AD. Since these molecules are known to have antioxidant, anti-inflammatory and anti-amyloidogenic potential, the current study investigates whether there is an association between plasma carotenoids and α-tocopherol levels, and neocortical amyloid-β load (NAL) prior to the onset of clinical dementia.

1.12 Aims

This thesis is focused on investigating the following aims:

1. The association of plasma carotenoids and α-tocopherol levels with neocortical amyloid-β load in cognitively normal individuals
2. The impact of clinical characteristics such as age, gender, BMI and *APOE* status, on the association between plasma carotenoids and α-tocopherol, and NAL
3. The association between plasma carotenoid and α-tocopherol levels and plasma amyloid-β levels in cognitively normal individuals
4. The associations of plasma carotenoid and α-tocopherol concentrations with dietary intake, cognitive performance and inflammatory markers in cognitively normal individuals

In order to address these aims, the following approaches were taken:

1. Cognitively normal individuals were categorised as high NAL (preclinical AD) or low NAL (at no apparent risk to AD) using PET imaging analysis. Carotenoids and α-tocopherol levels were determined using high performance liquid chromatography (HPLC). Analyses were carried out using general linear models and bivariate correlations.
2. General linear models were adjusted to control for clinical characteristics.
3. Plasma carotenoids and α-tocopherol levels were correlated with plasma amyloid-β, obtained using SIMOA.
4. Dietary information, obtained using the Cancer Council of Victoria's Food Frequency Questionnaire, was compared to plasma carotenoid and α-tocopherol concentrations using bivariate correlations.
5. Plasma carotenoids and α-tocopherol levels were correlated with inflammatory markers, wherein inflammatory marker concentrations were determined using ELISA analysis.
6. Plasma carotenoids and α-tocopherol levels were correlated with three measures of cognitive performance, obtained using a comprehensive neuropsychometric battery: 1. visual, verbal and episodic memory; 2. working memory and executive function, and 3. global cognition.

<u>Hypothesis:</u>

As seen in AD, the concentration of plasma carotenoids and α-tocopherol will be lower in the preclinical AD participants compared to those with no apparent risk of developing AD.

Chapter Two

2. Methods

2.1 Participants

Study participants were residents of Anglicare, New South Wales, Australia and belonged to the Kerr Anglican Retirement Village Initiative in Ageing Health (KARVIAH) cohort, recruited in 2015. All volunteers (n=206) were required to meet the set inclusion and exclusion criteria.

Inclusion criteria: aged between 65-90 years, good general health, English fluency, no known significant cerebral vascular disease, adequate vision and hearing to enable testing, no significant functional/behavioural impairment, no objective cognitive impairment determined by a Mini Mental State Examination (MMSE) score of ≥26 and a Montreal Cognitive Assessment (MoCA) score ≥26 [124].

Exclusion criteria: dementia diagnosis based on revised diagnostic guidelines [40], presence of acute functional psychiatric disorder including lifetime history of schizophrenia or bipolar disorder, history of stroke, severe or extremely severe depression determined by the Depression, Anxiety, Stress Scales (DASS) [125], uncontrolled hypertension (systolic blood pressure > 170mm Hg or diastolic blood pressure > 100mm Hg), and history (within last 2 years) of alcohol or drug abuse/dependence.

Inclusion and exclusion criteria were met by 134 volunteers, of which 105 agreed to undergo neuroimaging, neuropsychometric evaluation and blood collection, the remaining 29 declined these assessments or withdrew from the study. Of the 105 participants, 100 were considered to have no cognitive impairment. The study protocol required 500uL of plasma for HPLC analysis which was available for 99 participants. Therefore, the current study included these 99 participants.

The current study was approved by the Macquarie University Human Research Ethics Committee (reference number 5201701078, amendment number 5201826144153) and written informed consent was obtained from all participants.

2.2 *Blood collection and processing*

Participants fasted overnight (minimum 10h) prior to a blood draw of 80ml, using standard serological methods [126]. Immediately prior to blood draw, blood tubes for serum and plasma fractionation containing EDTA or lithium heparin were brought to ambient temperature, from 4°C. Following blood collection, tubes for plasma fractionation were placed on a rocker for approximately 20min, to prevent clotting, and enable homogenous mixing with the anticoagulant while tubes for serum fractionation were allowed to stand for 20min on the bench to allow clotting. Plasma fractionation tubes were centrifuged at 800g for 15min at 20°C, while serum fractionation tubes were centrifuged at 1800g for 15min at 20°C, following which plasma and serum were aliquoted and stored at -80°C within 3.5hrs of blood collection. 0.5ml of whole blood was kept separately for *APOE* genotyping. Plasma was used for the measurement of carotenoid and tocopherol, tumour necrosis factor (TNF)-α and interleukin (IL)-6, and serum was used for the measurement of C-reactive protein (CRP). Blood processing was completed in 2015 by another member of the Martin's research team.

2.3 Neuroimaging

Participants underwent neuroimaging at Macquarie Medical Imaging, Sydney within three months of blood collection (February to October 2015). Participants received a PET scan using the ligand ^{18}F-Florbetaben (FBB) administered slowly over 30sec, as an intravenous bolus, while participants were in a rested position. Images were acquired during a 20min scan, beginning 50min post-injection.

NAL was calculated as the mean standardized uptake value ratio (SUVR) based on the ratio of the ligand uptake in the region of interest comprising the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions to the ligand uptake in the reference region, the cerebellum, using image processing software, CapAIBL [127, 128]. The high NAL (n=35) group was categorised by an SUVR value ≥1.35 and low NAL (n=64) group was categorised by an SUVR value of <1.35. Participants with high NAL have a high likelihood of developing AD and can be thought of as in the preclinical-AD stage [129], though it is possible that some individuals in this stage may never develop AD in their lifetime [130].

Additionally, 95 (of the 99) participants underwent a brain MRI, performed on a General Electric 3 Tesla scanner (Model 750 W) using a 24-channel head coil. The scans included sagittal 3D T1 spoiled gradient recalled acquisition in steady state, and 3D T2 fluid-attenuated inversion recovery with axial dual echo Turbo spin-echo PD/T2 weighted sequences and axial T2 star gradient sequence scans. From the acquired images, hippocampal volume was calculated and normalised as a percent of the global intracranial volume including CSF, grey

matter, and white matter. Neuroimaging was completed in 2015 by another member of the Martin's research team.

2.4 C-reactive protein measurement and APOE genotyping

CRP levels (mg/L) in serum were measured using the COBAS Tina-quant immunoturbidimetric ADVIA wide range CRP (wr-CRP) assay at an independent clinical pathology laboratory (Laverty Pathology, NSW, Australia).

APOE genotype was determined from purified genomic DNA extracted from 0.5ml whole blood. DNA extraction was performed using the QIAamp DNA blood mini kit (Catalogue number 51106, Qiagen, Hilden, Germany). The presence of *APOE* variants (ε2, ε3, and ε4) was determined based on TaqMan 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). Briefly, this principle involved setting up 384 well plates containing single samples and duplicate control genotypes (ε2/ε2; ε3/ε3; ε3/ε4; ε4/ε4), with each containing 2-20ng DNA in 2µL per well. After blanking the real time-polymerase chain reaction machine, reagents were thawed on ice, protected from light. Next, 2µL template DNA was pipetted per well, then the master mix (genotyping buffer and probe) was aliquoted (4.15µL) per well. The plate was sealed, inverted three times and centrifuged. The absolute quantitation assay was performed, and an allelic discrimination plate read document set up, wherein a marker file was generated containing the two detector fluorescent dyes (corresponding to specific genotype). Finally, the wells were labelled, assigned markers, and allelic discrimination was performed, analysed and genotypes were determined. Genotyping was performed previously by another member of the research team, and data obtained was used in the current study.

2.5 Food frequency questionnaire and plasma carotenoid measurement

Dietary intake of carotenoids was determined using the Cancer Council of Victoria Food Frequency Questionnaire (CCV FFQ) [131]. This food frequency questionnaire assesses dietary intake over the previous 12 months, comprising a list of 74 food items with 10 frequency response options as well as photographs of scaled food portions. The questionnaire includes five types of intake (cereal foods, sweet and snacks; dairy products, meats and fish; fruit; vegetables; and alcohol) and output is calculated according to a wide range of food components including fat, protein, carbohydrates, cholesterol, carotenoids, glycaemic index/load and vitamins. Dietary intake information was obtained in 2015 by another member of the Martin's research team.

High performance liquid chromatography (HPLC) was used to determine carotenoid (lutein, β-cryptoxanthin, lycopene, α-carotene and β-carotene) and α-tocopherol concentrations in plasma, using a validated methodology [132, 133]. First to set up, chemical reagents were filtered, and samples allowed to thaw. To prepare the internal standard, first a 20mL glass scintillation vial and cap were prewashed with dichloromethane and allowed to dry in fume hood. Then, vial was covered with aluminium foil, as canthaxanthin is light sensitive. Next 5mL ethyl acetate, 25µL canthaxanthin and 100µL α-tocopherol acetate were pipetted into a vial and stored in an esky on ice. To begin the carotenoid measurement, first 500µL of each sample was pipetted into each test tubes (leaving two blanks filled with 500µL distilled water). Then 1mL ethanol was pipetted into each tube, and the tube was vortexed (approximately 5sec). Next, 1mL ethyl acetate was pipetted into 1 of the blank tubes. The internal standard (1mL) was pipetted into the sample tubes and the remaining blank tube, and the tubes were vortexed for 30sec and centrifuged at 4°C, spin of 3000xG for 5min. Following removal from the centrifuge, tubes were placed in a rack in the fume hood. Using a transfer pipette, the supernatant was decanted (leaving a small amount in the sample tube) to a duplicate tube, which was then stored on ice. The small pellet left in the sample tube was broken up gently and 1mL ethyl acetate was added to each sample tube. The tubes were then vortexed for 30sec, and centrifuged at 4°C, spin of 3000xG for 5min. The process of removal from the centrifuge and decanting the supernatant was repeated, and 1mL hexane was added to each tube. The tubes were then vortexed for 30sec, and centrifuged at 4°C, spin of 3000xG for 5mins. Next the tubes were removed from the centrifuge and this time, all the supernatant was decanted from each sample tube to the duplicate tube. 1mL of ultra-pure (Milli-Q) water was added to the pooled supernatant, the tube was vortexed for 30sec and centrifuged at 4°C, spin of 3000xG for 5min. Following removal from the centrifuge, the organic top layer was decanted into a clean glass tube which was placed and secured into slots on the carousel of a nitrogen evaporator. The evaporator pipette was lowered into each tube so that the pipette end was just above the liquid level. The evaporator valves were opened to allow gentle gas flow to evaporate the samples. Following complete evaporation, 100µL Dichloromethane:methanol (1:2 v/v) was added to reconstitute the sample and the tubes were vortexed for 1min. The re-suspended extract was transferred into a high recovery insert in an amber glass vial. The vial was sealed with cap, septa and parafilm tape, labelled and stored in a -80°C freezer until analysis.

HPLC analyses were performed on a Hypersil Octadecylsilane (ODS) column (100mm×2.1mm×5µm) (Thermo Fisher Scientific, MA, USA) with a flow rate of 0.3mL/min. Carotenoids and α-tocopherol were analysed using a mobile phase of acetonitrile:dichloromethane:methanol 0.05% ammonium acetate (85:10:5 v/v) and a diode

array detector (470 and 297nm). Sample carotenoid and α-tocopherol peaks were identified by comparing their retention times with a standard mixture of carotenoids and α-tocopherol and quantified using an Agilent 1200 Series High Performance Liquid Chromatograph instrument with Chemstations software (Agilent Corporation, Waldbronn, Germany).

The information that was collected by the detector during HPLC was converted into a chromatogram as seen in Figure 8. The peaks represent the absorbance amount for each analyte, the area under each peak was used to determine the concentration of the analytes in the sample, against the known internal standard, Canthaxanthin. Canthxanthin is an effective internal standard as it has minimal overlap with carotenoids present in human plasma at 450nm.

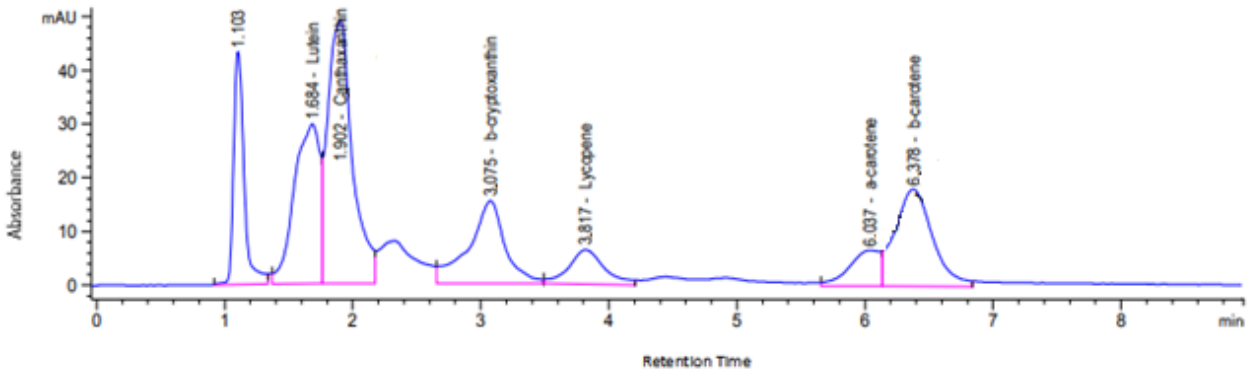


Figure 8. Example of HPLC output, showing each analyte and the standard canthaxanthin. Areas under the curve are calculated to provide concentration data.

HPLC was performed by myself with assistance from Dr Wood's team at Newcastle University. The HPLC data analysis was done by myself.

2.6 Inflammatory Marker assays

Interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) were assayed using the Quantikine Enzyme-linked Immunosorbent Assay (ELISA) protocol (IL-6 catalogue numbers: D6050, TNF-α catalogue numbers: DTA00D, R&D Systems, USA). Reagents and standards were prepared according to the manufacturer's instructions. Using a standard 96-well plate, first, Assay Diluent (100µL RD1W for IL-6 and 50µL RD1F for TNF-α) was added to each well. Then standards and thawed plasma samples were pipetted (100µL for IL-6 and 50µL for TNF-α) into separate wells, as seen in Figure 9. Following a 2hr incubation at room temperature (TNF-α on an orbital microplate shaker), the wells were aspirated and washed (a total of 4 times) using the provided wash buffer. Next, 200µL of the appropriate human cytokine conjugate was added, following which the plate was incubated for 2hrs at room temperature (TNF-α on an orbital microplate shaker), and then thoroughly aspirated and washed. Next,

200µL Substrate Solution, comprising two colour-reagents, 12mL of reagent A and 12mL of reagent B, was added and incubated for 20min for the IL-6 assay and 30min for the TNF-α assay. Following incubation, 50µL Stop Solution was added, enabling colour change. Finally, the plates were taken to a PHERAstar FS microplate reader (BMG Labtech, Germany), set at 450nm, to determine optical density. This reading was repeated at 540nm, which was then subtracted from the 450nm result, to correct for optical imperfections in the plate.

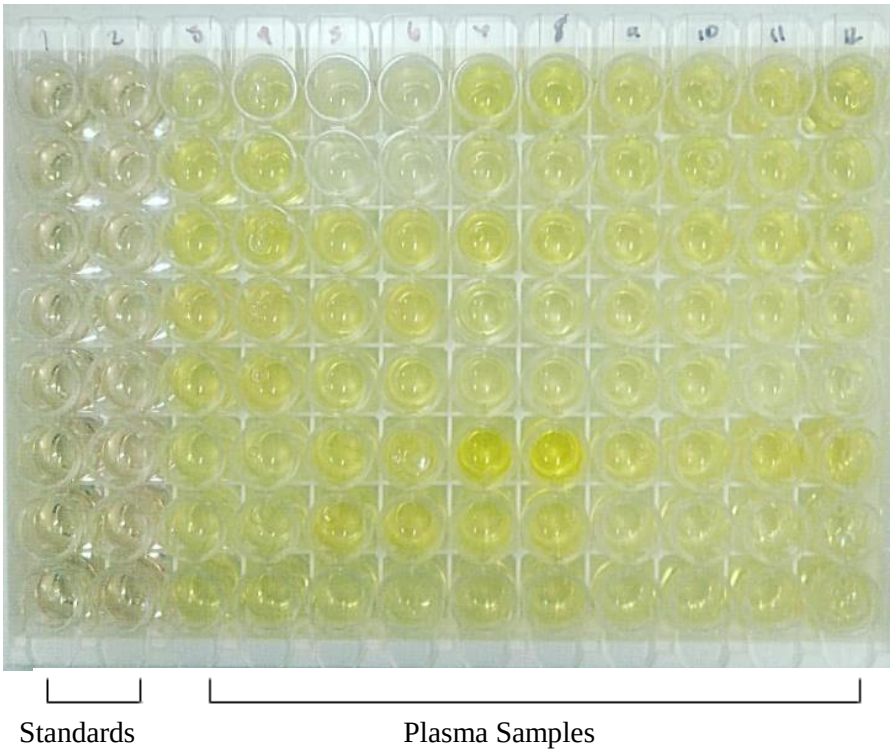


Figure 9. ELISA assay for IL-6. Image of ELISA plate taken during incubation step.

Standard curves were calculated from the values provided by the manufacturer (R&D Systems, USA) using the online software 'https://www.mycurvefit.com/index.html' from which concentrations for each sample were recorded (Figure 9a). The standard curve for IL-6 ranged from 3.13-300pg/mL. The standard curve for TNF-α ranged from 15.6-1000pg/mL. IL-6 and TNF-α ELISA assays were performed and analysed by myself.

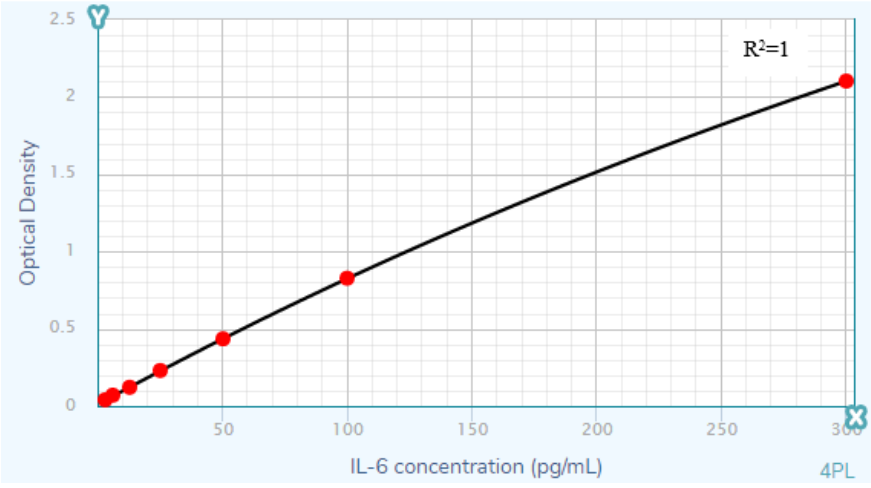


Figure 9a. Standard Curve for IL-6. Standard curve obtained from the ELISA assay for IL-6.

2.7 Plasma Aβ analysis

Plasma $A\beta_{40}$ and $A\beta_{42}$ were measured using the ultra-sensitive single-molecule array (SIMOA) platform (Quanterix Lexington, MA)[134-136]. The SIMOA assay allows dilution of plasma samples (1:4) minimizing matrix effects, increasing sensitivity and precision [136]. The SIMOA assays for $A\beta_{40}$ and $A\beta_{42}$ employed the same antibody capture agents to target the N-terminus and different biotinylated detection antibodies specific to each C-terminus. First, the antibody capture agents were attached to paramagnetic beads (diameter of 2.7µm). Next, the antibody-coupled capture beads, detection antibodies and plasma samples were combined. Washing was performed to remove non-specifically bound proteins and the beads are incubated with β-galactosidase–labelled streptavidin. A second wash was performed, substrate solution was added, and detection was carried out in a SIMOA array disc [136, 137]. Plasma amyloid data was obtained in 2015 by the Martin's research team.

2.8 Neuropsychological and clinical assessments

Study participants underwent a comprehensive battery of neuropsychological assessments. Within the comprehensive battery, the MoCA [113] and MMSE [120] were employed to define participants as 'cognitively normal' wherein MoCA scores >25 and MMSE≥26 were considered cognitively normal. The Memory Complaint Questionnaire (MAC-Q) [121] was used to categorise participants as subjective memory complainers (MAC-Q ≥25) and non-complainers (MAC-Q <25). The MoCA and MMSE assess a wide range of cognitive domains including attention, executive function, memory and orientation, with the MoCA more targeted to mild cognitive disturbance. The MAC-Q was used to assess self-reported memory complaints/concerns. The assessment battery also included the Rey Auditory Verbal Learning Test (RAVLT) [138], Logical Memory I and II (Wechsler Memory Scale-III; Story A only) [139], Rey Complex Figure Test (RCFT) [140], Digit Span, and Digit Symbol Substitution Test (DSST) (Wechsler Adult Intelligence Scale-Third edition, WAIS-III) [141], Delis-Kaplan Executive Function System (D-KEFS) Category Fluency (Boys Names) and Switching (Fruits and Furniture) Tasks [142], Controlled Oral Word Association Test (COWAT) [143], Stroop Test (Victoria version) [144], Boston Naming Test [145], Wechsler Test of Adult Reading (WTAR) [146] and the DASS [125]. Composite scores of verbal and episodic memory were generated from the mean of the z-scores of RAVLT List A, RAVLT short-delay, RAVLT long-delay, logical memory I, logical memory II, RCFT 3min and RCFT 30min. Composite scores of working memory and executive function were generated from the mean of the z-scores of Digit Span backward, DSST, D-KEFS Category Fluency and Switching tasks. A global composite score was created from the mean of the z-scores of the MMSE, RAVLT List A, RAVLT short-delay, RAVLT long-delay, logical memory I, logical memory II, RCFT 3min,

RCFT 30min, Digit Span backward, DSST, D-KEFS Category Fluency and Switching tasks. The Depression Anxiety Stress Scale (DASS) was used to determine the emotional state of the participants within three categories: depression, anxiety and stress [125].

Clinical assessment was undertaken to ensure participants complied with the study inclusion and exclusion criteria. The height and weight of participants was recorded to calculate their body mass index (BMI). Participants were asked if they were currently smoking cigarettes or had a history of smoking and the years in which they smoked. Smoker status was recorded as 'yes', if participants currently smoked (n=1) or if they had a history of smoking (n=25). Neuropsychological and clinical data was obtained at baseline in 2015 by the Martin's research team.

2.9 Statistical analyses

Statistical analysis was completed using the Statistical Package for Social Sciences (SPSS; Windows version 25). All analyses were two-tailed with significance taken at an alpha of 0.05. Between group analyses were conducted using independent samples t-test for normal variables, and Mann-Whitney for non-normally distributed variables. Nominal variables were analysed using the chi-squared test.

Continuous outcome variables were assessed for normality using the Shapiro-Wilk statistic and log transformed when required. Log-transformed data were obtained for lutein, β-cryptoxanthin, α-carotene, β-carotene, lycopene, α-tocopherol, IL-6, TNF-α and CRP. The transformed data was then used in further analysis. Univariate general linear models were used to compare continuous variables between the interest groups (i.e. high NAL and low NAL), with and without adjusting for covariates including age, gender, BMI, *APOE* status and years of education, as well as dietary carotenoids and α-tocopherol and supplement intake, and hippocampal volume.

Pearson's correlations were used to determine the associations between plasma carotenoid and α-tocopherol concentrations, and dietary carotenoid and α-tocopherol intake (FFQ), plasma Aβ (40, 42 and ratio of 42/40), cognitive performance (composite score of verbal, visual and episodic memory, working memory and executive function, and global cognition), cytokine concentrations (TNF-α, IL-6, and CRP) and hippocampal volume (left and right hemispheres). Intercorrelations were determined between plasma carotenoids and α-tocopherol, as well as between inflammatory markers. All statistical analyses were done by myself for this thesis.

Chapter Three

3. Results

3.1 Participant demographics

On average, as shown in Table 4, participants were aged 78.07 (±5.44), years were mostly female (n=68/99) and well-educated (14.40 years). The MAC-Q was used to determine if participants had subjective memory complaints (SMC) (MAC-Q≥25, n=75) or not (MAC-Q<25, n=24). The two groups, high NAL and low NAL, were not significantly different in gender, age, education, BMI, MMSE, MAC-Q, depression, anxiety, stress, hemispheric

Table 4. Descriptive characteristics of study participants

	Overall Cohort (n=99)	**Low NAL (<1.35) (n=64)**	**High NAL (≥1.35) (n=35)**	***t***	***p***
Gender, % female [a]	68.69	71.88	62.86	0.86	0.36
Age, years	78.07 (5.44)	77.44 (5.41)	79.23 (5.38)	-1.58	0.12
Education, years [b]	14.40 (3.26)	14.81 (3.39)	13.64 (2.92)	-1.61	0.11
BMI	27.60 (4.57)	27.34 (4.50)	28.05 (4.74)	-0.74	0.46
MMSE [b]	28.61 (1.15)	28.50 (1.17)	28.80 (1.11)	-1.27	0.20
MAC-Q	26.08 (2.72)	26.02 (2.57)	26.20 (3.00)	-0.32	0.75
SMC, % yes [a]	75.76	78.13	71.43	0.55	0.46
Depression	2.61 (3.42)	2.50 (2.96)	2.80 (4.17)	-0.38	0.71
Anxiety	2.45 (3.01)	2.44 (2.98)	2.49 (3.11)	-0.08	0.94
Stress	4.70 (4.52)	4.72 (4.28)	4.66 (4.99)	0.07	0.95
NAL SUVR	1.35 (0.32)	1.16 (0.09)	1.71 (0.26)	-	-
Left HV %	0.20 (0.02)	0.20 (0.02)	0.19 (0.02)	0.40	0.69
Right HV %	0.20 (0.02)	0.20 (0.02)	0.20 (0.02)	0.02	0.98
APOE, % ε4 present [a]	21.21	7.81	45.7	19.45	0.00*
Smoker Status, % yes [a]	26.27	26.56	25.71	0.01	0.93

Table 4 shows the comparison of demographic and clinical characteristics between the two outcome groups (low NAL and high NAL). Only the presence of *APOE* ε4 is statistically significantly different (p=0.000004). Data are presented as mean (standard deviation) or %; *p<0.05; statistics represent independent samples t-tests performed between low NAL and high NAL groups unless otherwise stated; [a] chi square statistic; [b] Mann-Whitney U statistic. Abbreviations: NAL, neocortical amyloid-β load; BMI, body mass index; MMSE, mini mental state examination; MAC-Q, memory assessment complaint questionnaire; SMC, subjective memory complainers; SUVR, standardised uptake value ratio; HV, hippocampal volume; *APOE*, Apolipoprotein E.

hippocampal volumes or smoker status (Table 4). There was a statistically significant difference in *APOE* status between groups, whereby high NAL participants were more likely (p=0.000004) to have the presence of ε4 (n=16/35) than low NAL participants (n=5/64).

3.2 Association of carotenoids and α-tocopherol with NAL: Plasma α-carotene is significantly higher, whereas plasma α-tocopherol is significantly lower in the preclinical AD group after adjusting for the study covariates

Carotenoid levels did not differ significantly between high and low NAL groups (Table 5), though there was a trend towards significance in the α-tocopherol levels (p=.078). The spread of individual carotenoids and α-tocopherol levels for participants in the high NAL and low NAL groups can be seen in Figure 10A-F. In the high NAL group, there was a non-significant trend of lower mean levels of lutein, β-cryptoxanthin, lycopene, α-tocopherol and higher levels of α-carotene and β-carotene. When carotenoids were investigated by polarity, there was no significant difference between the groups in levels of xanthophylls (p=0.736), or carotenes (p=0.448).

To further investigate the association between carotenoids and α-tocopherol with NAL, general linear models were used, using the following covariates in various combinations: age, gender, BMI, *APOE* ε4 status, years of education, dietary intake and supplement information. After controlling for these covariates separately and in combination, there were no significant associations between lutein, β-cryptoxanthin, β-carotene and total carotenoids, with regards to NAL (all p>0.05). Yet, when age, gender, BMI, *APOE* ε4 and years of education were controlled for, α-carotene in the high NAL group was significantly higher (p=0.035) shown as p^a in Table 5. This adjusted relationship between log-transformed carotenoids and NAL, controlling for age, gender, BMI, *APOE* ε4 and years of education, can be seen in Figure 11. After these adjustments were made, a non-significant trend towards higher levels of lutein, β-cryptoxanthin, lycopene and β-carotene in the high NAL group was revealed. Further, when dietary intake and supplement intake were added to this adjusted model, α-carotene in the high NAL group remained significantly increased (p=0.033). In addition, when combined dietary and supplement intake were controlled for, α-tocopherol levels were significantly lower in the high NAL group (p=0.044) shown as p^b in Table 6.

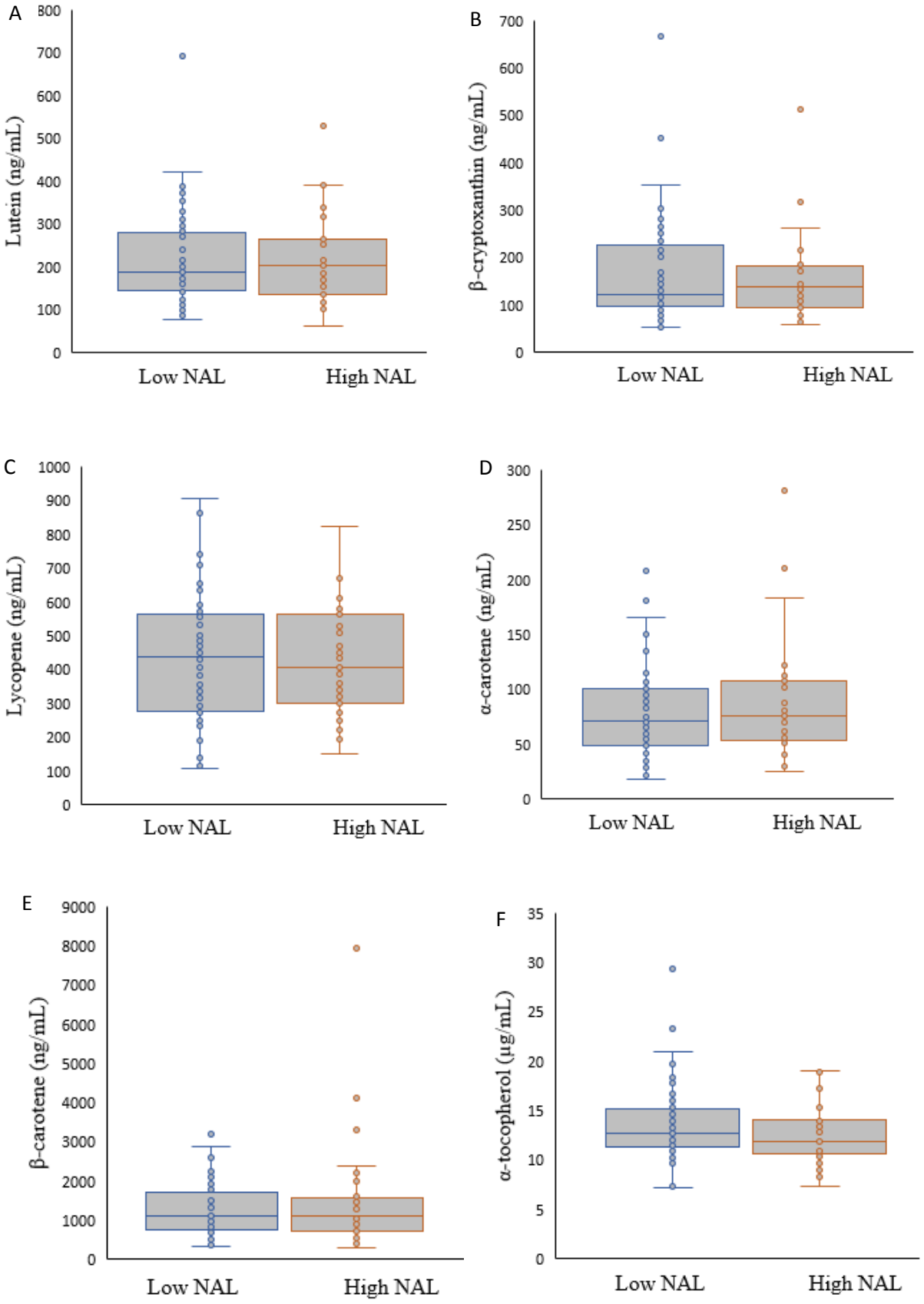


Figure 10A-F. Carotenoid and α-tocopherol levels in low neocortical amyloid-β load (NAL) and high NAL groups. Error bars represent the interquartile range of the data; line segments represent the median values.

Table 5. Plasma carotenoid and α-tocopherol levels in high NAL and low NAL groups

Carotenoids and α-tocopherol	Overall cohort (n=99)	NAL <1.35 (n=64)	NAL ≥ 1.35 (n=35)	p	p^a	p^b
Lutein	214.69 ± 102.63	214.95 ± 104.02	214.21 ± 101.53	0.897	0.293	0.824
β-cryptoxanthin	155.46 ± 99.62	159.01 ± 106.96	148.98 ± 85.69	0.736	0.392	0.513
Lycopene	428.97 ± 173.43	434.88 ± 182.60	418.16 ± 157.25	0.899	0.743	0.916
α-carotene	82.08 ± 46.49	80.02 ± 42.50	85.86 ± 53.48	0.406	0.035*	0.321
β-carotene	1320.83 ± 979.53	1256.93 ± 669.96	1437.68 ± 1382.80	0.861	0.157	0.972
α-tocopherol	13.14 ± 3.48	13.60 ± 3.78	12.29 ± 2.70	0.078	0.701	0.044*
Total Carotenoids	2202.03 ± 1126.24	2145.79 ± 890.91	2304.88 ± 1472.57	0.810	0.145	0.838

Table 5 shows the comparison of individual carotenoid and α-tocopherol levels, with the primary outcome groups (low NAL and high NAL). As shown, there are no statistically significant between-group differences. When adjustments were made for age, gender, BMI, *APOE* ε4 status and years of education (p^a), α-carotene is significantly higher in the high NAL group (p=0.035); when adjustments were made for diet and supplement intake (p^b) α-tocopherol was significantly lower in the high NAL group (p=0.044). Data are mean ± standard deviation; *p<0.05; statistics determined using General Linear Model; carotenoids reported as ng/mL, α-tocopherol reported as μg/mL. Abbreviations: NAL, neocortical amyloid-β load; *APOE*, apolipoprotein E.

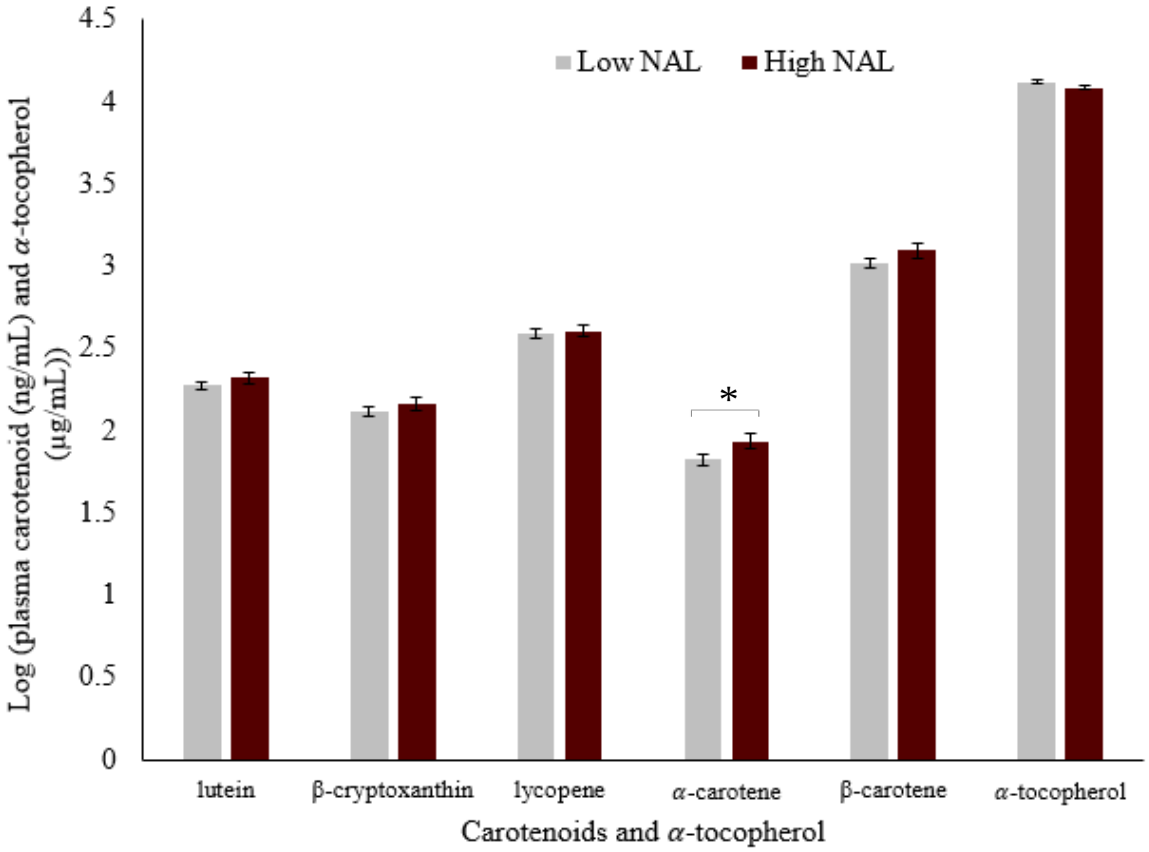


Figure 11. The relationship between plasma carotenoid and α-tocopherol levels and NAL, adjusted for age, gender, BMI, *APOE* ε4 status and years of education. α-carotene was significantly different between groups (p=0.035). The figure shows a non-significant trend of lower levels of lutein, β-cryptoxanthin, lycopene and β-carotene in the low NAL group present after adjustments to the model were made. Mean, log-transformed values for carotenoids and α-tocopherol have been used in the graph. *represents p<0.05. Error bars represent adjusted standard error.

3.2.1 Intercorrelation significance varies between carotenoids and α-tocopherol

Carotenoids were significantly intercorrelated, except lutein/lycopene (r=1.49, p=.140). The strongest correlations were found between β-carotene/total carotenoids (r=.968, p<.000), α-carotene/total carotenoids (r=.787, p<.000) and α-carotene/β-carotene (r=.748, p<.000). α-tocopherol was less strongly associated with most carotenoids, the strongest correlation being α-tocopherol/lutein (r=.432, p=.0.000012), and was not significantly correlated with β-cryptoxanthin (r=.163, p=.109) or lycopene (r=-.040, p= .697).

3.3 Associations between plasma carotenoid and α-tocopherol levels and dietary carotenoid and α-tocopherol levels: individual plasma carotenoids, except lycopene, were significantly positively associated with their respective dietary intakes

There were significant associations between plasma lutein (p=0.040), β-cryptoxanthin (p=0.003), α-carotene (p=0.000075) and β-carotene (p=0.030), and their dietary counterparts, as measured within the whole cohort, as shown in Table 6. However, lycopene (p=0.071) and α-tocopherol (p=0.087), as well as the combined total carotenoids (p=0.104), did not significantly correlate with the FFQ data on dietary intake.

Table 6. Correlation between plasma concentrations and dietary intake of carotenoids and α-tocopherol

Carotenoids and α-tocopherol	*r* (*p*)
Lutein	.207* (.040)
β-cryptoxanthin	.294** (.003)
Lycopene	.183 (.071)
α-carotene	.352** (.0.000075)
β-carotene	.219* (.030)
Total carotenoids	.164 (.104)
α-tocopherol	.087 (.391)

Table 6 shows that lutein, β-cryptoxanthin, α-carotene and β-carotene are significantly correlated between plasma and respective dietary intake. Data are Pearson's coefficient (p value) obtained using Bivariate Correlations; * p<0.05; ** p<0.01.

3.3.1 Dietary carotenoids and α-tocopherol were not significantly different between NAL groups

As seen in Table 7, the levels of dietary intake of carotenoids and Vitamin E were not significantly different between high NAL and low NAL groups ($p>0.05$).

Table 7. Dietary intake of carotenoids and α-tocopherol in the high NAL and low NAL groups

	Overall Cohort (n=99)	**Low NAL (<1.35) (n=64)**	**High NAL (≥1.35) (n=35)**	***t***	***p***
Lutein	877.67 ± 418.47	865.22 ± 445.38	900.43 ± 369.27	-.40	.69
β-cryptoxanthin	240.06 ± 175.88	227.11 ± 169.34	263.72 ± 187.44	-.99	.33
Lycopene	3159.22 ± 1761.33	3183.71 ± 1778.50	3114.45 ± 1754.35	.19	.85
α-carotene	788.36 ± 502.99	800.59 ± 462.14	766.00 ± 576.79	.33	.75
β-carotene	3697.66 ± 1718.64	3615.36 ± 1722.63	3848.15 ± 1725.99	-.64	.52
Vitamin E	6.13 ± 1.90	6.02 ± 1.85	6.33 ± 2.01	-.77	.44
Total Carotenoids	8762.97 ± 3459.33	8692.00 ± 3541.72	8892.75 ± 3350.12	-.28	.78

Table 7 shows that there are no significant between-groups differences in dietary intake of individual carotenoids or vitamin E. Data are mean ± standard deviation, carotenoid values in μg/day, α-tocopherol in mg/day; obtained using independent samples t-tests. Abbreviations: NAL, neocortical amyloid-β load.

3.4 Association of carotenoids and α-tocopherol with plasma Aβ: plasma lutein and β-carotene were significantly associated with plasma Aβ

Assessing the association between plasma analyte levels and plasma $A\beta_{40}$ and $A\beta_{42}$, revealed that of the five measured carotenoids and α-tocopherol, only the correlations between lutein and both plasma $A\beta_{40}$ and $A\beta_{42}$, and β-carotene and $A\beta_{40}$ were significant (Table 8). There were no significant associations between carotenoid and α-tocopherol levels and the ratio of plasma $A\beta_{42}$/ $A\beta_{40}$. The significant relationships can be seen graphically in Figure 12. The levels of plasma $A\beta_{40}$ and $A\beta_{42}$ did not statistically differ between low NAL and high NAL groups (Table 8a).

Table 8. Correlations between plasma analytes and plasma Aβ

Carotenoids and α-tocopherol	**Plasma $A\beta_{42}$**	**Plasma $A\beta_{40}$**	**Ratio $A\beta_{42}$/ $A\beta_{40}$**
Lutein	-.205* (.045)	-.227*(.025)	-.001 (.992)
β-cryptoxanthin	.062 (.552)	.014 (.891)	.055 (.600)
Lycopene	-.046 (.656)	-.055 (.591)	-.051 (.625)
α-carotene	-.091 (.382)	-.102 (.324)	-.023 (.824)
β-carotene	-.199 (.052)	-.220* (.030)	-.045 (.669)
Total	-.174 (.090)	-.194 (.056)	-.042 (.685)
α-tocopherol	-.053 (.609)	-.056 (.588)	.013 (.903)

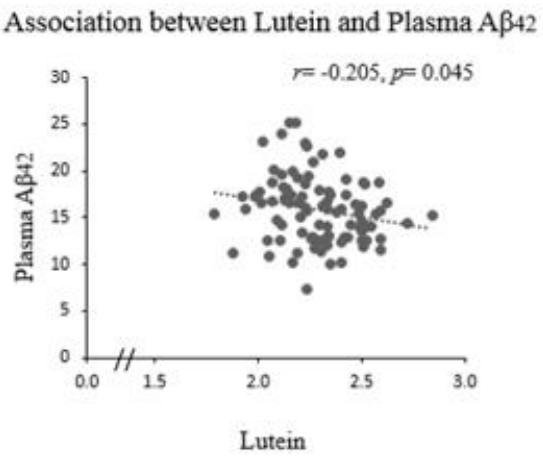
Table 8 shows that the correlations between lutein and plasma $A\beta_{40}$ and $A\beta_{42}$, and β-carotene and $A\beta_{40}$ were significant. Data are represented as Pearson's coefficient (*p* value); obtained using Bivariate Correlations; * $p<0.05$

Table 8a. Plasma Aβ levels in low NAL and high NAL groups

Plasma Aβ	**Overall Cohort (n=99)**	**Low NAL (<1.35) (n=64)**	**High NAL (≥1.35) (n=35)**	***t***	***p***
Plasma $A\beta_{40}$	315.41 ± 61.76	306.46 ± 54.04	331.27 ± 71.59	-1.78	.08
Plasma $A\beta_{42}$	15.79 ± 3.52	15.83 ± 3.56	15.71 ± 3.48	.16	.88

Table 8a shows that there are no significant differences between plasma $A\beta_{40}$ and $A\beta_{42}$, and brain Aβ. Data are mean ± standard deviation; *$p<0.05$; statistics determined using independent samples t-tests.

A



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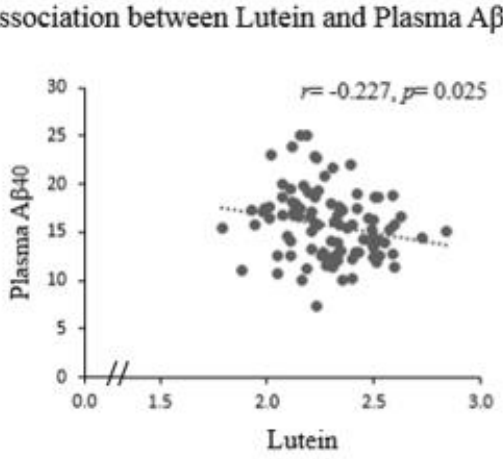

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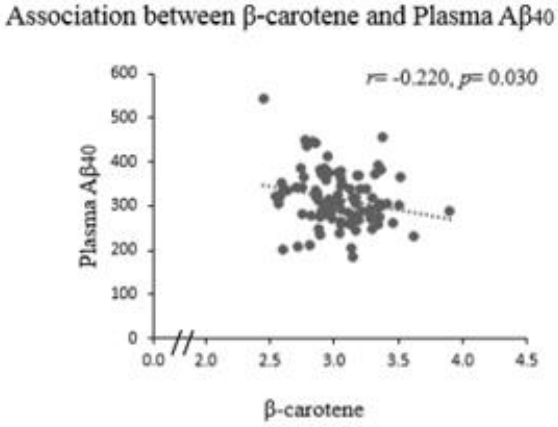

Figure 12A-C. Significant correlations of lutein and β-carotene with plasma $A\beta_{40}$ and $A\beta_{42}$. Pearson's coefficients were employed.

3.5 Associations of carotenoids and α-tocopherol with cognitive performance:

Cognitive performance was calculated as composite verbal, visual and episodic memory, working memory and executive function, and global cognitive performance.

3.5.1 Higher α-tocopherol levels were significantly associated with better verbal, visual and episodic memory

There was a trend towards correlation between higher levels of the individual carotenoids, lutein and β-carotene, and better performance in assessments of verbal, visual and episodic memory, analysed as one composite score represented in Table 9. Only higher levels of α-tocopherol were significantly associated with verbal, visual and episodic memory (p=0.046), although this was a weak correlation (r=.201).

Table 9. Correlations between plasma carotenoids and α-tocopherol, and a composite score of verbal, visual and episodic memory

Carotenoids and α-tocopherol	***r* (*p*)**
Lutein	.176 (0.081)
β-cryptoxanthin	.068 (0.508)
Lycopene	.096 (0.347)
α-carotene	.028 (0.785)
β-carotene	.159 (0.116)
α-tocopherol	.201* (0.046)

Table 9 shows the relative correlations between carotenoids and α-tocopherol and verbal, visual and episodic memory within the whole cohort. Only α-tocopherol correlated significantly with this composite cognitive score (p=0.046), though all correlations were positively inclined. Data are Pearson's coefficient (p value); *p< 0.05

3.5.2 Carotenoids and α-tocopherol were not significantly associated with working memory and executive function

Carotenoid and α-tocopherol levels were not associated with working memory and executive function as shown in Table 10. The correlation with β-cryptoxanthin shows that higher levels of β-cryptoxanthin are weakly and non-significantly correlated with poorer working memory and executive function performance, whereas higher levels of lutein, β-carotene and α-tocopherol showed a trend towards positive correlation. As the *p* values of lycopene and α-carotene are high, these statistics cannot be used to determine directionality of the causation.

Table 10. Correlations between plasma carotenoids and α-tocopherol, and a composite score of working memory and executive function

Carotenoids and α-tocopherol	***r* (*p*)**
Lutein	.164 (0.104)
β-cryptoxanthin	-.120 (0.240)
Lycopene	.039 (0.704)
α-carotene	.017 (0.868)
β-carotene	.181 (0.074)
α-tocopherol	.118 (0.246)

Table 10 shows no significant correlations between carotenoids and α-tocopherol and working memory and executive function. Data are represented as Pearson's coefficient (*p* value).

3.5.3 Higher levels of lutein, β-carotene and α-tocopherol were associated with better global cognitive performance

When cognitive performance was assessed using the calculated global composite score higher levels of lutein, β-carotene and α-tocopherol significantly positively correlated (p=0.024, p=0.024 and p=0.028, respectively), as shown in Table 11. These correlations were significant however, Pearson's coefficient was low, reflecting a weak association. Associations between higher cognition scores and plasma levels of β-cryptoxanthin, lycopene and α-carotene were non-significant.

Table 11. Correlations between plasma carotenoids and α-tocopherol, and a global cognitive performance

Carotenoids and α-tocopherol	*r* (*p*)
Lutein	.227* (0.024)
β-cryptoxanthin	.014 (0.891)
Lycopene	.082 (0.418)
α-carotene	.057 (0.575)
β-carotene	.227* (0.024)
α-tocopherol	.220* (0.028)

Table 11 shows that higher levels of plasma lutein, β-carotene and α-tocopherol were significantly correlated with better global cognitive performance. There were no significant correlations with β-cryptoxanthin, lycopene or α-carotene. Data are Pearson's coefficient (*p* value); * $p<0.05$.

3.6 Cytokine measurement and the association of carotenoids and α-tocopherol with inflammatory factors: cytokines were not significantly associated with carotenoids or α-tocopherol

Inflammatory factors (TNF-α, IL-6 and CRP) were detectable in the study participants at varied extents (TNF-α n=58, IL-6 n=70, CRP n=99) (Table 12). Serum CRP was present in the highest concentration (3.12 ± 6.11mg/L), followed by plasma TNF-α (6.22 ± 4.10pg/mL) and IL-6 (3.47 ± 2.72pg/mL). There was significant intercorrelation (Pearson's coefficient) between log-transformed TNF-α and IL-6 (r=.358, p=0.009), and TNF-α and CRP (r=.348, p=0.012) but no statistically significant correlations between IL-6 and CRP (r=.247, p=.064). Inflammatory markers were not investigated with regard to NAL, as this data was given to a colleague to use in her research.

Table 12. Detection and range of cytokines in plasma

Cytokine	Detection	Concentration (pg/mL)	Minimum	Maximum
TNF-α	58/99	6.22 (4.10)	0.06	16.68
IL-6	70/99	3.47 (2.72)	0.95	16.71
CRP	99/99	3.12 (6.11)[a]	0.00	52.53

Table 12 shows the relative detectability and concentration of TNF-α, IL-6 and CRP. TNF-α detected (n= 58) at a mean concentration of 6.22 pg/mL, IL-6 was detected (n=70) with a mean concentration of 3.47 pg/mL and CRP was detected in all participants (n=99) at the highest concentration (3.12 mg/L). [a] concentration in mg/L; Data are mean (SD); abbreviations: TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6; CRP, C-reactive protein.

There were no significant correlations between carotenoids and α-tocopherol and inflammatory markers (TNF-α, IL-6 and CRP) as shown in Table 13.

Table 13. Correlations of plasma carotenoids and α-tocopherol with inflammatory markers

Carotenoids and α-tocopherol	TNF-α	IL-6	CRP
Lutein	-.249 (0.072)	-.015 (0.912)	-.064 (0.528)
β-cryptoxanthin	.100 (0.478)	-.009 (0.946)	.068 (0.505)
Lycopene	-.116 (0.408)	-.125 (0.345)	.130 (0.201)
α-carotene	-.079 (0.574)	-.183 (0.168)	.039 (0.705)
β-carotene	-.090 (0.521)	-.016 (0.907)	.080 (0.435)
α-tocopherol	.090 (0.522)	.141 (0.286)	.136 (0.182)

Table 13 shows that there were no significant correlations between carotenoids and α-tocopherol and measures of inflammation. Data are Pearson's coefficient (*p* value).

3.6.1 Correlation between cytokines and hippocampal volumes: cytokines were not significantly correlated with hippocampal volumes

Table 14. Correlations of inflammatory markers with hippocampal volumes

Cytokines	Left HV	Right HV
TNF-α	-.059 (.678)	-.193 (.171)
IL-6	.184 (.168)	-.054 (.689)
CRP	-.070 (.501)	-.176 (.089)

Table 14 shows that there were no significant correlations between left or right hippocampal volume and measures of inflammation. Abbreviations: TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6; CRP, C-reactive protein; HV, hippocampal volume. Data are Pearson's coefficient (*p* value).

The association between each marker of inflammation and hippocampal volume (left and right) was determined using bivariate correlation analyses. This revealed no significant associations (Table 14).

3.7 Association of carotenoids and α-tocopherol with hippocampal volume: carotenoids and α-tocopherol were not significantly associated with hippocampal volume

The association between each analyte and NAL was investigated with regard to hippocampal volume. First, bivariate correlations were performed to determine the association between hemispheric hippocampal volume and each analyte, revealing no significant associations and a trend towards a weak positive association between lutein and right hippocampal volume (Pearson's correlation=0.193, p=0.061), shown in Figure 13.

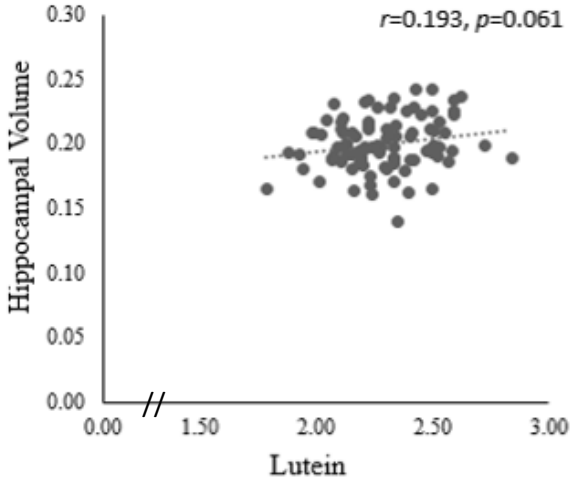

Figure 13. The relationship between right hippocampal volume and plasma lutein levels. The trend towards significance (p=0.061) and weak association is reflected by the trendline.

Next, a general linear model was constructed for each analyte, grouped by NAL, adjusted for hemispheric hippocampal volume. Again, the interaction between NAL and each analyte was not significant. α-tocopherol levels showed a trend towards being significantly lower in the high NAL group when adjustments were made for right hippocampal volume and left hippocampal volume (p=0.068 and p=0.073, respectively).

Chapter Four

4. Discussion

The current study findings suggest that plasma carotenoid and α-tocopherol levels are not significantly associated with neocortical amyloid-β load in preclinical AD. When demographic characteristics, dietary intake and supplements were adjusted for, α-carotene levels were significantly higher in the high NAL group (preclinical AD) compared to the low NAL group (cognitively normal individuals at no apparent risk to AD). This is, however, contrary to my initial hypothesis, which was that the concentration of plasma carotenoids and α-tocopherol would be lower in the preclinical AD participants compared to those with no apparent risk of developing AD. Furthermore, after these adjustments were made, all carotenoids, but not α-tocopherol, followed the pattern, though non-significantly, of higher mean concentrations in the high NAL group. Carotenoids levels are generally reported as lower in AD than healthy controls (Table 3). However, as has been shown previously for other protective molecules associated with AD, for example, plasmalogens (which are glycerophospholipids protective against ROS) [147] and brain derived neurotrophic factor (which is a protein involved in nerve growth, influential in Aβ processing) [148], the biochemical changes observed in preclinical AD may not continue the same trend in the clinical phase of AD. This could reflect the body's compensatory response to the increased Aβ deposition and oxidative stress. The bidirectional interaction of Aβ and oxidative stress is such that, Aβ has been shown to increase production of ROS, and conversely, elevated oxidative stress and damage can be observed prior to Aβ-deposition [149]. The increased plasma carotenoids could be reflective of increased active uptake of dietary carotenoids, strengthening the body's antioxidant capacity, required to compensate for the increase in ROS. The development of neuropathological changes in AD begins up to twenty years prior to clinical changes. Indeed, Aβ-deposition extensively progresses by the MCI stage [150]. It is therefore imperative to find potential modifiable associations with Aβ-deposition, such as dietary antioxidants, within the preclinical phase [151]. Furthermore, the identification of preclinical AD may have been made more accurate with the addition of tau-pathology to measures of brain Aβ [152].

Yet in contrast to the compensatory response hypothesis, explained above, after controlling for supplements and dietary intake, α-tocopherol levels were significantly lower in preclinical AD (high NAL group). Though no previous studies examined the association with NAL, Schmidt et al. found similar results with regard to cognitive performance, whereby after controlling for demographic and confounding clinical factors (such as age, gender, and smoking status), lower

α-tocopherol levels (and not other tocopherols or carotenoids) were suggestive of poorer cognitive performance in healthy middle-aged and older adults [15]. Furthermore, Amadieu et al. showed that higher levels of α-tocopherol levels were mildly associated with a higher risk of dementia at follow-up [118]. The relationship between vitamin E and dementia is one of contention. Studies examining the therapeutic efficacy of vitamin E in AD and with cognitive performance [153-158] showed inconclusive results (please see Supplementary Table 1 for a review of these studies). Though studies reported that vitamin E (or its isomer form of α-tocopherol) was beneficial to AD participants in that supplementation delayed AD progression [153, 156], it has also been reported that vitamin E may have no effect on AD clinical outcomes [155, 157], and it that it may have a detrimental effect on cognitive performance in individuals, reported when treatment did not affect markers of oxidative stress [157]. Notably, Sano et al. determined disease progression using a variety of outcomes including: loss of basic daily activity functioning, severe dementia diagnosis, institutionalisation and death, for which α-tocopherol treatment was beneficial, however for cognitive performance, the more commonly measured outcome, the treatment was not beneficial [156].

However, importantly, four of these five treatment studies [153, 155-157], did not control for dietary intake of vitamin E. Since diet can greatly influence cognition, as shown in Table 3, the effect of vitamin E supplements as reported in these studies may be mediated by dietary intake of vitamin E, or lack thereof. Importantly, vitamin E may also be harmful in higher doses. Over a 7-year follow-up study in 3 994 older adults with vascular disease or diabetes mellitus, the effect of vitamin E supplementation (400IU daily) on cancer and cardiovascular health was monitored [159]. This study found that compared with the placebo group, the supplementation group had higher rates of heart failure and increased related hospitalisations, and there was no significant between-groups difference on the outcome measures for cancer or major cardiovascular events. Similarly, a meta-analysis found that high-dosage vitamin E (≥400IU daily) was associated with increased all-cause mortality compared with low-dosage trials [160]. In light of these potential harmful effects, the significant association between vitamin E and preclinical AD found in the current study, other research findings regarding vitamin E and cognition or dementia and AD (Table 3), and treatments with vitamin E in both healthy individuals and dementia/MCI cohorts having inconclusive results (Supplementary Table 1), further investigation is required to determine if vitamin E levels are associated with future cognitive decline or if they have therapeutic potential.

The current study showed that plasma lutein, β-cryptoxanthin, α-carotene and β-carotene correlate significantly with their dietary counterparts (Table 6), yet, plasma lycopene and α-tocopherol were not significantly correlated with diet. These results are consistent with van

Kappel et al. who found modest correlations between serum carotenoids and dietary fruit and vegetable consumption (α-tocopherol not reported) [161]. Furthermore, when assessed by the CCV-FFQ, lycopene is typically reported as the highest mean intake per day, as opposed to other recall methods [162]. This suggests that lycopene may have been over-reported in the current sample. Other studies have also shown that plasma α-tocopherol is not reflective of dietary intake [163, 164]. A possible explanation for this is that α-tocopherol is most present in oils, typically used during the cooking process which are not well-represented in the CCV-FFQ. It should be noted that the CCV-FFQ does not determine individual tocopherol/tocotrienol measures for vitamin E, rather it combines the food sources of these isoforms into one outcome. Thus, the measure of dietary intake of α-tocopherol used in this study may encompass the other seven isoforms of vitamin E as well. Additionally, vitamin E bioavailability is influenced by demographic and clinical [165] characteristics as well as competing nutrients and amount of fat [166]. The bioavailability of carotenoids is difficult to determine based on dietary patterns as carotenoid bioavailability is dependent on the state of the carotenoid upon consumption such as raw vegetables, purified carotenoids or oils; as oils do not require release from the food matrix, they are more efficiently absorbed than carotenoids in foods, and often used in this form as supplements [165]. Additionally, heating plants before ingestion improves bioavailability, for example lycopene absorption from tomato products [167]. The efficiency of carotenoid release is also dependent on diet consumed with carotenoids, for example fats aid carotenoid absorption, the physical breakdown of food particles and digestive enzymes, and can vary inter- and intra-individually based on tissue uptake rate/release and metabolism [165]. Individual carotenoids demonstrate unique patterns of absorption, metabolism and transport in the bloodstream, dependent on isomerization and structural differences, particularly with regard to binding capabilities [167].

Furthermore, interindividual variations in the concentrations of carotenoids in blood and tissues are influenced by genetic differences. Specifically, single nucleotide polymorphisms in genes coding for scavenger receptor class B type 1 (SR-B1) and CD36 proteins, have been shown to affect carotenoid intestinal uptake, transport and metabolism [168]. Interestingly, research suggests that these proteins may also have relevance to AD. SR-B1 reduction enhanced fibrillar Aβ deposition and cerebral amyloid angiopathy in a mouse model [169]. Astrocytes in healthy and AD human brains have been shown to express SR-B1, which has been proposed to mediate interactions between astrocytes and fibrillar Aβ [170]. In late onset AD patients, genetic analysis has identified multiple polymorphisms on chromosome 12. This is the same chromosome at which the genes encoding for SR-B1 can be found [171]. Additionally, the links between polymorphisms in coding regions for CD36 and AD, as well as with cognitive

performance in controls have also been reported [172]. CD36 receptors interact with Aβ, causing its uptake and triggering microglia activation to initiate an inflammatory response, which in excess can progress to pathological neuroinflammation [173]. These studies suggest a potential link between carotenoids and AD in addition to the carotenoids' antioxidant, anti-inflammatory and anti-amyloidogenic activities. Importantly, this suggestion is speculative, regards clinical AD, and is based on genetic research which has largely been conducted in animal models or *ex vivo* with human tissue, and therefore requires further investigation in humans both in the clinical and preclinical stages of AD.

The most statistically significant correlations between diet and plasma carotenoids were found in β-cryptoxanthin and α-carotene. This is likely because these carotenoids are least commonly found in supplements, and so plasma concentrations are less confounded by supplements and most reflective of respective dietary intake. Plasma and dietary carotenoids typically intercorrelate strongly, yet this is dependent on the method of dietary record [162]. FFQs are less reliably associated with plasma carotenoid levels yet are the most commonly used method of dietary recall (over weighed record and 24h recalls) since they reflect the longest time-period (up to 12 months) and can be easily administered in large sample sizes. An alternate future study could validate the correlation between diet and plasma carotenoids by administering, to participants, a daily diet rich in specific carotenoids followed by a blood draw, repeating this measure for various carotenoids. This would control for errors from incorrect self-report of diet, fluctuations in individual nutrient consumption and bioavailability, and ultimately, reveal if what it is measured in the plasma is reflected in the diet. Additionally, though supplement use was included as a covariate, intake of vitamin supplements, individually or as complexes, were controlled for as one binary variable, i.e. as supplement intake or no supplement intake. This could be too broad and was reliant on subjective participant account and therefore may not have correctly accounted for actual supplement usage.

Since the bioavailability of carotenoids and α-tocopherol are difficult to determine from FFQ data, and FFQ data does not directly correlate for all plasma carotenoids (Table 6), plasma measures are a more accurate representation of the body's available nutrients, and thus more reflective of an influence on oxidative stress and therefore neurodegeneration and cognitive decline. However, it must be noted that plasma carotenoid and α-tocopherol levels may not reflect nutrient levels in the brain; the measurement of brain carotenoid and α-tocopherol concentrations could be argued to be more appropriate in AD. However, plasma and serum carotenoids are commonly used as proxies for brain carotenoids, due to their ease of assessment compared to human brain tissue. Indeed, when both serum and brain tissue carotenoid concentrations have been analysed, they significantly correlated with each other [11],

suggesting that serum carotenoid measures can be used to reflect brain carotenoid concentrations.

Similarly, although the measurement of brain Aβ deposition levels (for example by 11C-PiB-PET) reflects the development of AD pathology, the measurement method is expensive, difficult to implement and more invasive, and it is easier, cheaper and more practical to measure plasma Aβ. Researchers have tried to correlate plasma Aβ with the progression to dementia/AD [174, 175], however this has been reported as a weak interaction [176]; more successfully plasma Aβ has been correlated with brain Aβ [68]. The current study revealed significant inverse correlations between lutein and both plasma $A\beta_{40}$ and $A\beta_{42}$, as well as β-carotene and plasma $A\beta_{40}$. In contrast, a study investigating the association between plasma Aβ and several nutrients including β-carotene and vitamin E in older cognitively normal participants, did not find a significant association between β-carotene or vitamin E with plasma Aβ [74]. The finding of an inverse correlation between β-carotene and $A\beta_{40}$ in the current study may be attributable to the more sensitive plasma Aβ analysis technique used, namely SIMOA, as opposed to the more traditional sandwich ELISA used by Gu et al. [74]. The inverse relationship of β-carotene and plasma $A\beta_{40}$ levels could be explained with β-carotene's ability to bind to both Aβ peptides and fibrillar Aβ [107], which may help to remove Aβ from the circulation. However, this is currently speculation, and further studies are needed to determine why these levels might correlate. It was also the most abundant carotenoid in the plasma in the current cohort, which could explain the ability to correlate with the relatively low levels of plasma Aβ. Although no previous study examined lutein levels with plasma Aβ, lutein has been shown to decrease binding of Aβ to red blood cells, which in turn aids the resistance of red blood cells to oxidative damage induced by Aβ [177].

Although changes in plasma Aβ are likely to reflect changes in brain Aβ as they have been reported to exist in dynamic equilibrium [178], plasma Aβ is also likely to contain Aβ peptides from elsewhere than from the central nervous system such as from platelets [179]. The significant correlations of lutein and β-carotene with plasma Aβ levels, but not with brain Aβ levels may be attributable to this difference in origin. However, the more obvious difference is that brain Aβ measured by ^{18}F-FBB-PET represents an accumulating deposition of fibrillar Aβ [180], whereas the blood levels change dynamically with production and breakdown, and there is no static accumulation. The practical significance of carotenoids associating with plasma Aβ levels is that there may be therapeutic value if it is established that altering plasma carotenoid levels can reduce neurotoxicity associated with brain Aβ, however these results do not provide evidence of any direct effect of carotenoids on Aβ deposition in the brain. As there is limited

data on the association between plasma Aβ and nutrients, future research should continue to investigate this relationship.

As neuropsychological tests to assess cognitive decline are more commonly used as the primary outcome for dementia progression, as opposed to cerebral or plasma Aβ, this measure was included in the current study. The plasma concentrations of nutrients were assessed in association with three outcomes of cognitive performance: 1. verbal, visual and episodic memory; 2. working memory and executive function, and 3. global cognitive performance. Only α-tocopherol was significantly positively correlated with a composite score of verbal, visual and episodic memory. Interestingly, episodic memory deficits are some of the first symptoms of AD, and have been shown in the preclinical AD phase [181, 182]. Previously, though non-significantly, higher α-tocopherol levels (measured in ventricular CSF) have been associated with better performance in various cognitive domains including episodic memory [183].

In this study, an association between plasma carotenoid levels and visual, verbal and episodic memory was determined within the whole cohort. It is likely that the early deficits in episodic memory in the high NAL group, (most probably un-noticeable by the individuals concerned) are likely to be driving this significant association. These results as well as the previous study findings [183], justifies further research into this association. No plasma nutrient levels were found to correlate with working memory and executive function. This was to be expected, as the current cohort includes individuals who are at the preclinical stages of AD, and controls who are at no apparent risk to AD, yet working memory and executive function decline are expected to arise later in the AD trajectory [184]. The small variation in these cognitive domains within the current cohort would hence not be significant enough to drive an association with carotenoids, and it is therefore unsurprising that there were no significant correlations with carotenoids or α-tocopherol in these domains. Lutein, β-carotene and α-tocopherol correlated significantly with global cognitive performance. Similar carotenoid results have been reported by Wang et al. [120] and similar vitamin E results by Morris et al. [109]. Antioxidants are commonly reported to correlate with cognitive decline, due to the interaction between cognitive decline and oxidative stress [185], however, this cognitive decline is often defined using a broad cognitive assessment such as the MMSE [120]. In the current study, participants were all screened to have an MMSE score ≥26, which is recognised as cognitively normal, however the significant associations revealed when a more sensitive outcome measure of global cognitive performance was used, speak to the importance of including multiple measures of cognitive performance when reporting on cognitive decline.

As carotenoids and vitamins have anti-inflammatory properties, the plasma nutrients were investigated in association with three markers of inflammation: TNF-α, IL-6 and CRP. There were no significant associations of the nutrients with the inflammatory markers, however, there was a trend towards an inverse relationship between 1. lutein and all three inflammatory markers, 2. β-cryptoxanthin and IL-6, and 3. lycopene, α-carotene and β-carotene with TNF-α and IL-6. Though non-significant, this trend suggests that these carotenoids may have anti-inflammatory potential. Since the detection of TNF-α and IL-6 were low, relative to the cohort sample size, there may have not been enough statistical power to determine a significant relationship. To conclusively determine if inflammation is influential in the interaction between nutrients and NAL, further investigation should include a larger sample size for the cytokine covariates.

This study may be limited in that it used plasma samples that were frozen at -80°C, stored for up-to-3 years and then thawed. Yet, a previous study's analysis of the stability of micronutrients including carotenoids and α-tocopherol at -70°C for multiple years (up-to 4.3 years) has determined that micronutrients were largely unaffected by this processing, and plasma concentrations remained near constant during this time [186]. It is therefore possible, though unlikely, that the plasma concentrations of carotenoids and α-tocopherol were compromised by storage.

However, it is likely that the plasma cytokine analyses were limited, in that plasma cytokines may have been partially degraded at the time of analysis. Cytokines remain stable in -80°C storage for up-to-2 years, however their stability decreases after this time, such that IL-6 has been shown to be at 50% of its baseline concentrations 2-3 years after storage [187]. In addition, the ELISA kit employed provided no controls for the plasma matrix. The plasma matrix can affect binding of the antibody to proteins by blockage with other substances and changes to the sample conditions including viscosity and pH. Controls could be useful in determining if there was a matrix effect. Further investigations involving shorter sample storage time, repetition of analyses using both serum and plasma, and alternate kit types are required.

Furthermore, cytokine concentrations were likely affected by medication use. The strict project inclusion/exclusion criteria ensured that the participants had no serious comorbid illnesses, however, as this cohort were of older age, there was a high level of medication use. Medications which involved an anti-inflammatory mechanism were controlled for, however this control was not dose-frequency or duration dependent, and therefore may not have encompassed the medication use adequately. Untreated inflammation is also more likely to be present in the elderly, simply due to the greater incidence of low-level chronic conditions in this cohort [188].

There were no significant associations between hemispheric hippocampal volume and plasma carotenoids or α-tocopherol. There was a trend, however, towards significance between higher lutein levels and higher hippocampal volume. Hippocampal volume is often used as a clinical diagnostic marker for AD, often in conjunction with MRI-determined global atrophy [23]. Since plasma analytes did not correlate with NAL, the lack of correlation with hippocampal volume is unsurprising. However, the Rotterdam study of cognitively normal older adults found that serum carotenoid levels were inversely associated with levels of less severe periventricular white matter lesions, particularly in smokers [189]. Since these white matter lesions may be involved in dementia pathology [189], further investigation into the association of carotenoids with neuropathological changes in preclinical cohorts would help clarify these results.

Longitudinal studies should also be carried out to determine whether individuals with high carotenoid levels have a slower rate of change in Aβ levels in the preclinical stages, indicating a therapeutic value in boosting dietary carotenoid or antioxidant supplement intake. A recent study has determined that saliva Aβ levels can distinguish between AD and control subjects with great accuracy [190]. Validation of these results will revolutionise the diagnosis of AD, and further studies are needed to characterise the changes in saliva Aβ levels during the transition from control to MCI to AD, and to correlate longitudinally with brain Aβ deposition levels. Following this, a study to determine whether there are any correlations between saliva Aβ levels and plasma (and/or saliva) carotenoid levels, would help determine whether longitudinal changes in Aβ levels correlate with changes in carotenoid levels.

Plasma carotenoid levels may change over time as a response to developing Aβ pathology or associated increased oxidative stress. They may be an indicator of future rate of AD pathogenesis, may reflect individual metabolic differences and/or other demands for antioxidants. They will also depend on, and be an indicator of, the overall quality of the diet and any supplements taken.

This study is limited in that is has a cross-sectional design. This enables investigation of one timepoint, of participants likely to develop and less likely to develop AD, yet it does not enable a longitudinal follow-up of these participants or investigation of the association of carotenoids and α-tocopherol with AD biomarkers over time. Other studies have captured longitudinal data but have not included brain Aβ as a measure of disease progression. Regarding protection against cognitive decline, studies have suggested an association with vitamin E [109], lutein [117] and a possible association with vitamin C [109], though there has also been a report of no association between plasma carotenoids and tocopherols with cognitive decline over ten years

[14]. Given the time-restraint of the Master of Research program, a longer project was not feasible, yet more informative data would be obtained from follow-up during MCI and AD.

A further limitation is the relatively small sample size of high NAL participants (n=35) compared with the moderately sized low NAL cohort (n=64). Perhaps more robust findings would have been determined in a larger cohort, as they were reported in previous studies [8, 13, 15, 121] To more thoroughly investigate the study aims in individuals at high likelihood of developing AD, future studies should recruit more participants with high NAL, albeit the challenge of finding individuals with advanced NAL and no cognitive impairment. In addition, *APOE* genotype has been shown to influence β-carotene and lutein metabolism in mice, such that it seems to mediate increased hepatic cleavage rather than intestinal carotenoid metabolism in *APOE* ε4 compared to ε3 [191]. However, carotenoid metabolism is varied in human and mice, such that in humans 40% of ingested dietary carotenoid have been reported to reach circulation, whereas in rodents, intestinal cleavage is far more efficient [191]. Repetition of the current study with a larger cohort would allow statistical evaluation of the *APOE* ε4 modulatory effect on all carotenoid correlations previously discussed.

5. Conclusion

There were minimal associations found between plasma carotenoids, in particular α-carotene and α-tocopherol levels, and cerebral Aβ levels. There appears to be an inverse association between plasma levels of carotenoids, particularly lutein and β-carotene, and plasma levels of Aβ. Further investigation is required to determine if this association is reproducible in other preclinical AD cohorts, and to determine if the carotenoids interact directly with these Aβ peptides or whether they influence Aβ peptide levels indirectly. This would help determine the potential therapeutic benefit of increasing carotenoid content in the diet to slow the development of AD pathological changes and cognitive decline.

6. References

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7. Appendix

Supplementary Table 1. Investigations of vitamin E supplementation in association with cognitive decline

Study	Participants	Antioxidants investigated	Key Findings
Dysken et al. [153]	613 mild to moderate AD	α-tocopherol (2000 IU daily) alone and combined with Memantine	After 4-year treatment, α-tocopherol slowed outcomes of clinical AD progression
Galasko et al. [154]	66 mild to moderate AD	α-tocopherol (8 IU daily) plus Vitamin C and α-lipoic acid, coenzyme Q3 or placebo	After 16-week treatment, combined vitamins E, C and α-lipoic acid treatment accelerated cognitive decline and decreased an oxidative stress marker by 19%; treatment type did not influence CSF $A\beta_{42}$, tau, or phosphorylated-tau
Kang et al. [158]	5 073 older women (by end of follow-up)	Vitamin E (600 IU every other day) or placebo	Vitamin E supplements were not associated with cognitive performance after up to 4 years. Vitamin E was associated with more favourable cognitive change in participants with lower dietary vitamin E, who exercised less frequently, and who didn't have diabetes
Lloret et al. [157]	33 AD 18 matched healthy controls	Vitamin E (800 IU daily)	After 6-months vitamin E treatment, oxidised glutathione (marker of oxidative stress) was reduced in 9/19 participants, and their cognitive ability was maintained. However, in 10/19 participants glutathione remained constant, yet cognitive function significantly decreased

Petersen et al. [155]	769 amnestic MCI	Vitamin E (2000 IU daily) or donepezil	During the 3-year trial, Vitamin E supplements had no effect on progression to AD or on cognition and function
Sano et al. [156]	3 141 moderate AD	α-tocopherol (2000 IU daily) alone and combined with Selegiline	After 2-years, α-tocopherol treatment (alone and when combined with Selegiline) delayed progression of AD, yet did not improve cognitive performance test scores

Supplementary table 1 shows the main publications investigating the association between vitamin E supplementation and cognition/dementia. Vitamin E is reported as having a protective effect, no effect and a detrimental effect on cognition. Abbreviations: AD, Alzheimer's disease; MCI, Mild cognitive impairment; CSF, cerebrospinal fluid.

Supplementary Table 2. Plasma levels of carotenoids and tocopherols obtained from select studies regarding the effect of carotenoids and vitamin E on cognition and Alzheimer's disease

Study	Participants	Plasma levels of investigated analytes
Akbaraly et al. [13]	589 older adults (age=73.55, range= 68-79)	Raw data not provided
Amadieu et al. [118]	666 older adults (age=73.3)	Incident dementia cases: lutein: 161.9 ± 84.3, zeaxanthin: 38.3 ± 26.1, β-cryptoxanthin: 150.9 ± 107.8, lycopene: 232.2 ± 150.9, α-carotene: 86.6 ± 64.4, β-carotene: 338.6 ± 226.0, α-tocopherol: 13.9 ± 3.6, γ-tocopherol: 0.6 ± 0.4, retinol: 502.2 ± 135.8, and 25(OH)D: 28.4 ± 13.1. No incident dementia cases: lutein: 168.1 ± 87.9, zeaxanthin: 40.2 ± 23.6, β-cryptoxanthin: 168.4 ± 127.7, lycopene: 274.9 ± 170.9, α-carotene: 99.0 ± 78.1, β-carotene: 407.2 ± 304.2, α-tocopherol: 13.4 ± 3.3, γ-tocopherol: 0.6 ± 0.3, retinol: 511.0 ± 145.3, and 25(OH)D: 36.3 ± 18.9. (Data are mean ± SD, carotenoids and retinol in μg/L, tocopherols in mg/L, 25(OH)D in nmol/L).
Devore et al. [116]	5 395 older adults (age=67.7, range=55+)	Only dietary data provided
Feart et al. [117]	1 092 older adults (age=74.4)	Incident dementia cases: α-carotene: 94 ± 78, β-carotene: 364 ± 273, lycopene: 227 ± 155, lutein: 153 ± 71, zeaxanthin: 37 ± 22, and β-cryptoxanthin: 155 ± 114. No incident dementia cases: α-carotene: 98 ± 84, β-carotene: 398 ± 315, lycopene: 240 ± 167, lutein: 166 ± 87, zeaxanthin: 40 ± 24, and β-cryptoxanthin: 167 ± 132. (Data are mean ± SD in μg/L)
Foy et al. [122]	79 AD 37 VaD 18 PDem 58 controls	Controls: α-carotene: 0.04, β-carotene: 0.31, lycopene: 0.09, vitamin A: 1.77, vitamin C: 41 and vitamin E: 25.6 AD: α-carotene: 0.04, β-carotene: 0.31, lycopene: 0.09, vitamin A: 1.41, vitamin C: 17 and vitamin E: 23.5

	(overall age median=73, range=62-85)	VaD: α-carotene: 0.03, β-carotene: 0.18, lycopene: 0.08, vitamin A: 1.20, vitamin C: 16 and vitamin E: 20.8 PDem: α-carotene: 0.02, β-carotene: 0.18, lycopene: 0, vitamin A: 1.47, vitamin C: 13 and vitamin E: 22.8. (Data are median in μmol/L)
Jiménez-Jiménez et al. [113]	38 AD 42 controls (age=70.95)	AD: α-carotene: 0.06 ± 0.06, β-carotene: 0.21 ± 0.14 and retinol: 1.60 ± 0.4 Controls: α-carotene: 0.07 ± 0.06, β-carotene: 0.32 ± 0.26, and retinol: 1.89 ± 0.53. (Data are mean ± SD in μmol/L).
Johnson et al. [11]	78 octogenarians (age=84.2, range=80-89), 220 centenarians (age=100.4, range=98+)	Octogenarians: Lutein: 316 ± 255, zeaxanthin: 53 ± 32, cryptoxanthin: 159 ± 105, α-carotene: 80 ± 110, β-carotene: 568 ± 855, lycopene: 629 ± 443, α-tocopherol: 31.9 ± 16.8 and retinol: 2.14 ± 0.07. Centenarians: Lutein: 199 ± 177, zeaxanthin: 49 ± 37, cryptoxanthin: 148 ± 125, α-carotene: 63 ± 61, β-carotene: 460 ± 432, lycopene: 369 ± 337, α-tocopherol: 25.5 ± 12.9 and retinol: 1.84 ± 0.62. (Data are mean ± SD, carotenoids in nmol/L, α-tocopherol and retinol in μmol/L).
Kang et al. [14]	858 older women (age=65)	Retinol: 621, carotenoids: 1122, tocopherol: 15779. (Data are mean in µg/L)
Lindbergh et al. [115]	43 older adults (age=72, range=65-86)	Lutein and zeaxanthin: 0.31 ± 0.17 (Data are mean ± SD in μmol/L)
Min et al. [121]	6 958 older adults (25% aged 50-59, 32% aged 60-69, 25% aged 70-79, 17% 80-89, 2% ≥90)	With AD mortality: Lutein/zeaxanthin: 24.63 ± 0.37, β-cryptoxanthin: 9.87 ± 0.23, α-carotene: 5.51 ± 0.13, β-carotene: 25.30 ± 0.58, and lycopene: 20.07 ± 0.34 Without AD mortality: Lutein/zeaxanthin: 19.33 ± 1.24, β-cryptoxanthin: 8.49 ± 0.76, α-carotene: 5.37 ± 0.38, β-carotene: 24.33 ± 2.03, and lycopene: 15.41 ± 1.00. (Data are mean ± SE in µg/dL).

Mullan et al. [8]	251 AD 308 controls (age=78.1, range=65+)	AD: Lutein: 42.5 ± 25, zeaxanthin: 10.3 ± 5.54, β-cryptoxanthin: 11.9 ± 10.0, α-carotene: 19.6 ± 15.6, β-carotene: 103 ± 74.9, retinol: 1.44 ± 0.47, lycopene: 0.32 ± 0.43, α-tocopherol: 16.0 ± 5.3, γ-tocopherol: 4.92 ± 2.32 Controls: Lutein: 53.3 ± 30.2, zeaxanthin: 13.7 ± 7.65, β-cryptoxanthin: 17.8 ± 14.3, α-carotene: 26.9 ± 20.9, β-carotene: 139 ± 105, retinol: 1.57 ± 0.78, lycopene: 0.54 ± 0.60, α-tocopherol: 19.1 ± 10.2, γ-tocopherol: 4.14 ± 1.99. (All data mean ± SD, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene in mmol/L, retinol, tocopherols and lycopene in μmol/L).
Polidori et al. [112]	63 AD 23 VaD 55 controls (age=77)	AD: Vitamin A: 2.0 ± 0.38, vitamin C: 25.9 ± 8.9, vitamin E: 37.8 ± 5.8, lutein: 0.36 ± 0.18, zeaxanthin: 0.06 ± 0.02, lycopene: 0.61 ± 0.22, β-cryptoxanthin: 0.17 ± 0.14, α-carotene: 0.06 ± 0.03, β-carotene: 0.57 ± 0.28. VaD: Vitamin A: 2.0 ± 0.37, vitamin C: 26.6 ± 11.3, vitamin E: 36.4 ± 4.7, lutein: 0.34 ± 0.1, zeaxanthin: 0.07 ± 0.05, lycopene: 0.61 ± 0.23, β-cryptoxanthin: 0.17 ± 0.12, α-carotene: 0.06 ± 0.03, β-carotene: 0.53 ± 0.27. Controls: Vitamin A: 2.7 ± 0.3, vitamin C: 52.4 ± 16.4, vitamin E: 50.2 ± 10.2, lutein: 0.67 ± 0.35, zeaxanthin: 0.15 ± 0.08, lycopene: 0.78 ± 0.36, β-cryptoxanthin: 0.41 ± 0.42, α-carotene: 0.12 ± 0.12, β-carotene: 0.55 ± 0.34. (Data are mean ± SD in μmol/L).
Rinaldi et al. [123]	25 MCI 63 AD 56 controls (age=76)	MCI: Vitamin A: 2.3 ± 0.2, vitamin C: 24.9 ± 2.4, vitamin E: 41.0 ± 5.0, lutein: 0.51 ± 0.18, zeaxanthin: 0.11 ± 0.04, lycopene: 0.84 ± 0.38, β-cryptoxanthin: 0.45 ± 0.36, α-carotene: 0.063 ± 0.07, β-carotene: 0.56 ± 0.29. AD: Vitamin A: 2.1 ± 0.4, vitamin C: 25.9 ± 8.9, vitamin E: 37.7 ± 5.8, lutein: 0.37 ± 0.19, zeaxanthin: 0.06 ± 0.03, lycopene: 0.62 ± 0.23, β-cryptoxanthin: 0.17 ± 0.14, α-carotene: 0.065 ± 0.03, β-carotene: 0.59 ± 0.28.

		Controls: Vitamin A: 2.6 ± 0.3, vitamin C: 52.4 ± 16.5, vitamin E: 50.2 ± 10.2, lutein: 0.69 ± 0.34, zeaxanthin: 0.16 ± 0.08, lycopene: 0.79 ± 0.36, β-cryptoxanthin: 0.43 ± 0.42, α-carotene: 0.13 ± 0.12, β-carotene: 0.57 ± 0.33. (Data are mean ± SD in μmol/L).
Schmidt et al. [15]	1769 middle-aged and older adults (age= 62, range=50-75)	Lutein/zeaxanthin: 0.56 ± 0.24, cryptoxanthin: 0.25 ± 0.23, canthaxanthin: 0.12 ± 0.08, lycopene: 0.20 ± 0.17, α-carotene: 0.09 ± 0.11, β-carotene: 0.49 ± 0.45, retinol: 1.93 ± 0.74, α-tocopherol: 30.57 ± 10.43, γ-tocopherol: 2.45 ± 1.27, and ascorbate: 56.57 ± 20.27. (Data are mean ± SD in μmol/L).
Wang et al. [120]	13 mild AD 13 moderate AD 10 severe AD 10 controls (age=74.25)	Mild AD: Lutein: 15.47 ± 5.38, zeaxanthin: 2.79 ± 1.55, β-cryptoxanthin: 10.88 ± 10.61, α-carotene: 9.19 ± 7.51, β-carotene: 40.22 ± 22.58, and lycopene: 34.24 ± 16.11. Moderate AD: Lutein: 17.97 ± 9.91, zeaxanthin: 3.50 ± 2.39, β-cryptoxanthin: 10.91 ±7.95, α-carotene: 6.64 ± 5.81, β-carotene: 26.30 ± 27.41, and lycopene: 34.49 ± 27.33. Severe AD: Lutein: 9.78 ± 5.15, zeaxanthin: 2.40 ± 1.76, β-cryptoxanthin: 8.76 ± 8.39, α-carotene: 5.12 ± 4.34, β-carotene: 16.83 ± 11.21, and lycopene: 31.23 ± 8.46. Control: Lutein: 14.41 ± 8.03, zeaxanthin: 2.24 ± 1.37, β-cryptoxanthin: 8.02 ± 7.01, α-carotene: 7.16 ± 6.31, β-carotene: 35.13 ± 27.40, and lycopene: 31.34 ± 15.85. (Data are log-transformed mean ± SD in μg/dL).

Supplementary Table 2 shows the plasma levels of analytes from publications which investigated the association between carotenoids and vitamin E with cognition. Abbreviations: AD, Alzheimer's disease; VaD, Vascular dementia; PDem, Parkinson's disease and dementia; SD, Standard deviation. Age given as mean, unless otherwise specified. Range given if provided in original article.