The effects of docosahexaenoic acid combined with supporting nutrients and physical activity on mobility and cognitive function in older women

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August 2021

Abstract

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There is a complex interplay between mobility and cognition in older adults. It has previously been shown that a high DHA multi-nutrient supplement improves habitual walking speed, verbal memory, and psychomotor response latency in older women. Exercise also improves mobility and cognition in older adults, and omega-3 fatty acids and exercise share a range of overlapping biological effects. The primary aim of this thesis was to conduct a randomised semi-blinded placebo controlled study exploring the effects of a high DHA multi-nutrient supplement and aerobic exercise, alone and in combination, on mobility and cognition in older women.

One the novel aspects of the research conducted within the thesis is the use of a multi-nutrient-intervention which includes both omega-3 polyunsaturated fatty acids (PUFA) and B vitamins. There is now evidence to suggest that there may be an interaction between B vitamins and omega-3 polyunsaturated fatty acids, with suggestions that optimising intake of both nutrients being key to eliciting benefits to cognition within older adults. As such, a systematic review was conducted to investigate whether supplementation with a combination of omega-3 PUFAs and B vitamins can prevent cognitive decline in older adults. Randomised control trials conducted in older adults that measured cognitive function were retrieved. The included trials provided a combination of omega-3 PUFAs and B vitamins alone, or in combination with other nutrients and trials which tested for interactions between omega-3 PUFAs and B vitamins by providing omega-3 PUFA alone and also measuring B vitamin status or provided B vitamin supplementation alone and measured omega-3 PUFA status. The databases searched were The Cochrane Library, EMBASE, CINAHL, Scopus, and MEDLINE. A total of 14 papers were included in the analysis (n=4913; age: 60-70 y; follow up 24 weeks to 4 years). The meta-analysis results found a significant benefit of nutrient formulas, which included both omega-3 PUFAs and B vitamins, versus placebo on global cognition assessed using composite scores from a neuropsychological test battery (G=0.23, p=0.002), global cognition using single measures of cognition (G=0.28, p=0.004) and episodic memory (G=0.32, p=0.001). The results indicate that providing a combination of omega-3 PUFA and B vitamins benefits cognition in older adults versus a placebo, the potential for an interaction between these key nutrients should be considered in future experimental work.

The first aim of the program of experimental work was to assess feasibility and establish appropriate methods for testing of cognitive function and mobility. These aims were addressed through a series of pilot and feasibility studies. Five young healthy adults (mean age 29 y, SD 3) and four healthy older adults (mean age 70 y SD 8) were taken through a testing battery which

consisted of tests of both gait and cognitive function. Gait testing was performed using opal inertial sensors and was conducted under single and dual task conditions. Two different dual-task scenarios were used, a 3-back and 7-back condition. Cognitive testing consisted of assessment of verbal memory, spatial working memory, executive function and interference control. There was evidence for floor effects of the 7-back dual task scenario as such only the 3-back condition was taken forward into the subsequent clinical trial. There was no evidence of floor or ceiling effects in the cognitive testing and thus no adjustments were made to the difficulty of these tasks. In conclusion this pilot/feasibility study allowed the researcher to familairise themselves with the testing protocols, established timings and protocols for clinical visits and allowed for the selection of mobility and cognitive testing that were at an appropriate level of difficulty for the target population.

The second phases for the research process was to conduct a pilot study to establish a reliable method for assessing whole blood fatty acids on the research site. Five blood samples were taken from the fingertip of a volunteer from a single puncture using an automated lancet. Blood spots were collected on filter paper which had been pre-treated with 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene, BHT) diluted in ethanol at 2 mg/ml. Fatty acid methyl esters (FAME) were extracted from these blood samples. FAMES were quantified using a gas chromatograph with flame ionisation detector (GC-FID). The coefficient of variation for each fatty acid was calculated. To be deemed reliable a coefficient of variation of <10% for the main fatty acids of interest, EPA, DHA and AA and <15% for all other measured fatty acids would need to be achieved. The coefficient of variance for EPA, DHA and AA was 5.1%, 7.2% and 9.63% respectively, all other fatty acids were <15%. In conclusion, this method was successfully established and a standard operating procedure was produced for the research site. Results indicate that the method used, storage conditions, sample handling and user proficiency are reliable and can produce consistent results.

To address the primary aim of thesis a randomised semi-blinded placebo controlled study was conducted Women (mean age 67 y, SD 8) were assigned to the following groups: multi-nutrient (1 g DHA, 160 mg EPA, 240 mg *Ginkgo biloba*, 60 mg phosphatidylserine, 20 mg d-α tocopherol, 1 mg folic acid, and 20 µg vitamin B12 per day, N=13), multi-nutrient and exercise (spin class twice per week, N=14), exercise and placebo (N=12), or placebo (N=12). The multi-nutrient was given for 24 weeks, and exercise for 12 weeks. Trial registration: NCT03228550.

Baseline correlational analysis performed on mobility outcomes revealed a significant positive relationship between whole blood DHA and dual-task gait speed (R=0.318 p=0.018) and dual task effect on gait speed (R=0.312 p=0.019). No other relationships were identified for EPA, DHA:AA or serum homocysteine with any other mobility outcome. Following the post intervention period no treatment effects were observed for the primary outcome, habitual

walking speed. Improvements in verbal memory and executive function were seen for all treatments groups versus placebo (all, $p<0.05$). Significant improvements in self-reported emotional wellbeing were seen with multi-nutrient and exercise groups versus placebo ($p=0.03$). Per-protocol analysis revealed benefits of aerobic exercise on habitual and fast walking gait speed and the combined multi-nutrient supplement and aerobic exercise lead to improved time on the five times sit to stand test (all, $p<0.05$).

The results suggest that a high DHA multi-nutrient supplement and aerobic exercise produce similar improvements in cognitive function to aerobic exercise in healthy older adults. It was not possible to establish whether combining the interventions lead to additive or synergistic benefits. Combining the multi-nutrient supplement with aerobic exercise did elicit positive effects on emotional wellbeing and ability to rise from a chair, which were not observed for each intervention separately, offering the intriguing prospect that the combination of a high DHA multi-nutrient supplement and exercise may have a broader impact across multiple healthy ageing outcomes.

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List of Abbreviations

5, 10 Methylenetetrahydrofolate (5, 10-methylene THF)

5-Methyltetrahydrofolate (5-MTHF)

Alpha Linolenic Acid (ALA)

Alzheimer's Disease (AD)

Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-cog)

Arachidonic Acid (AA)

Body Mass Index (BMI)

Bovine Serum Albumin (BSA)

Brain Derived Neurotrophic Factor (BDNF)

Butylated Hydroxytoluene (BHT)

Consolidated Standards of Reporting Trials (CONSORT)

Cystathionine Beta Synthase (CBS)

Docosahexaenoic Acid (DHA)

Docosapentaenoic Acid (DPA)

Dual Task Effect (DTE)

Eicosapentaenoic Acid (EPA)

Enzyme-Linked Immunosorbent Assay (ELISA)

Fatty Acid Methyl Esters (FAME)

Five Times Sit to Stand (FTSTS)

Flame Ionisation Detector (FID)

Food Frequency Questionnaire (FFQ)

Gas Chromatograph with Flame Ionisation Detector (GC-FID)

Habitual Gait Speed (HGS)

Hazard Ratio (HR)

Interleukin (IL)

Linoleic Acid (LA)

Methionine Synthase (MSy)

Methylated (ME)

Methylenetetrahydrofolate Reductase (MTHFR)

Mild Cognitive Impairment (MCI)

Mini Mental State Examination (MMSE)

Multi-nutrient Supplement Group (MS)

Multi-nutrient Supplement and Exercise Group (MS+EX)

Muscle Protein Synthesis (MPS)

National Adult Reading Test (NART)

National Diet and Nutrition Survey (NDNS)

Omega-3 Polyunsaturated Fatty Acids (omega-3 PUFA)

Peroxisome Proliferator-Activated Receptor Gamma (PPARγ)

Phosphate Buffered Saline (PBS)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylethanolamine Methyltransferase (PEMT)

Phosphatidylserine (PS)

Phosphatidylserine Synthase (PSS)

Phosphatidylserine Synthase 1 (PSS1)

Phosphatidylserine Synthase 2 (PSS2)

Physical Activity Scale in the Elderly (PASE)

Placebo Group (P)

Placebo and Exercise Group (P+EX)

Randomised Control Trial (RCT)

Rey's Auditory Verbal Learning Test (RAVLT)

S-Adenosylhomocysteine (SAH)

S-Adenosylmethionine (SAM)

Scientific Advisory Committee on Nutrition (SACN)

Standard Form 36 Questionnaire (SF-36)

Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT)

Template for Intervention Description and Replication (TIDieR)

Tetrahydrofolate (THF)

The Community Health Activities Program for Seniors (CHAMPS)

World Health Organisation (WHO)

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet the requirements for an award at this or any other higher education institution. To the best of my knowledge and belief this thesis contains no material previously written by another person except where due reference is made.

Paul Fairbairn

August, 2021

Preface

An occupational workplace health and safety assessment was undertaken for this project. Referencing was completed in accordance with Bournemouth University Harvard style throughout the thesis. Parts of this project have been already been disseminated.

Publications

Fairbairn, P., Tsofliou, F., Johnson, A. & Dyall, S. C., 2020. Effects of a high DHA multi-nutrient supplement and exercise on mobility and cognition in older women (MOBILE): A randomised semi-blinded placebo controlled study. *British Journal of Nutrition*, 124, 2.

Fairbairn, P., Tsofliou, F., Johnson, A. & Dyall, S. C., 2019. Combining a high DHA multi-nutrient supplement with aerobic exercise: Protocol for a randomised controlled study assessing mobility and cognitive function in older women. *Prostaglandins Leukot Essent Fatty Acids*, 143, 21-30.

Conference Proceedings

Fairbairn, P. & Tsofliou, F., 2021 The combined effects of Omega-3 Polyunsaturated Fatty Acids and B vitamins on Cognition in the older adult: A Systematic Review and Meta-analysis. *The Nutrition Society Summer Conference: Nutrition in a Changing World*. July 2021, Southampton, United Kingdom. (Appendix 1)

Fairbairn, P., Tsofliou, F., Johnson, A. & Dyall, S. C., 2018 Preliminary analysis suggests a high DHA multi-nutrient supplementation and aerobic exercise produce similar improvements in verbal memory in older females. *13th International Society for the Study of Fatty Acids and Lipids Congress*. May 2018, Las Vegas, United States of America. (Appendix 2)

Fairbairn, P., Tsofliou, F., Johnson, A. & Dyall, S. C., 2018 Circulating DHA levels as a predictor of gait performance under single and dual-task conditions in older females. *13th International Society for the Study of Fatty Acids and Lipids Congress*. May 2018, Las Vegas, United States of America. (Appendix 3)

Fairbairn, P., Tsofliou and Dyall, S.C., 2017. The relationship between circulating omega-3 polyunsaturated fatty acids, cognitive function and dual task gait speed is different in non-frail and pre-frail older women. *In: Lipids and Brain IV* 8-11 October 2017 Nancy, France. (Appendix 4)

Fairbairn, P., Tsofliou, F., Johnson, A. & Dyall, S. C., 2017. A Feasibility Study to Develop Dual Task Gait and Cognitive Testing Protocols in Women Aged ≥60 years. *Bournemouth University Postgraduate Conference* April 2017 Bournemouth, United Kingdom (Appendix 5)

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Dr Fotini Tsofliou for her continuous support throughout my PhD study and related research, for her patience, motivation, and immense knowledge. Her guidance helped me throughout the research and writing of this thesis.

Besides my supervisor, I would also like to thank Dr. Andrew Johnson, Dr Tom Wainwright and Dr. James Gavin for their assistance in the design and execution of my research.

I would like to thank Isabell Nessel for her support and training within the laboratory, as without her patience and expertise my research would not have been possible.

My sincere thanks also go to all participants who volunteered their time, and occasionally their blood, to take part in my research. It was a genuine pleasure to meet and work with all the volunteers.

To all my friends and family I thank you for your support, belief and encouragement. Finally, thank you to Beth Dyer, whose love, support and understanding, and imperturbability helped me remain on a steady pathway to completion.

Funding for my PhD studentship came from Efamol Ltd. and Bournemouth University and grants from the Sylvia Waddilove Foundation Trust.

Chapter 1- Introduction and Literature Review

1.1 Healthy Ageing

In Europe, the proportion of adults aged 65 years and over is expected to rise from 16.1 to 22% by 2031 (Soong et al. 2015). Age related health conditions have been estimated to account for 51.3% of all global burden of disease among adults, based on the sum of all disability-adjusted life-years across 92 identified age related diseases (Chang et al. 2019). Given that population health care spending increases with the proportion of older adults, it is expected that the trend towards an ageing population will have profound implications for the health care systems for decades to come. The UK spent £153 billion on health care in 2013 (Office for National Statistics 2015). The Office for Budget Responsibility has indicated that health care costs are projected to rise from 7.6% of gross domestic product to 13.8% by 2065 with the ageing population being identified as a major driving factor behind this increased spending (Office for Budget Responsibility 2018). Ageing is associated with a progressive decline across multiple health domains with mobility and cognitive function being of particular importance to the older adult. Reduced cognitive function and mobility can lead to a number of age related health conditions including frailty, cognitive impairment and sarcopenia (Cesari et al. 2014). Whilst it is expected to observe some decline in mobility and cognitive function with age (Ko et al. 2010; Harada et al. 2013), early interventions aimed at reducing the trajectory of this decline and promoting what is referred to as “healthy” or “successful” ageing or the healthy ageing phenotype (HAP) (Lara et al. 2013) **(**Figure 1**)** gained interest within the literature as we seek to develop methods to enhance health and quality of life in an ageing population.

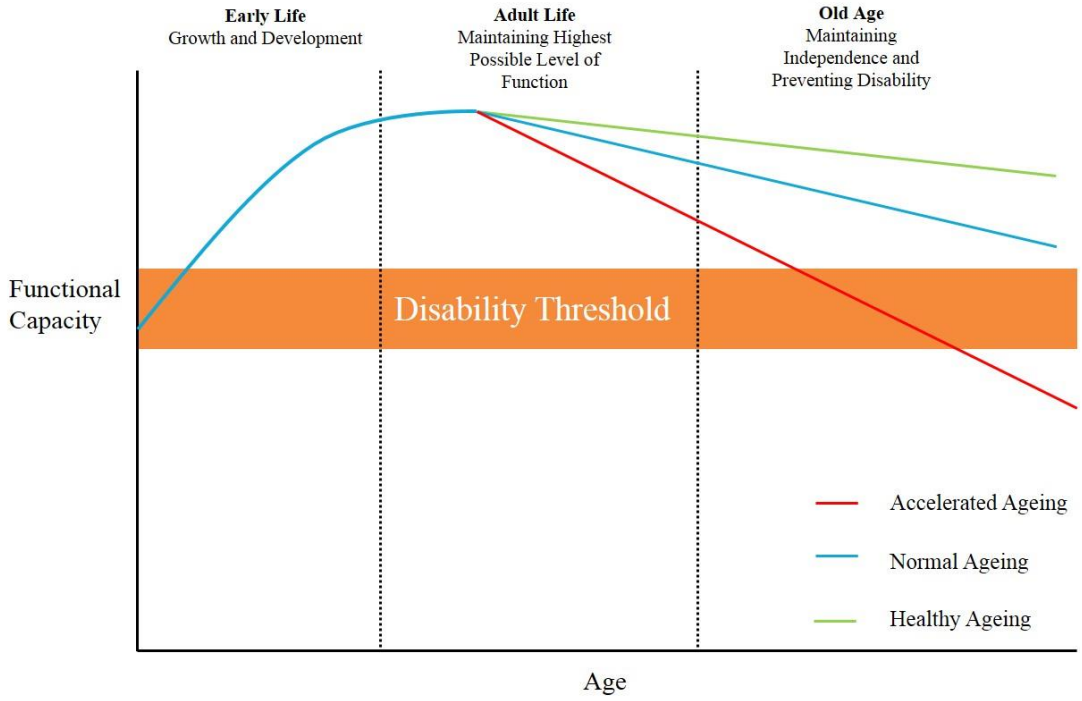


Figure 1 *A display of ageing trajectories adapted from Kalache and Kickbusch (1997). Functional capacity peaks during adulthood and then starts to decline into old age. The trajectory of this decline can be influenced by a number of factors including diet and exercise. Accelerated ageing has a steep decline and leads to functional disability. Even those who age successfully do experience some decline in functional capacity, but are able to maintain their independence late into old age.*

The HAP is defined as having highly preserved metabolic, hormonal and neuroendocrine function as well as having a high degree of physiological functioning into old age (Franco et al. 2009). Trajectory towards the HAP would be characterised by the ability to adapt to daily environmental stressors, which translates into the absence of age related health conditions and the maintenance of important aspects of human functioning, including mobility and cognitive function (Lara et al. 2013). The HAP presents an advantageous target for intervention by targeting high functioning older adults, with the aim of maintaining their health. A trajectory towards the HAP would allow individuals to maintain their ability to perform activities of daily living such as personal hygiene, dressing, and feeding, as well as instrumental activities of daily living such as preparing meals, shopping and managing medications (Cooper et al. 2011; Mlinac and Feng 2016). By maintaining their ability to conduct these activities older people are more likely to maintain their independence and have a greater quality of life, thus reducing the burden upon the health care systems (Millan-Calenti et al. 2010).

Given the complexity, and the heterogeneity, of the ageing process, it is highly unlikely that any single measure will be capable of encapsulating healthy ageing in any individual. Therefore,

intervention studies, especially those with lifestyle interventions, should include a range of measures focusing on cognition, mobility, biochemical outcomes and quality of life (Lara et al. 2015) (*Figure 2*)

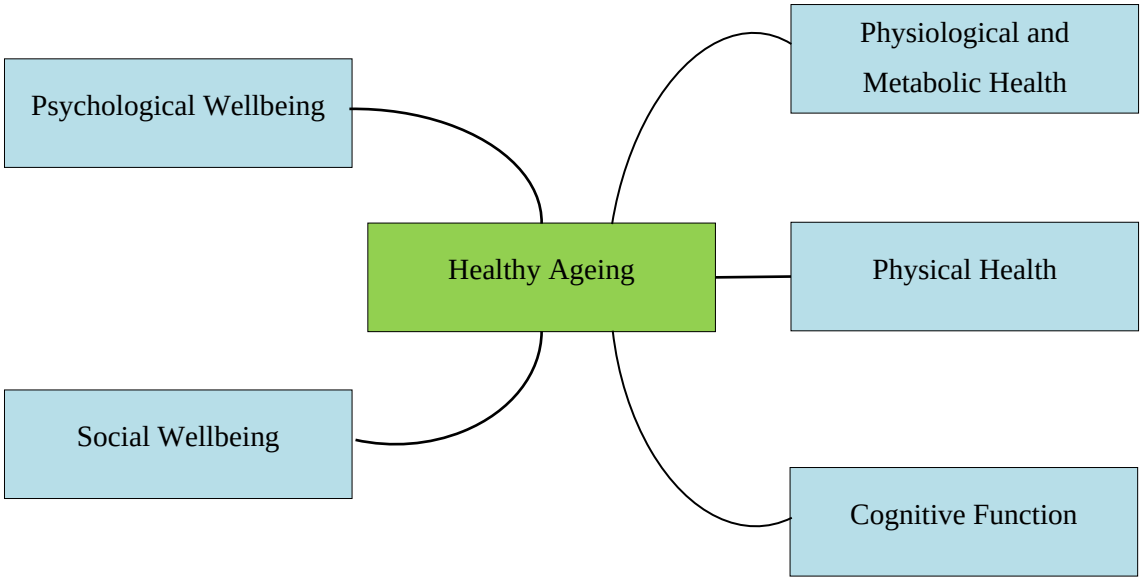


Figure 2 *Diagram of the multiple health domains that contribute towards the healthy ageing phenotype. To fully encapsulate the effects of any intervention on preventing age related decline and promoting healthy ageing, it is important to consider a number of domains which can impact the health and quality of life of older adults. These include psychological wellbeing, social wellbeing, physiological and metabolic health, physical health and cognitive function. Amended from Lara et al. (2013).*

Previous intervention studies have largely focused on either physical or cognitive endpoints in older adults, therefore, limiting the ability to assess the impact on healthy ageing as a whole. The emergence of the HAP has clearly highlighted the requirement for multicomponent outcomes, thus this PhD thesis has utilised measures of cognition and mobility as well as biochemical outcomes and health related quality of life to assess the effectiveness of a dietary supplement and exercise in older women.

1.2 Physical and Cognitive Decline in Healthy Ageing

Mobility and cognitive function are two key functional domains upon which preventative strategies should be targeted towards older adults (Davis et al. 2015). Reduced mobility and cognitive function are both common in older adults and are independent risk factors for morbidity, disability, and mortality (Aguero-Torres et al. 2002; Sachs et al. 2011; Studenski et al. 2011; Perera et al. 2016).

1.2.1 Cognitive Function In Old Age

It has been estimated that in the year 2015, 688, 300 people in England had dementia with 6.7% of older adults having the condition (Wittenberg et al. 2019). Due to the insidious onset, cognitive impairment often goes unnoticed for several years, with clinical diagnosis being made late into the disease progression, thus it is likely that a much higher number of older adults are currently living with some form of cognitive impairment (Bradford et al. 2009). Cognition is critical for functional independence as people age, including whether someone can live independently, manage finances, take medications correctly, and drive safely. In addition, intact cognition is vital for humans to communicate effectively, including processing and integrating sensory information and responding appropriately to others (Murman 2015).

The human brain grows and develops until it reaches its peak in the third to fourth decade of life, after which volumetric declines occur (Raz et al. 2005). The neural atrophy that proceeds following the peak in development is not linear or regionally uniform (Buckner 2004; Raz et al. 2005) and by the age of 60 years regionally specific brain tissues loss has been detected in both grey and white matter (Resnick et al. 2000). While there is inter-individual variability in the rate of age related brain atrophy, the prefrontal cortex, parietal and medial temporal lobes in particular have been demonstrated to show higher rates of atrophy in healthy older adults (Raz et al. 2005), indicating that these regions may be of particular importance when we consider interventions to promote a healthy ageing trajectory. Despite these documented changes in brain physiology with ageing, the extent to which these changes translate to changes in cognition is contentious. Cognitive function is a complex behaviour and the relationship between individual brain regions and specific cognitive domains is still being established.

Several cognitive domains have been demonstrated to be impacted even during a healthy ageing trajectory including memory, visuospatial ability, executive function and interference control (Harada et al. 2013). These domains are commonly placed under the umbrella term of fluid cognitive abilities or fluid intelligence, which refers to an individual's capacity to process and integrate information, act, and solve novel problems (McDonough et al. 2016). Whilst the domains associated with fluid intelligence appear to be susceptible to age related decline crystallised abilities that are reliant upon skills, ability, and knowledge that is learned, well-

practised, and familiar for example vocabulary and general knowledge appear to be more resilient to ageing (McDonough et al. 2016).

The most widely seen cognitive change associated with ageing is that of memory. Memory function can be broadly divided into four sections, episodic memory, semantic memory, procedural memory, and working memory. Studies have proposed that episodic memory, which is defined as the ability to recall and mentally re-experience specific episodes from one's personal past, as the most robust predictor of pathological cognitive ageing. The decline in episodic memory is greater than that associated with the normal age-related processes and also detectable 7–15 years before diagnosis of AD (Mortamais et al. 2017). As well as changes in episodic memory declining executive function has also emerged as an important cognitive domain with deficits being detected up to 18 years prior to dementia diagnosis (Rajan et al. 2015). When we consider that the main principles behind healthy ageing are preservation of function and absence of disease (Lara et al. 2013) these two cognitive domains may be of particular importance when assessing the effectiveness of interventions to promote healthy ageing.

Whilst the literature within the area of normal versus pathological cognitive ageing provides some valuable insight into the regions of the brain and cognitive domains that are impacted during the ageing process there some consistent limitations. Firstly it has been well established that it is difficult to clearly separate participants into non-pathological or pathological categories with regards to cognition (Edmonds et al. 2016). Often this categorisation is performed through brief tests such as the MMSE and/or by the absence of a clinical diagnosis, and sometimes by exclusion using a standardised psychiatric interview (Mortamais et al. 2017). There are concerns that this form of testing is not always sensitive enough to pick up milder forms of dementia and thus leading to the misclassification of participants. Secondly, there is a wide variety in the specific tests and testing batteries that are being used to measure cognitive function, as well as methods for scoring and calculating composite scores. Taken together with the large inter-individual differences in rates of cognitive decline in healthy adults (Harada et al. 2013), this makes it difficult to establish normative data for the decline within a specific cognitive domain or apply normative data from test battery to another.

Overall, it is clear that cognitive decline occurs within healthy ageing across multiple domains, particularly amongst fluid cognitive abilities. Deficits in episodic memory and executive functioning are perhaps of particular importance with regards to predicting future risk of progression to dementia.

1.2.2 Physical Function and Mobility in Old Age

Physical function and mobility are key hallmarks of healthy ageing. Mobility is necessary for accessing commodities, making use of neighbourhood facilities, and participation in meaningful social, cultural, and physical activities (Ferrucci et al. 2016). Two conditions that are strongly linked to mobility and physical function within older adults are frailty and sarcopenia.

Frailty is a clinically recognised condition of increased vulnerability to poor resolution of homeostasis following stress, which increases the risk of adverse outcomes including falls, delirium and disability (Clegg et al. 2013). Frailty is most commonly defined using the Fried frailty criteria (Fried et al. 2001), or the frailty index (Mitnitski et al. 2001). The Fried criteria primarily focuses on physical function which includes low muscle strength, self-reported exhaustion, slow gait speed, low levels of physical activity, and unintentional weight loss. Meeting three or more of these criteria would classify an individual as frail, meeting 1-2 would be pre-frail (a subset at high risk of progressing to frailty) and 0 non-frail. Whilst frailty measurements do largely focus on physical function, the frailty index assesses a wider variety of health domains and is determined by counting the number of deficits accumulated over time including disability, diseases, physical and cognitive impairments, psychosocial risk factors, and geriatric syndromes. Data from the English longitudinal study of ageing has estimated that the prevalence of frailty amongst the population aged 60 years greater, assessed using the Fried frailty criteria, is 14% (Gale et al. 2015).

Sarcopenia is a clinically recognised condition characterised by a loss of both muscle quantity and quality, which leaves an individual with increased susceptibility for falls, fractures, hospitalisation, morbidity/mortality and reduced quality of life and is a contributing factor towards the development of physical frailty (Cruz-Jentoft et al. 2010; Cruz-Jentoft et al. 2019). Recently the European Working Group on Sarcopenia in Older People updated their guidelines for the cut-off points for assessing sarcopenia and recommended that low muscle strength be used as the primary parameter of sarcopenia (Cruz-Jentoft et al. 2019). Some concerns have been raised regarding this recent change in criteria with the new guidelines on assessment leading to reduced prevalence statistics for sarcopenia, which could, in turn, lead to a lack of identification of those who still may be at risk of adverse health outcomes (Phu et al. 2019; Reiss et al. 2019), with one study in participants from the UK Biobank cohort showing a drop in prevalence from 8.14% to 0.36% when comparing the 2010 to 2019 criteria (Petermann-Rocha et al. 2020). This change in the definition does present some challenges in terms of accurately assessing the prevalence of sarcopenia in older adults, although previous cohort studies based on the 2010 criteria have suggested that 4.6% and 7.9% of older community-dwelling men and women in the U.K. respectively are affected (Patel et al. 2013).

1.2.3 Cognitive Frailty - The Link between Cognition and Mobility within Older Adults

It is clear that declining physical and cognitive function are two key areas of concern for older adults. In addition, there is now emerging evidence that the two domains are linked with changes in grip strength and gait speed being associated with cognitive function in older adults in longitudinal ageing cohorts (Clouston et al. 2013; Zammit et al. 2019). This apparent link between the physical and cognitive domains has contributed to the emerging entity of cognitive frailty, defined as a heterogeneous clinical manifestation characterised by the simultaneous presence of both physical frailty and cognitive impairment defined as having a clinical dementia rating of 0.5 indicating very mild dementia (Kelaiditi et al. 2013).

The relationship between cognition and physical function could be mediated through multiple different mechanisms. Firstly human movement requires the coordinated integration of widespread brain regions, which are also involved in higher level cognitive tasks (Rosano et al. 2012; Rosso et al. 2013), therefore damage to the brain through age related conditions such as AD could simultaneously present in deficits to both cognitive function and mobility. This downstream effect on physical function as a result of pathology within the brain is referred to as the brain-driven hypothesis, and it is supported by data that has found that common brain pathologies including AD pathology, macroinfarcts and nigral neuronal loss can predict progression of physical frailty and slow gait speed (Buchman et al. 2013; Del Campo et al. 2016). The other overarching hypothesis is referred to as the body-driven hypothesis, which states that the presence of one or multiple chronic diseases could result in deficits in both cognition and physical function. For example cardiovascular and respiratory risk factors, as well as insulin resistance and type 2 diabetes mellitus, have all been associated with cognitive function, and physical frailty in older adults (Gottesman et al. 2017; Kleipool et al. 2018; Xue et al. 2019). Furthermore, these chronic conditions may exacerbate the symptoms from brain-driven conditions such as AD. Data from 456 older adults, measured post mortem, found a significant interaction between frailty index score as a marker of systemic burden and AD pathology for predication of AD diagnosis (Wallace et al. 2019). The data indicated that those with less frailty were better able to tolerate AD pathology, whereas those who were frailer were more likely to have diagnosable AD symptoms. This relationship between systemic burden and cognitive symptoms could explain partially why some older adults with high levels of AD pathology show only mild or no cognitive impairment at all (Aizenstein et al. 2008). An important distinction is that these two hypotheses that underpin the link between cognition and mobility are not mutually exclusive. Multi-morbidity, defined as having at least two chronic conditions, is common in older adults with some evidence suggesting prevalence could be as high as 88.6% in those aged 60 years and older (Calderón-Larrañaga et al. 2017). As a result

both brain-driven and body-driven declines in mobility and cognition could occur simultaneously.

1.2.4 The Biological link between Cognitive and Physical Decline in Older Adults

Cognitive decline and physical frailty are both heterogeneous conditions, for which the underlying causes are multifactorial and involve multiple systems. Although some research has been conducted on the dynamic association between physical function and cognitive impairment, there is still no comprehensive list of biomarkers for cognitive frailty and the precise biological mechanisms are still unclear. It has been suggested that there are common underlying processes that link to both neurodegenerative conditions as well as frailty and sarcopenia including chronic low-grade inflammation, hormonal deficit and increased oxidative stress (Okereke et al. 2006; Forman et al. 2008; Verghese et al. 2011; Welmer et al. 2013; Wichmann et al. 2014; Revel et al. 2015; Doi et al. 2016; Sargent et al. 2018).

Advancing age is associated with an increase in innate immune system function, a process that has been referred to as inflammageing (Franceschi et al. 2018). A low-grade pro-inflammatory status, driven by visceral obesity, genetic predisposition and environmental factors such as smoking and psychological stress, is typical of the ageing process, even in healthy older individuals with no risk factors or overt clinical conditions (Singh and Newman 2011; Newman et al. 2016). The relationship between inflammatory markers, such as interleukin-6, C-reactive protein and tumour necrosis factor alpha, and health outcomes in older adults appears to be non-specific with positive relationships being shown for both physical and cognitive outcomes (Wichmann et al. 2014; Soysal et al. 2016), and has been implicated in the development of a number of pathological conditions including cardiovascular disease, diabetes, sarcopenia and dementia (Beyer et al. 2012; Lopez-Candales et al. 2017; Franceschi et al. 2018).

Reactive oxygen species are active molecules generated during enzymatic reactions that play a physiological role under controlled conditions. However, they can generate undesired damaging and oxidising effects with molecules such as proteins, DNA, and lipids (Kruman et al. 2000; Agostinho et al. 2010). The intensity of this redox activity is termed “oxidative stress” and an abnormally increased oxidative stress is believed to be a major pathophysiological mechanism underlying disease and ageing (Álvarez-Satta et al. 2020). Observational studies in humans support a positive association between biomarkers for increased oxidative stress with both frailty and pre-frailty (Soysal et al. 2017), as well as cognitive decline (Hajjar et al. 2018).

Reductions in sex steroid hormones and important growth factors such as insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF), have also been implicated in the development of age related health conditions (Aleman et al. 2000; Erickson et al. 2010; Eichholzer et al. 2012). BDNF is a member of the neurotrophic growth factor family and an

important molecular mediator of brain neuroplasticity as well as neuronal protection and survival (Lu 2003; Miranda et al. 2019), and decreased circulating BDNF has been associated with cognitive decline (Shimada et al. 2014) and frailty (Coelho et al. 2012). IGF-1 is a growth factor that is essential for muscle regeneration and maintenance of muscle integrity (Song et al. 2013). Furthermore it is known that IGF-1 can cross the blood-brain barrier (Nishijima et al. 2010) and although its relationship with cognition in humans is still unclear (Tumati et al. 2016), animal models have shown that IGF-1 modulates the effect of BDNF on memory functions (Ding et al. 2006). Although there are multiple lines of evidence supporting relationship between declining sex steroid production and physical function in older males (Eichholzer et al. 2012; Nam et al. 2018), there is a lack of consensus of the effect of endogenous sex hormones on cognition in the older adult (Boss et al. 2014). Furthermore, the effects of declining hormone production in females on healthy ageing outcomes is less clear with studies on oestrogen replacement therapy yielding inconclusive results for cognitive function (Maki and Henderson 2012; Imtiaz et al. 2017), although studies on muscle strength are more promising (Greising et al. 2009; Laakkonen et al. 2017).

As well as being independently associated with both physical and cognitive function there is evidence to suggest a link between inflammatory markers and growth factor production and function in older adults. Low-grade inflammation is associated with reduced synthesis and function of growth factors including IGF-1 and BDNF (Barbieri et al. 2003; Tong et al. 2008; Calabrese et al. 2014). In a sample of 1002 older women the presence of simultaneous low IGF-1 and high interleukin-6 was associated with physical disability (OR, 5.14; CI95%, 1.85–14.25) and impaired ability to walk (OR, 10.77; CI95%, 1.68–69.11) over a three year follow up period, further supporting a link between the endocrine and immune systems (Cappola et al. 2003). Data from animals provide a further mechanistic link with transgenic mice that overexpress interleukin-6 having low levels of IGF-1 compared to wild-type mice (De Benedetti et al. 1997). Although this may suggest that interventions that target inflammation may also elicit effects on growth factor production and function and vice versa, RCTs within this area have been inconclusive. For example 12 weeks of resistance exercise in forty older adults lead to reductions in serum interleukin-6 but not serum BDNF (Forti et al. 2014). More research is required to better understand the complex relationship between the nervous and endocrine system, but the data is suggestive that interventions that are able to resolve low-grade inflammation and deficits in IGF-1 and BDNF could be efficacious in preventing age related decline in cognitive and physical function.

Taken together there is a complex and dynamic link between cognitive and physical decline in older adults. Importantly cognitive frailty has been proposed to be an important target for preventative intervention (Panza et al. 2017) and presents the interesting concept that

interventions targeted towards physical health may influence cognition and vice versa, possibly due shared pathological and underlying biological mechanism (Figure 3). At present the precise biological mechanisms that underpin the development of cognitive frailty are not well understood. Whilst identifying a unique biomarker would seem appealing, considering the complex nature of the development of both physical and cognitive decline alongside high degrees of inter-individual variability in regard to age related pathologies, it is more likely that integrating multiple biomarkers has the potential to help us better understand the complex relationships between physical and cognitive decline. Moving forward there is a need for long term prospective cohort studies to consider both physical and cognitive decline to help better understand this complex area.

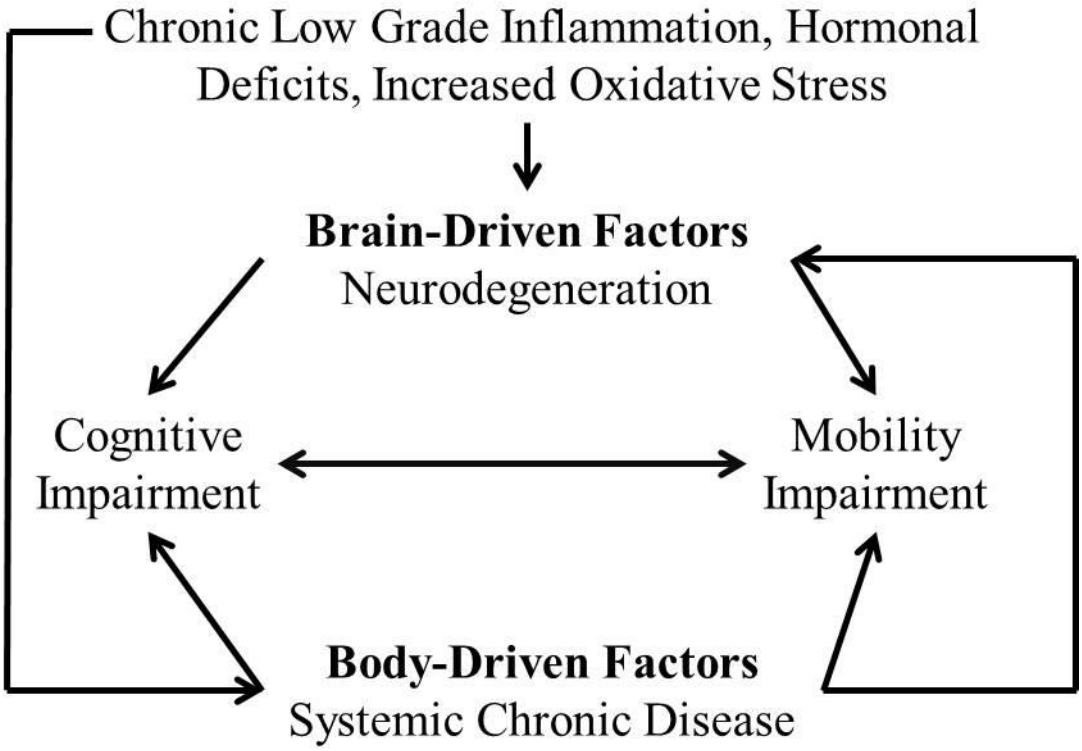


Figure 3 *Summary of the shared underlying factors that contribute towards both cognitive decline and mobility impairment. Brain-driven factors refer to neurodegenerative health conditions that can manifest in both cognitive impairment and declines in motor skills, such as gait. Body-driven factors include chronic health conditions that increase the risk for physical and cognitive decline and may also exacerbate the effects of the brain-driven conditions. Factors related to both the brain and body driven factors have been linked to common biological mechanism including chronic low-grade inflammation, hormonal deficits and increased oxidative stress. Amended from (Grande et al. 2019).*

1.3 Gait Assessment in Older Adults – A window into both Cognitive and Physical Function

Mobility limitations increase with advancing age and are often a sign of further functional decline (Rantakokko et al. 2013). Habitual and fast gait speeds are both examples of widely used performance based indicators of mobility (Verghese et al. 2009; Artaud et al. 2015). Gait speed is an established clinically relevant marker in older adults, due to strong associations with physical functioning, falls and disability (Montero-Odasso et al. 2005; Perera et al. 2016). Furthermore, in over 17,000 older adults baseline gait speed was associated with mortality with a pooled hazard ratio of 0.88 per 0.1 m/s (CI95%: 0.87-0.90) (Studenski et al. 2011).

Besides speed there are other gait variables that are relevant within research, these are summarised in Table 1. Of these measures, stride to stride variability is of particular importance. Greater stride variability has been reported in older adults with both mild cognitive impairment (MCI) and AD (Muir et al. 2012), as well as physical frailty (Montero-Odasso et al. 2011). Gait variability was also shown to predict future falls among 262 community-living older adults during 2 years of follow-up (Herman et al. 2010). Importantly, gait speed did not predict falls in this study indicating that gait variability could be a more sensitive measure for detecting falls, thus making it a relevant target for intervention trials for healthy ageing.

Table 1 Summary of relevant gait variables for the assessment of older adults, and their definitions (Nadkarni et al. 2009).

Gait Variable	Description
Stride Length Variability	The coefficient of variation of stride length [(standard deviation/mean) x 100]
Stride Length	The distance between two successive placements of the same foot.
Cadence	The number of steps per minute
Double Support Phase Percentage	The percentage of time spent with both feet in contact with the ground, during a gait test

1.3.1 Gait Assessment to Evaluate Physical Function in Older Adults

Increased risk of falls and loss of muscle mass are linked to increasing age and are both associated with impairment of physical function including gait, thus gait has primarily been used to assess the physical function of older adults (Visser et al. 2000).

Falls in older adults are associated with fractures, limited mobility, reduced quality of life, and mortality (Phelan et al. 2015). Habitual gait speed (HGS) has consistently been demonstrated to be a reliable predictor for fall risk in older adults (Luukinen et al. 1995; Cesari et al. 2005; Montero-Odasso et al. 2005; Abellan van Kan et al. 2009), with a proposed cut off of 1.0 m/s being proposed as a cut of for fall prediction with data suggesting a threefold increase in fall risk below this threshold (Kyrdalen et al. 2019)

As well as being a reliable indicator of fall risk gait speed has also been associated with the quantity and quality of muscle in the lower extremities in older adults (Visser et al. 2000; Visser et al. 2002), with slow gait speed being proposed as a mediating factor in the effect sarcopenia has on loss of independence (Perez-Sousa et al. 2019). As such, gait speed is recommended by the European Working Group on Sarcopenia in Older People as part of the diagnostic criteria for sarcopenia, with a cut off of 0.8 m/s indicating severe sarcopenia (Cruz-Jentoft et al. 2010; Cruz-Jentoft et al. 2019).

1.3.2 Gait Assessment to Evaluate Cognitive Function in Older Adults

Previous studies have used gait and cognitive assessments separately when evaluating older adults. The latest evidence now suggests that walking is a complex task with a significant cognitive aspect (Savica et al. 2016). Neuroimaging studies using functional magnetic resonance imaging have shown that motor control and cognitive processes have common neural substrates, in particular in the prefrontal, parietal and temporal areas (Rosano et al. 2012; Rosso et al. 2013). Changes in several gait parameters including speed, variability, cadence, stride length, and time spent in the double support phase coexist with or precede the onset of cognitive decline in older adults (Savica et al. 2016). A recent meta-analysis including data from 8699 older adults with a mean follow up of 6.6-14.5 years found that a yearly gait speed decline of 0.05 m/s or greater, a value that has been demonstrated to be clinically meaningful (Kwon et al. 2009), independent of memory decline was associated with increased risk of dementia (HR 2.24, CI95% 1.62-3.09) (Tian et al. 2020).

Specific cognitive domains appear to have greater importance in controlling gait related outcomes. Early declines in attention, executive function and visuospatial memory are associated with slower gait and gait instability (Montero-Odasso et al. 2009; Ansai et al. 2017).

Executive function is the cognitive domain which is most consistently associated with gait stability (Coppin et al. 2006; Holtzer et al. 2006; Springer et al. 2006; Kearney et al. 2013; Martin et al. 2013a). However, there is also evidence for the involvement of processing speed (Bruce-Keller et al. 2012). Executive function is the term given to the management of cognitive processes, including working memory, reasoning, task flexibility, and problem solving, which is central to planning, goal-directed action and coordination of complex locomotion (Diamond 2013). Executive function and processing speed were both associated with gait speed, stride length, stride length variability and double support phase percentage in a sample of 422 adults aged 72±7 years (Martin et al. 2013b).

1.3.3 Dual-Task Gait Assessment in Older Adults

The role of cognitive function in relation to walking is increasingly important in older adults when required to conduct a simultaneous secondary task (dual-task paradigm). This was first discovered by Lundin-Olsson et al. (1997) who found that, in a sample of 58 older adults living in a nursing home, 83% (10/12) of those who stopped walking when required to start a conversation had a fall over the preceding six months. This was a significantly higher fall rate when compared with the 24% (11/46) observed in those who did not pause whilst starting a conversation. In a dual-task scenario, a participant would be asked to perform an attention demanding task while walking so comparisons can be made to the single task walking condition. This is commonly referred to as the dual task effect (DTE) or dual task cost (Plummer and Eskes 2015). Modifications to both gait speed and variability have been observed in older adults without cognitive impairment under dual-task conditions (Springer et al. 2006; Hausdorff et al. 2008). The role of evaluating DTE to predict the risk of falls in older adults has been investigated and a systematic review of prospective cohorts found that an increased DTE was associated with a greater risk of falls, although due to the heterogeneity in the testing protocols it was not possible to perform a meta-analysis to gain an overall effect size on the strength of the associations across the body of literature (Muir-Hunter and Wittwer 2016). Furthermore, there is evidence to suggest that dual-task gait assessments are more sensitive for predicting falls in cognitively healthy older adults versus single task protocols (Beauchet et al. 2008b; Herman et al. 2010)

In addition, a study in 1,038 older adults found that a DTE of 18% or greater predicted falls in participants with gait speeds above 0.95 m/s, indicating that DTE could be a useful measure for predicting falls even in those who do not have impaired gait in accordance with the Fried frailty criteria (Yamada et al. 2011). As well as showing promise for indicating the risk of falling, assessing DTE in gait assessments could indicate subtle cognitive impairment. Patients with AD

and MCI have been shown to have a greater DTE on speed than age matched controls with no cognitive impairment (Sheridan et al. 2003; MacAulay et al. 2017).

1.4 Nutrition and Exercise Interventions to Promote Healthy Ageing

Most of the pharmacological approaches used in age related health conditions have been met with limited success, and this is likely due to the multifactorial aetiology underlying these conditions. These include amongst others, varying physical and neurological pathologies as well as factors such as inflammation, metabolism, and genetics (Chen et al. 2014). Development of lifestyle interventions to reduce the burden of age related health conditions would be highly advantageous considering the poor efficacy of the current pharmaceutical options (Cesari et al. 2015). Dietary compounds and exercise have been shown to separately act on a broad spectrum of health outcomes in older adults including gait speed, cognition and muscle strength and function (Liu and Latham 2009; Gomez-Pinilla and Hillman 2013; Strike et al. 2016). The capacity to act on multiple outcomes makes these lifestyle interventions particularly valuable in the prevention of age related cognitive and physical impairments. Some of the lifestyle interventions that have shown promise in combatting age related declines in mobility and cognition include aerobic exercise and the dietary compounds omega-3 polyunsaturated fatty acids (omega-3 PUFAs), vitamin E, phosphatidylserine (PS), B vitamins, and *Ginkgo Biloba* (Harber et al. 2009; Reay et al. 2013; Dysken et al. 2014; Tan et al. 2015; Strike et al. 2016). As well as showing promise when administered separately, there is now evidence suggesting interactions or even synergies between these specific nutrients themselves and between the nutrients and resistance and aerobic exercise on both mobility and cognition in older adults (Wu et al. 2008; Kobe et al. 2016; Oulhaj et al. 2016; Da Boit et al. 2017).

Overall the apparent link between the physical health with cognitive function in older adults provides a host of potential new targets and pathways for intervention to prevent or ameliorate progressive frailty in this population (Panza et al. 2018). Interventions targeted towards cognition could have a downstream effect on physical function, and similarly, those which target physical health could influence cognition (Kelaiditi et al. 2013). Interventions, such as omega-3 PUFA supplementation, that can target cognition have been also shown to improve gait speed compared to placebo supplementation (Hutchins-Wiese et al. 2013; Strike et al. 2016). Taking this into consideration, gait speed, in particular, has emerged as a particularly important outcome for older adults. Gait speed was already a long established clinically relevant marker, which has now consistently shown links with several cognitive domains. Therefore, gait assessment can be used as a window into both physical and cognitive function. In particular, dual-task gait is a novel method for the assessment of both domains (Montero-Odasso et al. 2012b).

Previous work in our laboratory found that a supplement containing a daily of dosage of 1000 mg docosahexaenoic acid (DHA, 22:6n-3), 160 mg eicosapentaenoic acid (EPA, 20:5n-3), 20 µg vitamin B12, 1 mg folic acid, 124 mg PS, 20 mg vitamin E and 240 mg *Ginkgo Biloba* standardized leaf extract increased HGS, verbal memory, and processing speed in older women versus placebo (Strike et al. 2016). The focus of this PhD thesis will be to examine the effects of this supplement as well as an exercise intervention alone and in combination using HGS as a primary outcome. Secondary outcomes will be related to mobility, cognition and health related quality of life, to fully encapsulate the effects on the HAP (Lara et al. 2013). Therefore, the focus of this literature review will be on the effects of and interactions between these nutrients and exercise on factors related to the HAP.

1.5 Omega-3 Polyunsaturated Fatty Acids

Fatty acids are identified by the presence of a carboxyl group at one end and a methyl group, commonly referred to as "omega" or "n", at the other end of a carbon chain. The presence of one or more double bonds occurring between adjacent carbon atoms results in an unsaturated fatty acid. Unsaturated fatty acids can be further divided based on the number of double bonds that occur, with a single double bond being found in monounsaturated fatty acids and two or more being found in PUFAs. PUFAs can then be further subdivided according to the position of the first double bond from the methyl end. A PUFA in which the first double bond occurs between the third and fourth carbon linkages are known as omega-3 PUFA. Likewise, a PUFA with a double bond occurring between the sixth and seventh carbon linkages is referred as an omega-6 PUFA. Individual omega-3 and omega-6 PUFA types can be identified based on the length of their carbon chains and number of double bonds (Cunnane 2003).

1.5.1 Dietary Sources and Nutrient Status in Older Adults

Omega-3 PUFAs are particularly enriched in fish and seafood but are also contained in seeds and nuts (Nettleton 1991). Marine sources contain preformed EPA and DHA, whereas plant sources contain the shorter chain omega-3 PUFA alpha-linolenic acid (ALA). The majority of the literature to date has focused on the effects of the longer chain PUFAs EPA and DHA (Stark et al. 2016a). The Scientific Advisory Committee on Nutrition (SACN) recommends two portions of fish per week, one white and one oily to provide 0.45g omega-3 PUFA per day (SACN, 2004). According to data from National Diet and Nutrition Survey (NDNS), the current consumption of oily fish in older adults is well below recommendations in the U.K., with an intake of 84 grams of oily fish per week, with one portion being defined as 140 grams (NDNS, 2018). This insufficient intake is reflected in levels of circulating lipids as people who live in the UK had mean values of ≤4% total EPA+DHA when measurements were combined

from plasma, whole blood plasma phospholipids and erythrocytes, which was categorised as very low levels (Stark et al. 2016b). 2

1.5.2 Omega-3 PUFA Metabolism

Humans are not able to endogenously synthesise ALA or the omega-6 polyunsaturated fatty acid (omega-6 PUFA) linoleic acid (LA), thus making these essential dietary fatty acids. Despite not being able to synthesise omega-3 PUFAs, the human body is capable of metabolising ALA, through a series of elongation and desaturation reactions to produce EPA and DHA (Figure 4). Human metabolic studies using stable isotopes of ALA show a limited conversion of ALA to DHA, typically below 1%, however, the conversion may depend on the dosage of ALA consumed as well as the amount of linoleic acid, which competes for the same elongase and desaturase enzymes (Brenna et al. 2009). Furthermore there is evidence of age related decline in the conversion of ALA to DHA, especially in women, caused by a decline in Delta-6-desaturase activity (Bolton-Smith et al. 1997). Consuming preformed EPA and DHA has been shown to be a more efficient method for increasing tissue levels than consuming precursors (Barcelo-Coblijn and Murphy 2009). EPA intake increases blood levels of EPA and DPA n-3, with only preformed DHA significantly influencing levels of DHA (Brenna et al. 2009). As a result of the age related decline in conversion of ALA in older women, this group, in particular, should consider consuming preformed DHA through diet or supplementation (Geppert et al. 2006; Talahalli et al. 2010).

Omega-3 PUFAs have many potential fates in the body, including undergoing β-oxidation, storage in adipose tissue or incorporation into phospholipids. These form the major structural components of all cellular membranes and serves as a pool of PUFAs which can be made available for further metabolism to various bioactive lipids (Surette 2008). Cellular membranes from some tissues including the retina and the brain are particularly enriched in omega-3 PUFAs, specifically DHA (Brenna and Diau 2007b; Weiser et al. 2016). DHA is the primary omega-3 PUFA in the brain, concentrated at levels of about 10,000 nmol/g brain (10–15% of brain fatty acids or about 5 g in an adult brain (Martinez 1992), and levels are at least 250-300 fold greater than EPA (Chen et al. 2013). DHA is especially concentrated in the grey matter (Brenna and Diau 2007a). It is stored primarily in phosphatidylethanolamine (PE) and PS membrane phospholipids, with smaller amounts, also found in phosphatidylcholine (PC) (Rapoport 2001). DHA is enriched in membrane structures found at synaptic terminals, mitochondria and endoplasmic reticulum (Suzuki et al. 1997). The observation that these cells have developed to preferentially incorporate DHA into their membranes and that synthesis of DHA from docosapentaenoic acid (DPA) is more energy costly suggest that this particular

omega-3 PUFA plays a key role in the proper function of these cells (Surette 2008). In support of this, DHA has been shown to promote hippocampal neurogenesis *in vitro* and *in vivo* by stimulating neuronal differentiation of neural stem cells (Calderon and Kim 2004; Kawakita et al. 2006; Katakura et al. 2009) and promoting neurite growth in hippocampal neurons *in vitro* (Calderon and Kim 2004). Specifically, with regards to ageing, dietary supplementation of aged rats with an omega-3 PUFA enriched diet for 12 weeks partially reverses age related decline in neurogenesis in the dentate gyrus, as assessed by doublecortin expression (Dyall et al. 2010). As DHA is so highly enriched and appears to have an important role within the brain, this is the primary factors for why it is thought that DHA is the most biologically relevant omega-3 PUFA in terms of neurodegeneration (Dyall 2015a).

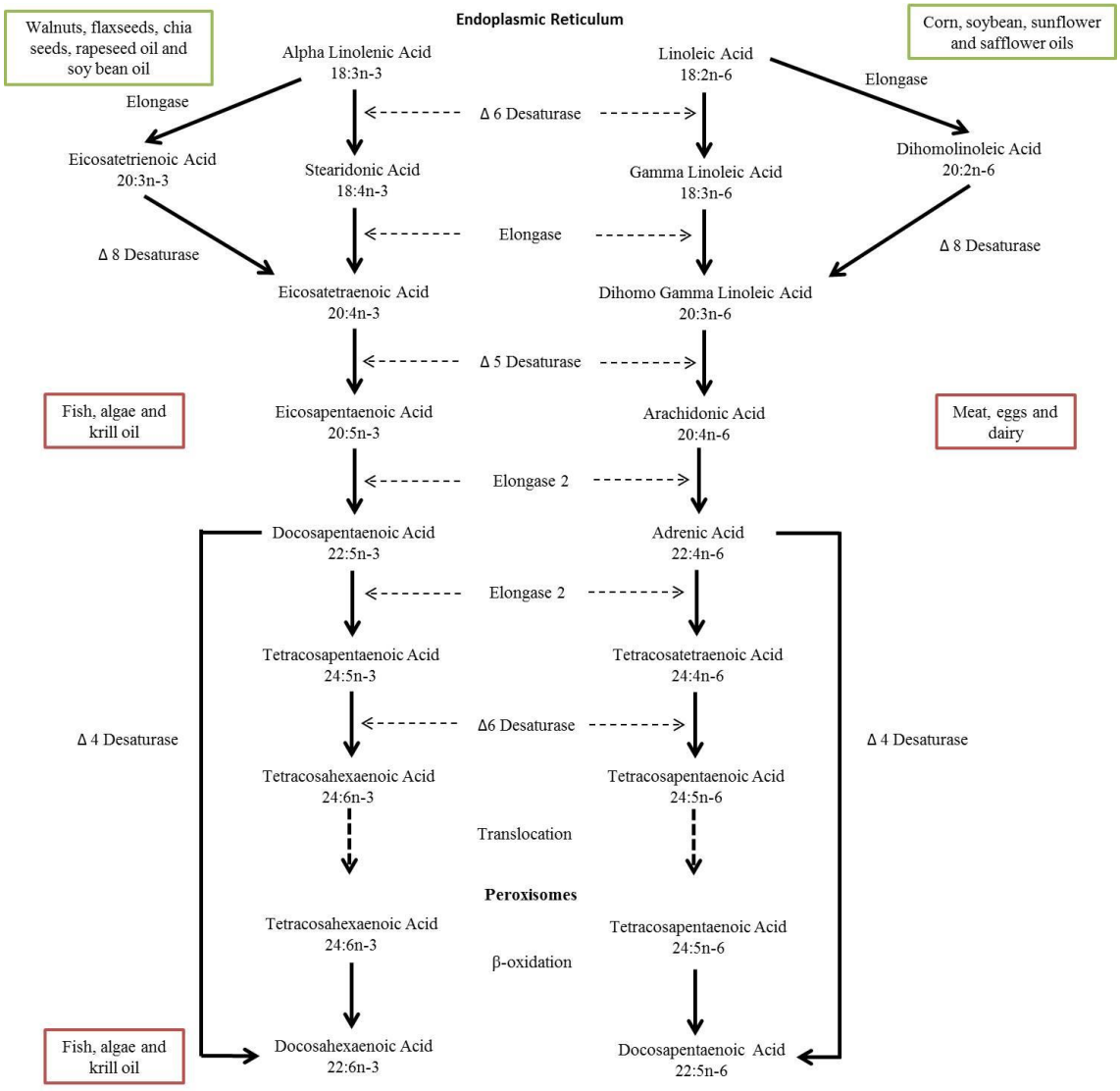


Figure 4 *Summary of omega-3 polyunsaturated fatty acid and omega-6 polyunsaturated fatty acid biosynthetic pathways. The pathways include a series of desaturation and elongation steps*

taking place in the endoplasmic reticulum. Tetracosahexaenoic acid 24:6n-3 and tetracosapentaenoic acid 24:5n-6 are than translocated to the peroxisome where they are shortened through beta-oxidation to form docosahexaenoic acid 22:6n-3 and docosapentaenoic acid 22:5n-6. Amended from Dyall and Michael-Titus (2008).

1.5.3 Mechanisms of Action of Omega-3 PUFAs in Relation to Healthy Ageing

Omega-3 PUFAs have been shown to affect a number of metabolic processes including membrane fluidity, cellular signalling, membrane protein function, gene expression and inflammation (Litman and Mitchell 1996; Mitchell et al. 1998; Akbar et al. 2005; Huang et al. 2012). Furthermore, it has been proposed that DHA could play an essential role in the process of electron tunnelling (Crawford et al. 2013), a process that underpins the function of microtubules in regulating synaptic function and cohesion across brain regions and therefore consciousness (Ekert et al. 1998).

1.5.3.1 Membrane Integrity

Physical membrane properties are influenced by their lipid composition (Maulucci et al. 2016). The double bonds in DHA introduce kinks that lower the packing density of the acyl chains and inhibit transition of the membrane from a fluid to a solid gel phase, therefore increasing membrane fluidity (Holthuis and Menon 2014). Adult mice fed a diet composed of sardine oil, containing 13.8% EPA and 9.8% DHA, for 12 months showed increased membrane fluidity of the synaptic plasma membranes, as well as improved spatial learning and memory versus an omega-3 PUFA deficient diet of palm oil (Suzuki et al. 1998).

Lipid rafts are liquid ordered microdomains comprised primarily of sphingolipids and cholesterol, which serve as platforms for protein activity (Simons and Ikonen 1997). The function of lipid rafts is to allow the compartmentalisation of the membrane into functional domains which facilitate protein-protein and lipid-lipid interactions involved in signal transduction and trafficking (Laude and Prior 2004). *In vitro* studies have shown that omega-3 PUFAs modulate the structure and composition of lipid rafts (Shaikh et al. 2009). These structural changes have been shown to affect protein localisation within raft and non-raft regions *in vitro* (Wong et al. 2009; Langelier et al. 2010). The current literature largely focuses on *in vitro* models of breast and colorectal cancer (Duraisamy et al. 2007; Schley et al. 2007; Altenburg and Siddiqui 2009; Rogers et al. 2010); therefore it is unclear whether any effect on mobility or cognitive outcomes in human trials is a result of lipid raft alteration. However, the DHA content of lipid rafts has been shown to be significantly lower in the frontal cortex of AD patients versus healthy controls (Martin et al. 2010). The decrease in DHA enhanced interactions between amyloid precursor protein and β-secretase versus healthy controls, thus promoting β –amyloid protein synthesis, which is a key factor in the development of AD

(Fabelo et al. 2014). This DHA specific difference is likely due to the differential incorporation of omega-3 PUFAs into sphingomyelin/cholesterol-rich lipid rafts. DHA has been shown to be more highly enriched than EPA (Williams et al. 2012), and in the study by Martin et al. (2010) made up over 95% of the omega-3 PUFA content in lipids rafts.

1.5.3.2 Neurotransmission

Proteomic studies in the hippocampus of aged rats focusing on the synaptic proteome have revealed age-related changes in the expression of proteins involved in neurotransmitter vesicle exocytosis and recycling dynamics in the synapses (VanGuilder et al. 2010). A large subset of synaptic proteins including dynamin 1, hippocalcin, postsynaptic density protein 95, synaptosome associated protein 25, syntaxin 1, synapsin 2, synaptophysin and vesicle-associated membrane protein 2, which represent both effectors and regulators of neurotransmission were found to change during ageing, indicating an age-based deterioration of hippocampal neurotransmission.

DHA is a major structural component of grey matter neuronal membranes, with a particularly high concentration of DHA being found in synaptic membranes (Suzuki et al. 1997). The enrichment of DHA appears to play an essential role in neurotransmission through altering the synaptic plasma membrane proteome. A DHA deficient diet comprised of 0.09 % DHA of total fatty acids in aged mice exacerbated the age related decline in the expression of multiple synaptic plasma membrane proteins in the cortex, as well as leading to a decline in recognition memory versus aged rats fed a diet enriched with 0.9% DHA (Sidhu et al. 2016). These findings are further supported by previous pre-clinical work in aged rats that found that DHA enriched diets ameliorated the age related decreases glutamate receptor 2 and glutamate receptor subunit epsilon-2, which are associated with learning and memory (Dyall et al. 2007). The proteins influenced by dietary DHA are known to play an important role in neurotransmission and have been demonstrated to be down regulated during ageing, thus the effects of DHA provides a molecular basis for the impact of nutritional DHA on learning and memory function and preservation of cognition in old age.

1.5.3.3 Cell Signalling

Omega-3 PUFAs influence a number of signalling proteins, which are related to healthy ageing by modulating inflammation, muscle protein synthesis (MPS), neuronal survival, neurotransmission and synaptic plasticity. In particular, DHA increases activation of peroxisome proliferator-activated receptor gamma (PPARγ) and reduces activation of nuclear factor kappa-light-chain-enhancer of activated B cells in human dendritic cells *in vitro and in vivo* (Zapata-Gonzalez et al. 2008; Kong et al. 2010). This leads to reduced secretion of the inflammatory cytokines interleukin (IL) 12 and 27 under lipopolysaccharide stimulation.

Furthermore, DHA has been shown to increase the PS content of neuronal membranes, which leads to increased activation of the phosphoinositide 3-kinase/protein kinase-B pathway and a reduction in neuronal apoptosis (Akbar et al. 2005). Increases in this signalling pathway modulated by both EPA and DHA have also been observed in muscle tissue in rats (You et al. 2010; Marzuca-Nassr et al. 2016); however, this was a model of physical inactivity and may not translate to a mobile and active population. Moreover, an increase in the activation of mechanistic target of rapamycin complex 1 and ribosomal protein S6 kinase beta-1 was observed in muscle biopsies from the quadriceps femoris of sixteen healthy adults aged 71±2 years supplemented with 1.86 g EPA and 1.5 g DHA for eight weeks, as well as an increase in MPS (Smith et al. 2011a). Interestingly, no effects of supplementation on the inflammatory markers tumour necrosis factor alpha, C-reactive protein or interleukin-6 were observed, suggesting that this effect was indeed driven by the effect on cell signalling, rather than any influence on systemic inflammation which has been implicated in the development of sarcopenia (Beyer et al. 2012). Furthermore, these results were achieved during a hyperaminoacidemic-hyperinsulinemic clamp and not under basal conditions, indicating that omega-3 PUFAs, alone, may not be sufficient to elicit an anabolic response, and may require other stimuli such as exercise or amino acids from dietary protein to have an effect. This may reduce the likelihood of an impact in free-living older adults, as malnutrition prevalence in community-dwelling older adults has been reported to be as high as 15% in developed countries (Gil-Montoya et al. 2013). Under more physiological relevant conditions where the anabolic stimulus came from resistance exercise, a study in 27 healthy older adults aged found that daily supplementation with 2100 mg EPA and 600 mg DHA for 18 weeks did not affect protein S6 kinase beta-1 activity or rate of MPS versus placebo. This study did differ from the previous work by Smith et al. (2011a) using a measure of free-living MPS which incorporated samples under basal conditions and two hours following resistance exercise, which may have reduced the capacity to detect a change in MPS. There is evidence to suggest that this effect of omega-3 PUFA supplementation on MPS may be driven by EPA with an *in vitro* study on C2C12 myotubes which showed incubation with EPA resulted in increases in MPS and decreases in muscle protein breakdown, whereas DHA had no effect on either outcome (Kamolrat and Gray 2013). This effect was accompanied by an upregulation of protein S6 kinase beta-1 phosphorylation, however this was independent of upstream signalling pathways including protein kinase B and mechanistic target of rapamycin complex 1.

1.5.3.4 Inflammation

It is well established that omega-3 PUFAs influence the inflammatory response by acting as pre-cursors for bioactive lipid mediators (Norling et al. 2017). The influence of omega-3 PUFAs on inflammation could be a key underlying mechanism in healthy ageing as chronic low-grade

inflammation has been proposed to be part of the pathology for a number of age related degenerative conditions including frailty, dementia and sarcopenia (Beyer et al. 2012; Cunningham and Hennessy 2015; Soysal et al. 2016).

To synthesise lipid mediators, firstly, phospholipids are hydrolysed by specific phospholipases to release individual fatty acids. Lipid mediators are then produced from these fatty acids by three enzyme systems: cyclooxygenase, lipoxygenase and cytochrome p450. These enzymatic systems are shared by the omega-6 PUFA arachidonic acid (AA), LA, DPA and dihomo-γ-linolenic acid as well as both omega-3 PUFA and omega-6 PUFA derived endocannabinoids (Culp et al. 1979; Dyall 2017). Lipid mediators include prostaglandins, thromboxanes and leukotrienes as well as specialised pro-resolving mediators (Bannenberg and Serhan 2010) and electrophilic fatty acid oxo-derivatives (Cipollina et al. 2014). Lipid mediators, derived from EPA and AA are commonly referred to as eicosanoids and the DHA derived mediators are called docosanoids. The influence of EPA and DHA on inflammation may firstly be by displacing AA, thus decreasing the production of AA derived mediators. Frequently, although not always, omega-3 PUFA derived mediators have lesser inflammatory effects than the mediators derived from AA (Wada et al. 2007). Diets higher in omega-6 PUFAs and lower in omega-3 PUFAs result in higher quantities of omega-6 PUFAs being enriched in cell membrane phospholipids at the sn-2 position and vice versa, thus altering the availability of substrates for synthesis of bioactive lipid mediators (Lands et al. 1992; Healy et al. 2000). The increase in the production and consumption of vegetable oils over the last century has led to a dramatic rise in intake of LA in Western populations with total energy derived from LA rising from 2.79% to 7.21% in the United States between 1909 and 1999 (Blasbalg et al. 2011). It is estimated that Western populations have an omega-6 PUFA to omega-3 PUFA ratio of 15-16.7:1, which is far removed from 1:1 ratio which is said to have occurred throughout the evolutionary history of man (Eaton and Konner 1985; Kuipers et al. 2010). Secondly the omega-3 PUFA derived specialised pro resolving mediators resolvins, protectins and maresins have been shown to have a unique ability to resolve inflammation and modulate immune function (Dalli et al. 2013; Serhan 2017).

The resolution of inflammation is initiated by a change in eicosanoid signalling that shifts from a pro-inflammatory to a pro-resolution, anti-inflammatory state, which is characterised by the biosynthesis of specific pro-resolving mediators (Serhan et al. 2000; Hong et al. 2003). During the initiation phase of the acute inflammatory response, mediators derived from AA become up-regulated (Levy et al. 2001). Following this, there is an active resolution phase. During this phase prostaglandins, which are involved in the initial acute inflammatory response, activate the translation of mRNAs encoding enzymes that are required for the production of pro-resolvin mediators, a process referred to as lipid mediator class switching (Levy et al. 2001; Serhan et al.

2015). Furthermore, an increase in the production of omega-3 PUFA derived specialised pro-resolving mediators also occurs, promoting an active resolution to the inflammatory response (Serhan et al. 2008). These specialised pro-resolving mediators include the resolvin D series compounds, protectins/neuroprotectins, and maresins, which are derived from DHA, as well as the resolvin E series compounds derived from EPA (Figure 5 and Figure 6). In addition it has been observed that acetylsalicylic acid can trigger the resolution phase by initiating the production of specialised pro-resolvin mediators (Serhan et al. 2002). This interaction may be of particular importance in older adults as acetylsalicylic acid intake occurs in up to 30% of the population (Stuntz and Bernstein 2016).

The pro-resolving effects of the lipoxins and omega-3 PUFA derived specialised pro-resolving mediators act by suppressing polymorphonuclear leukocytes, promoting non-phlogistic monocyte recruitment, and increasing macrophage-mediated clearance of apoptotic polymorphonuclear leukocytes (Buckley et al. 2014). It is hypothesised that a lack of resolution of inflammation can occur in older adults leading to a state of chronic low-grade inflammation (Xia et al. 2016). By increasing the production of specialised pro-resolving mediators, omega-3 PUFA supplementation could have a significant impact on this state of chronic low-grade inflammation, which underpins the pathology for a number of age related diseases (Beyer et al. 2012; Cunningham and Hennessy 2015; Soysal et al. 2016).

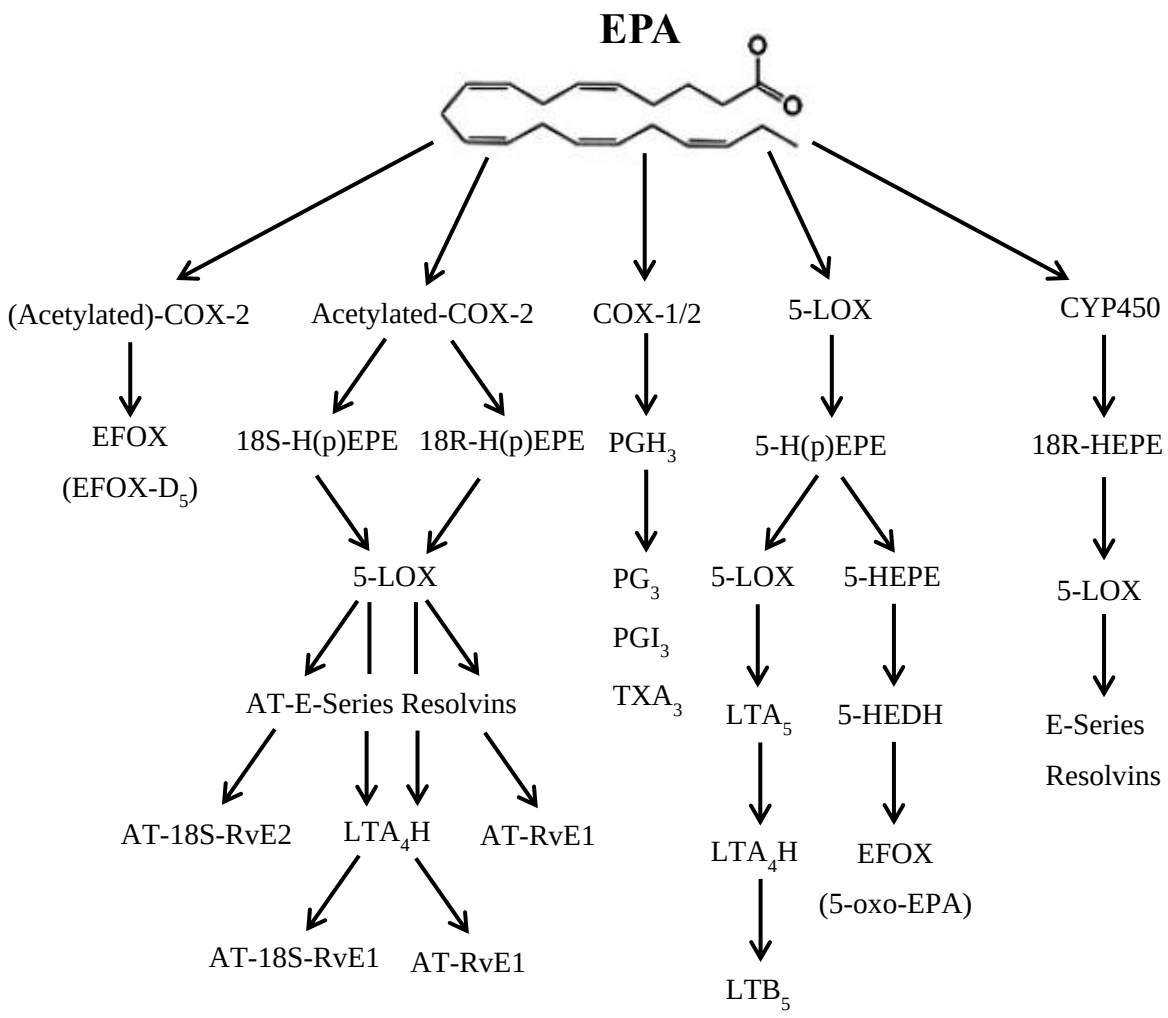


Figure 5 *Summary of the lipid mediators produced form eicosapentaenoic acid (EPA). In the cyclooxygenase (COX)-1/2 pathway EPA is enzymatically converted to the 3 series prostaglandin, prostacylcins or thromboxanes. EPA can also be converted by 5-lipoxygenase (5-LOX) to 5-hydroperoxyeicosapenataenoic acid (5-H(p)EPE). 5-H(p)EPE can then be converted by 5-LOX to leukotriene A5 (LTA5) and then by Leukotriene A4 Hydrolase (LTA4) to leukotriene B5 (LTB5) or to 5-hydroxyeicosapentaenoic acid (5-HEPE), which is then converted into 5-oxo-EPA by 5-hydroxyeicosanoid dehydrogenase (5-HEDH). The cytochrome P450 (CYP450) pathway converts EPA to 18R-hydroxyeicosapentaenoic acid (18R-HEPE), which is then converted to the E-series resolvins (RvE) by 5-LOX. COX-2 can also convert EPA to the electrophilic fatty acid oxo-derivative electrophilic fatty acid oxo-derivates (EFOX)-D5, in a process enhanced by aspirin acetylation of COX-2. Aspirin acetylation of COX-2 also produces 18S- and 18R-hydroperoxyeicosapentaenoic acids (18S-, or 18R-HETE) from EPA, which are either converted by 5-LOX to aspirin-triggered 18S-resolvin E1 and resolvin E1 (AT-18S-RvE1 and AT-RvE1), respectively or through an extra step by LTA4H to AT-18S-RvE2 and AT-RvE2. Amended from Dyall (2015a).*

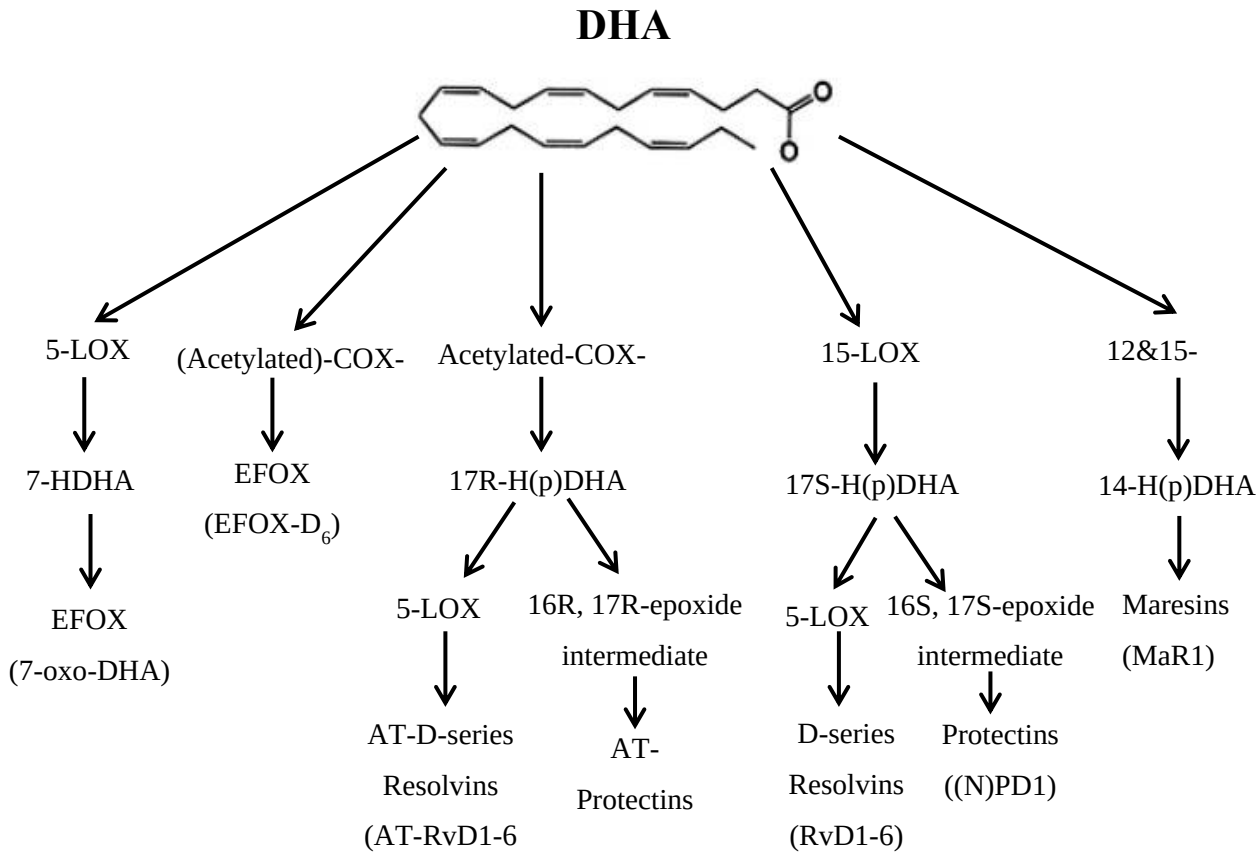


Figure 6 *Summary of the lipid mediators produced from docosahexaenoic acid (DHA). DHA can be converted by 15-lipoxygenase (15-LOX) to 17S-hydroperoxydocosahexaenoic acid (17S-H(p)DHA), which is converted by 5-lipoxygenase (5-LOX) to D-series resolvins (RvD), or enzymatically hydrolysed to (neuro)protectin D1 ((N)PD1). DHA can also be converted by 12 or 15-LOX via 14-hydroperoxydocosahexaenoic acid (14-H(p)DHA) to the maresins. 5-LOX can also directly convert DHA to 7-hydroxydocosahexaenoic acid (7-HDHA) which is then converted to 7-oxo-DHA by dehydrogenase. Similarly to EPA, production of DHA derived mediators can also be enhanced by aspirin acetylation where it is converted by cyclooxygenase-2 (COX-2) to EFOX-D6, or by producing 17R-hydroperoxyDHA which can then be converted to aspirin triggered resolvins and protectins. Amended from (Dyall 2015a).*

There is evidence to support that omega-3 PUFAs may exert their positive effects on outcomes related to the HAP through multiple mechanisms. These mechanisms are dependent on enrichment with omega-3 PUFAs within cell membranes where they can impact cell signalling and act as precursors for the production of pro-resolvin lipid mediators. Within muscle tissue EPA appears to be the most relevant biologically active omega-3 PUFA (Kamolrat and Gray 2013) and may be driving the increase in MPS through changes in cell signalling rather than through any effects in inflammation (Smith et al. 2011a). Conversely we know that DHA is

highly enriched within brain tissue where it can impact cell signalling, cell survival (Akbar et al. 2005). More data is now suggesting that EPA and DHA should be treated as two biologically distinct molecules with separate functionalities. Arguably if the goal is to promote healthy ageing as a whole adequate levels of EPA and DHA would need to be provided to target both muscle and cognitive function. Whilst this mechanistic data does provide some promising insight it is largely dependent on animal or *in vitro* models and thus is considering preliminary in nature. Furthermore, whilst mechanistic understanding is certainly important this may not always translate into clinically meaningful benefits. The following section will explore the observational and experimental data on omega-3 PUFA intake on mobility and cognition within older adults.

1.5.4 The Effects of Omega-3 PUFAs on Mobility and Cognitive Function in Older Adults

1.5.4.1 Observational Evidence

The observational evidence measuring the association between omega-3 PUFA intake or fish consumption and cognitive outcomes has largely yielded positive results. A meta-analysis of 21 longitudinal observational studies including 181, 580 participants (follow-ups ranging between 2.1 and 21 years) found that increasing fish consumption by one serving per week was associated with a significantly reduced risk for dementia (Relative risk (RR): 0.95; 95% CI: 0.90, 0.99) and AD (RR: 0.93; 95% CI: 0.90, 0.95). In comparison with participants who had no fish consumption, there was a significant reduction in risk for AD (RR: 0.79 CI: 0.66, 0.95) for 2 servings of fish per week which is currently the recommended intake for the UK (SACN, 2004). The same analysis also found that a 0.1g/day increment in DHA intake was inversely associated with risk of developing dementia (RR: 0.86; CI95%: 0.76, 0.96) and AD (RR: 0.63; CI95%: 0.51, 0.76). However, there were no significant relationships found for EPA or ALA for cognitive outcomes (Zhang et al. 2015). This is in support of some of the previously highlighted mechanistic data where DHA has been demonstrated to play an essential role in the brain, providing a level of biological plausibility, and underpins the theory that EPA and DHA have distinct functions within the human body.

The dietary guidelines within the countries of all the included cohorts have recommendations to encourage fish consumption with all the European and North American cohorts having guidelines that encourage PUFA consumption or displacement of saturated fat with PUFA. Few of these studies controlled for other dietary factors such as fruit and vegetable consumption and saturated fat intake. As such it is impossible to elucidate whether it is the omega-3 PUFA intake alone that is driving this association, or whether increased omega-3 PUFA intake is just indicative of an overall healthier diet which also may be accompanied by other health seeking behaviours. Furthermore, with the increased EPA and DHA intake within these cohorts largely

coming from fish consumption it becomes difficult to again establish whether the association is a result of increased omega-3 PUFA intake alone or whether the other nutrients within fish have also contributed. Fish and seafood is also a source of other essential micronutrients including B vitamins, which will be covered in sequent sections of this literature review, and may contribute towards the protective associations observed between fish consumption and cognition in the older adult (Oulhaj et al. 2016).

Omega-3 PUFAs have been linked with cognitive function in the older adult for decades (Kalmijn et al. 1997; Conquer et al. 2000), more recently, however, there have been several lines of evidence that have found an association between omega-3 PUFAs and mobility within the same demographic. A cross-sectional analysis of 417 participants in the Tokyo Oldest Old Survey on Total Health cohort found that lower habitual intakes of EPA and DHA were associated with greater time taken on the timed up and go test in men only (Takayama et al. 2013). Intakes of omega-3 PUFAs within Japanese cohorts are typically higher than studies in Europe and North America. The mean intake of EPA+DHA in this particular analysis was 1155 mg per day so caution must be applied when translating these results to western population groups that typically have notably lower omega-3 PUFA intakes. A longitudinal analysis among 1273 older adults involved in the Invecchiare in Chianti, ageing in the Chianti area study found that lower baseline omega-3 PUFAs levels were associated with an increased risk for decline in Short Physical Performance Battery, which consisted of tests of fast walking speed, standing balance and ability to rise from a chair, to a score of less than or equal to nine (OR:0.21; CI95%:0.08, 0.53), a score that indicates low physical performance. (Abbatecola et al. 2009). This relationship appeared to be driven by EPA and DHA which both showed separate significant inverse relationships with risk for impaired physical performance, with LA and AA showing no relationship. Furthermore, this analysis also found that a higher omega-6 PUFA/omega-3 PUFA ratio was associated with a higher risk of poor physical performance (OR:5.23; CI95%:2.02, 13.51). A prospective cohort study in 5,764 older men and women in Reykjavik, found that higher baseline phospholipid levels of total omega-3 PUFAs and DHA alone in plasma was associated with a reduced risk for self-reported mobility disability over 5 years in women only (Reinders et al. 2015). Odds ratios were 0.48 (CI95% 0.25, 0.93) for every standard deviation increase of total omega-3 PUFA and 0.45 for every standard deviation increase in DHA (CI95% 0.24; 0.83). An important factor to note with this study is that 62% of the participants reported taking fish oil supplements daily and the mean percentage weight of long-chain omega-3 PUFAs was considerably higher than in the majority of countries worldwide, with total circulating EPA+DHA of ≈9% (Stark et al. 2016b). Similar to the Japanese cohort, it would be difficult to extrapolate these results beyond this demographic, as the relationship may not be linear and could change across lower levels of intake typical of the

U.K. Furthermore this study did not appear to consider cognitive function as either an exclusion criterion or a confounding factor in their analysis. Considering the close relationship between cognitive function and gait in older adults this would have been an important consideration as any participants experiencing cognitive decline could subsequently see declines in their physical performance. A recent cross-sectional analysis of 982 older adults from the Three-City-Bordeaux study found that greater level of total omega-3 PUFAs, as well as EPA and DHA independently, in plasma were associated with lower odds of having a low gait speed (<0.63m/s). These associations were independent of many potential confounders (sex, age, educational level, energy and protein intakes, physical activity, and comorbidities) (Frison et al. 2017). The omega-3 PUFA levels in plasma in this cohort were more typical of a European population with mean total percentage weight of EPA+DHA being 3.2%. The threshold for low gait speed was established at 0.63 m/s based on being in the lowest quartile of the studied population. The cut off criteria for low gait speed in frailty and sarcopenia is 0.8 m/s (Fried et al. 2001; Cruz-Jentoft et al. 2010), therefore it is unclear whether this relationship between omega-3 PUFA status and low gait speed would have been consistent for a more clinically relevant cut off point.

In contrast, a study in 247 older adults found no association between self-reported intake of omega-3 PUFAs and any measure of mobility which included rising from a chair, grip strength and gait speed (Rousseau et al. 2009). Initially, the analysis showed a weak association between omega-3 PUFA and leg strength (r=.0205). However, after correcting for protein intake this relationship was no longer significant. A secondary analysis from the placebo group of the Multidomain Alzheimer's Disease Trial found that higher baseline levels of total omega-3 PUFAs in erythrocytes was associated with a slower decline in gait speed over three years. However, in the fully adjusted model that controlled for age, gender, level of education, mini mental state examination score (MMSE), depressive symptoms, body mass index (BMI), physical activity and hand grip strength, this relationship was no longer significant (Fougere et al. 2017). The statistical model used in this study compared those in the lowest quantile of omega-3 PUFA with the combined 2^{nd} to 4^{th} quintile, this method is limited as it is only comparing the trajectory of gait speed in those considered to have low levels of omega-3 PUFAs (omega-3 index of ≤4.89%) versus those not at low levels instead of across a range of omega-3 PUFA levels. Furthermore, as per the inclusion criteria of the study upon which this secondary analysis took place, all participants had a subjective memory complaint, thus these results cannot be extrapolated to a cognitively healthy population.3

There are some consistent limitations of methodologies used in the epidemiological studies investigating the relationships between omega-3 PUFAs and mobility in the older adult. These studies have similar drawbacks as the observational literature on cognitive outcomes with

regards to healthy user bias and a lack of causal inference. Furthermore, it is common in large population studies to use dietary assessment methods to quantify omega-3 PUFA intake, including diet diaries and food frequency questionnaires. Firstly, fish tends to be the primary source of omega-3 PUFAs in the diet (Meyer et al. 2003), however, it may not be consumed every day or even every week, thus a diet diary would be an inefficient method to assess intake. Although dietary intake is the predominant driving factor for erythrocyte levels of omega-3 PUFAs, with an analysis of 704 adults aged 62±12 years finding that omega-3 PUFA intake quantified from self-reported fish oil supplement and fish intake explained 47% of the variance in erythrocyte omega-3 index, other factors such as gender, age and apolipoprotein epsilon genotypes can also influence blood levels (Block et al. 2008). Ideally studies should directly measure fatty acid status to have the most accurate assessment of incorporation and metabolism of omega-3 PUFAs when the aim is quantify the biological effects of omega-3 PUFAs. In the aforementioned population studies that looked at the relationship between omega-3 PUFAs and mobility, the FFQ used by Takayama et al. (2013), had a significant correlation with erythrocyte omega-3 PUFA levels with R values for EPA and DHA of 0.51 and 0.42 respectively. This moderately strong correlation indicates that the assessment method lacks the sensitively to detect subtle differences in omega-3 PUFA levels that direct biochemical measures would be cable of. This could, in turn, influence the results of any correlation analysis. The study by Rousseau et al. (2009) used a diet diary to assess omega-3 PUFA intake, and the authors do not indicate how many days were recorded. Therefore, it cannot be ruled out that the low reported intakes of omega-3 PUFA in this study are a result of inaccuracies in the collection method.

Several of the previously mentioned studies used objective methods of fatty acid quantification by measuring the levels in erythrocytes (Fougere et al. 2017) or plasma (Abbatecola et al. 2009; Reinders et al. 2015; Frison et al. 2017). Despite this method being more reliable than dietary assessment methods, plasma PUFA concentrations are only indicative of PUFA intake over a few days prior to a sample being taken (Hodson et al. 2009), and can be influenced by PUFA intake within the 24 hour period prior to collection (Hodson et al. 2009). Therefore these measures could be influenced by short term fluctuations in diet as a result of social desirability bias. Measuring fatty acids in erythrocytes is recommended as this reflects intake over several months, and has less biological variability than plasma PUFAs (Harris 2007; Harris and Thomas 2010).

Although the epidemiological evidence indicates that there is a relationship between omega-3 PUFAs, mobility and cognition within the older adult, the limitations that have been discussed and the heterogeneity of the studied populations particularly the varying levels of omega-3 PUFA exposure make it difficult the draw definitive conclusions. High quality randomised control trials (RCTs) are clearly required to examine whether this is a causal relationship. These

key limitations along with the lack of causal inference from observational data highlight the requirement for experimental work to test whether supplementation or increased dietary intake of omega-3 PUFAs can indeed improve cognitive outcomes in older adults.

1.5.4.2 Human Intervention Trials

Results of RCTs on omega-3 PUFA supplementation have not been consistent, with some studies showing no effect of omega-3 PUFA on cognitive function (Stough et al. 2012; Jaremka et al. 2014). Furthermore, two separate Cochrane systematic reviews found no effect of omega-3 PUFA supplementation on cognitive function in older adults with AD (Burckhardt et al. 2016) and without cognitive impairment (Sydenham et al. 2012).

There are several major limitations and inconsistencies in previous RCTs investigating the effects of omega-3 PUFA supplementation, with regards to cognition. For example, some studies have included a population group that has already been diagnosed with AD or dementia (Freund-Levi et al. 2006; Kotani et al. 2006; Freund-Levi et al. 2008; Sinn et al. 2012). These participants would have clinically diagnosed memory deficits, thus will have already sustained significant neurodegeneration, which would likely reduce their responsiveness to supplementation (Tarawneh and Holtzman 2012; Cespedes et al. 2017). Omega-3 PUFAs appear to be more effective as a preventative measure as they exert a positive effect on those who are less cognitively impaired (Freund-Levi et al. 2006; Cederholm et al. 2013). Another consideration is the dosage of DHA used in RCTs with supplemental dosages ranging between 156-2000 mg per day. When pooled together the evidence would suggest that higher DHA dosages are more likely to exert a positive effect on cognition in older adults (Figure 7). It must also be noted that these studies varied in other ways beyond the dosage of DHA including the participant demographics, intervention length and the specific domains of cognition that were tested. There is currently limited dose-response data from randomised trials on omega-3 PUFA supplementation on cognitive outcomes. A meta-analysis of the published literature including fifteen RCTS that supplemented healthy older adults and those with mild memory complaints with EPA+DHA does provide some insight into a potential dose-response for omega-3 PUFA supplementation. The authors found that combined dosages of 1 g EPA+DHA including a minimum of 580 mg DHA were required to elicit positive effects on episodic memory in adults with memory complaints and without (Yurko-Mauro et al. 2015). Although this finding cannot be extrapolated beyond this single cognitive domain, this study provides evidence that higher dosages of supplementation, particularly DHA, appear to show greater efficacy in preserving cognition in older adults. Certainly more work is required to further establish any dose threshold for effect detection or the shape of the dose-response curve for omega-3 PUFA supplementation on cognitive outcomes. Given, the high enrichment of DHA in the brain, the previously

discussed pre-clinical work establishing unique mechanisms of DHA on neuronal function and this data from human RCTs, a dosage of 1 mg EPA+DHA including a minimum of 580 mg DHA should be used in future experimental work. Although this level of intake would require individuals to exceed current recommendations set by SACN (SACN, 2004), other countries such as Japan have demonstrated this is achievable and safe with many people consuming over 1000 mg of preformed EPA+DHA per day (Matsuoka et al. 2017).

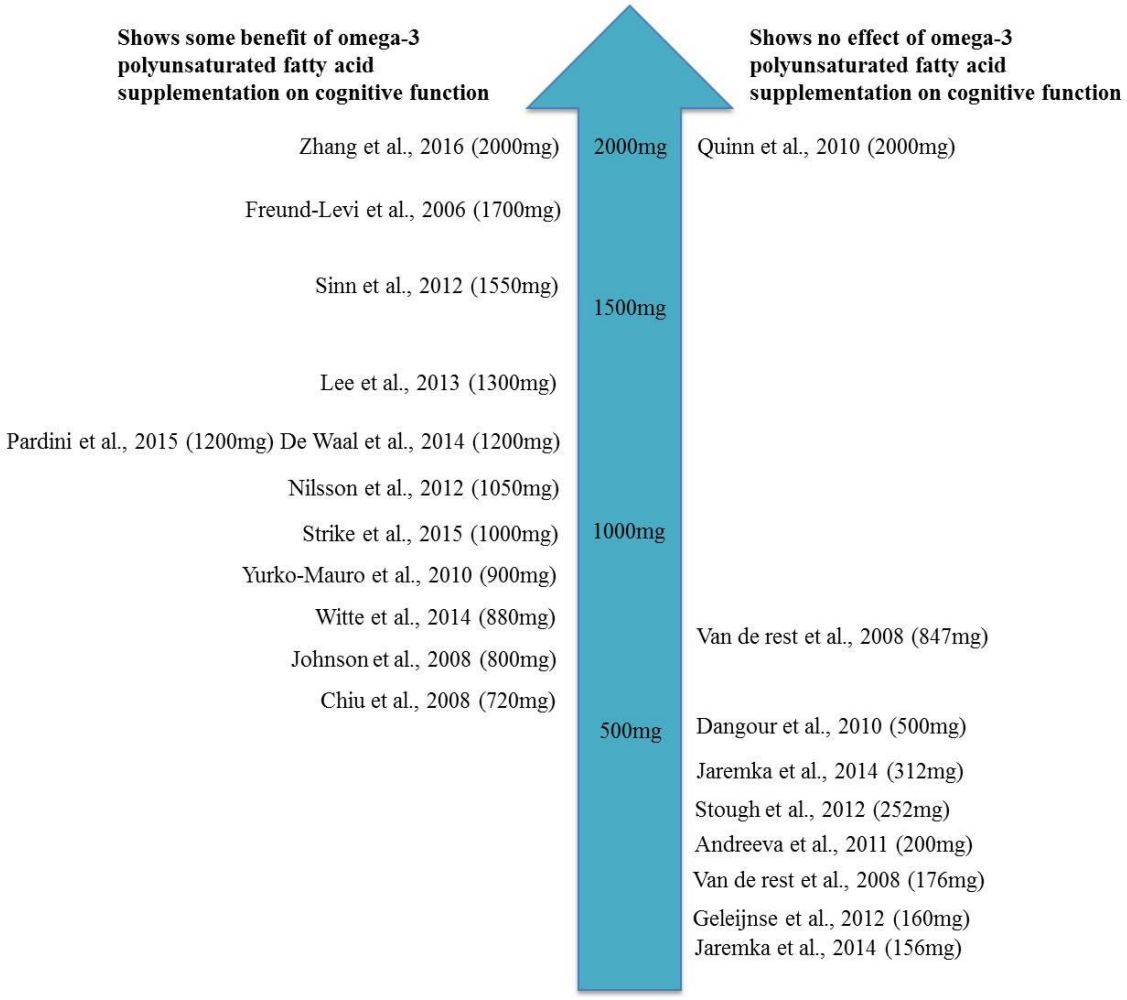


Figure 7 *Studies investigating the effects of omega-3 polyunsaturated fatty acids on cognitive function in older adults sorted by their docosahexaenoic acid content. Dosage of docosahexaenoic acid appears to have an influence on whether a positive result on an area of cognitive function is detected. Higher doses appear to be more likely to have beneficial results. Figure adapted from Ismail (2015).*

The benefit of omega-3 PUFAs on cognition, evidenced in clinical trials has largely been limited to a few domains including verbal memory, immediate recall and attention (Yurko-

Mauro et al. 2010; Sinn et al. 2012; Lee et al. 2013; Zhang et al. 2016b; Hooper et al. 2017); however, this does not diminish the role omega-3 PUFA could have on health outcomes as these cognitive processes are associated with gait speed and activities of daily living (Bruce-Keller et al. 2012; de Paula et al. 2015). There is some evidence for a modulation of other cognitive domains, as 26 weeks of supplementation with 1320 mg EPA and 880 mg DHA omega-3 PUFA improved executive function by 26% versus placebo in a group of 65 adults aged 64±7 (Witte et al. 2014). The potential to have a positive influence on executive function would be important within the context of healthy ageing, particularly considering the strong link between this domain and habitual and dual-task gait outcomes (Doi et al. 2014).

Data from trials of omega-3 PUFAs on gait and other mobility outcomes are scarce, however, thus far research has provided some promising results. An RCT in 126 post-menopausal women assigned to receive 720mg EPA and 480mg DHA or placebo over six months found that supplementation led to a significant 0.06m/s increase in gait speed versus placebo (Hutchins-Wiese et al. 2013). It is important to note that additionally to reaching statistical significance, it also met the criteria for clinically meaningful changes with a medium effect size (Kwon et al. 2009). Furthermore, the study showed that omega-3 PUFA supplementation maintained participants above a gait speed of 1.0m/s which is suggested as the threshold for healthy ageing (Studenski et al. 2011).

There is some evidence which suggests that omega-3 PUFAs could be a potentially useful therapeutic agent for the prevention of muscle atrophy in older adults. A cross-sectional and retrospective cohort study analysed the relationship between diet and grip strength in 2,983 older men and women (Robinson et al. 2008). The consumption of each additional portion of fatty fish per week determined an increase in grip strength, a commonly used measure of muscle strength in older adults, of 0.48 kg in women. Animal and human studies have indicated that the effect on muscle strength could be mediated by an increase in MPS (Gingras et al. 2007; Smith et al. 2011a; Kamolrat et al. 2013). There evidence to suggest that the effect on muscle strength and MPS may be limited to older adults who commonly experience anabolic resistance, a phenomenon where muscle tissue becomes less responsive to normal anabolic stimuli (Breen and Phillips 2011). A study in young healthy adults yielded null effects of 8 weeks of omega-3 PUFA supplementation (3500 mg EPA and 900 mg DHA), following resistance exercise and ingestion of 30 g of whey protein (McGlory et al. 2016).

An RCT in 60 older adults examined the effects of omega-3 PUFA supplementation (1.86g EPA and 1.5g DHA) or placebo for six months. Supplementation did not affect body weight, total body fat mass or intramuscular fat, but lead to greater thigh muscle volume, grip strength and one repetition maximum muscle strength (assessed via sum of scores on leg press, chest press, knee extension and knee flexion) versus placebo (Smith et al. 2015). These results were

achieved despite a lack of exercise intervention, and participants were required to take part in less than 90 minutes of exercise per week as per the exclusion criteria. However, no restrictions were placed on physical activity and this was not monitored so it is unclear whether there were differences in activity between groups, or whether similar results could have been achieved in a completely sedentary population. Furthermore, other dietary factors were not controlled for, which is important considering the role energy balance and dietary protein plays in muscle atrophy in older adults (Weinheimer et al. 2010; Arciero et al. 2013).

Overall the evidence from human intervention trials suggests that omega-3 PUFA particularly DHA may exert a neuroprotective effect in older adults who are not already cognitively impaired. In regards to cognition the dosage of DHA appears to be of particularly importance, this is in agreement with the previously discussed mechanistic and observational literature, with dosages exceeding 580 mg per day being required to elicit positive effects on memory. Whilst the data on cognition is promising it is important to note that omegs-3 PUFAs may only be effectively for specific cognitive domains, with effects being shown on verbal memory, immediate recall and attention. Emerging evidence also suggests a role for omega-3 PUFAs in the prevention of muscle atrophy with mechanistic underpinnings related to cell signalling (Smith et al. 2011a; Smith et al. 2015). A positive influence on both cognitive and muscle function could result in clinically relevant changes in gait speed as shown by Strike et al. (2016) and Hutchins-Wiese et al. (2013), and thus greatly influence the functional trajectory of older adults.

1.6 Phosphatidylserine

1.6.1 Synthesis and Function

It is well established that the lipid bilayer is essential to both the structure and function of the cell. Alterations in the phospholipid subclasses, as well as the fatty acids enriched within these phospholipids, can alter the function of a cell by influencing membrane fluidity as well as changing the composition of lipid rafts which in turn affects protein docking sites and cell signalling (Kim et al. 2004; Kim et al. 2010). Phospholipids have a polar head group and two hydrophobic hydrocarbon tails. The tails are usually fatty acids with a saturated fatty acid at the sn-1 position and an unsaturated fatty acid at the sn-2 position. PS is the major acidic phospholipid class that forms part of the lipid bilayer of human membranes and accounts for 13-15% of the phospholipids in the human cerebral cortex (Kim et al. 2014).

Throughout the human body, PS is a structural component of endoplasmic reticulum, nuclear envelopes, Golgi apparati, cytosolic leaflets of plasma membranes, outer mitochondrial membranes, and myelin. PS is highly enriched in the brain, particularly at synaptic membranes,

the hippocampus and the olfactory bulb (Kim et al. 2014). The PS in the brain is typically in the form of 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoserine, (stearic acid at the sn-1 position and DHA at the sn-2 position (Akbar et al. 2005; Kim 2007) (*Figure 8*).

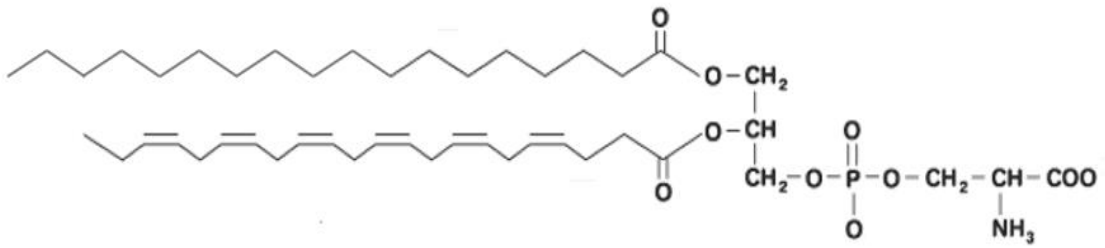


Figure 8 *Structure of 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoserine, the most abundant form of phosphatidylserine in the human brain. Amended from National Center for Biotechnology Information (2018).*

In mammalian tissues, PS is exclusively synthesized in the endoplasmic reticulum from either PC or PE by calcium dependent base-exchange reactions where the head group of the substrate phospholipids is replaced by serine. These base-exchange reactions are catalysed by phosphatidylserine synthases (PSS). There are two different PSS isoforms, PSS1 and PSS2. PSS1 catalyses the conversion of PC and PSS2 converts PE (Vance and Tasseva 2013) (Figure 9).

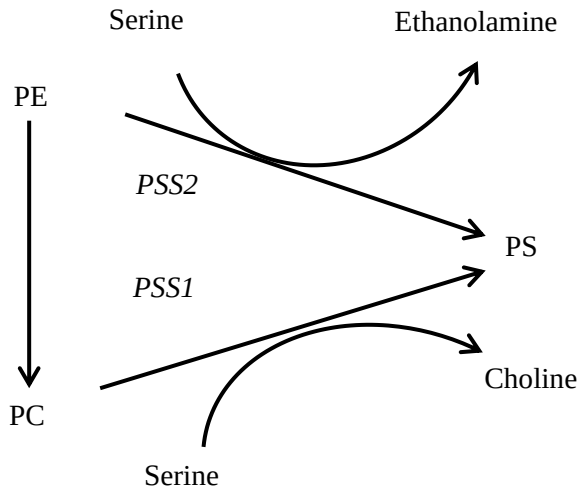


Figure 9 *Processes by which phosphatidylserine (PS) is synthesised from phosphatidylcholine and phosphatidylethanolamine, through base-exchange reactions catalysed by phosphatidylserine synthase 1 (PSS1) and phosphatidylserine synthase 2 (PSS2) (Kim et al. 2014).*

PS participates in key signalling pathways in the neuronal system. The functions of PS are dependent on it being enriched in cellular membranes, where it binds and activates cytosolic proteins involved with neuronal signalling including protein kinase B, protein kinase C and proto-oncogene serine/threonine-protein kinase which are known to stimulate neuronal survival, neurite growth and synaptogenesis (Newton and Keranen 1994; Kim et al. 2000; Huang et al. 2011). DHA is highly enriched in PS and makes up over a third of total fatty acids and 80% of the PUFA in grey matter (Kim et al., 2014), as a consequence the incorporation of PS into cell membranes is reliant on the availability of both PS and DHA (Kimura and Kim 2013).

Supplemental DHA has been shown to increase levels of PS in neuronal tissues (Akbar et al. 2005; Guo et al. 2007). PS has also been shown to be integral to the neuroprotective effects of DHA, thus enrichment into PS could be key to DHAs effects on cognitive function in older adults (Akbar et al. 2005; Kim et al. 2010), and a reduction in the DHA content of PS mid-frontal cortex and superior temporal cortex is associated with the progression of MCI to AD (Cunnane et al. 2012).

1.6.2 The Effects of Phosphatidylserine on Mobility and Cognition in Older Adults

Due to its enrichment in the human brain, studies on supplemental PS have largely focused on its effects on cognitive function. Early supplement trials used PS derived from bovine cortex and found positive results in participants with dementia (Delwaide et al. 1986) and memory complaints (Crook et al. 1991).

More recently daily supplementation for six weeks with 300 mg/day PS from soy lecithin containing 37.5 mg of EPA+DHA has been shown to improve verbal memory in adults aged ≥60 years with subjective memory complaints (Richter et al. 2010). This study was conducted as a pilot study and had only eight participants, thus statistical analysis to assess treatment effects should not have been conducted. Furthermore, the study had a total of seventeen cognitive outcomes and the authors did not correct for multiple comparisons in their statistical model. Improved verbal memory, assessed using the Rey's auditory verbal learning test (RAVLT), was also observed in a double blind, placebo controlled clinical trial in 152 older adults with subjective memory complaints supplementing with 300mg of PS combined with 78mg of EPA and DHA for 15 weeks (Vakhapova et al. 2010). Furthermore, an open label extension of this trial in which all participants received 100mg PS with 26mg EPA and DHA per day for 15 weeks, found that those who continued to supplement did not show any additional cognitive benefits but did maintain their performance and the former placebo group had an improvement in sustained attention and memory recognition assessed using the computerised neuropsychological assessment tool, NexAde^{tm} (Vakhapova et al. 2014). A similar double blind study was performed in 78 Japanese adults aged between 50-69 years with subjective memory complaints, supplementing with 100 mg and 300 mg of soy lecithin derived PS for six months. Overall there was no difference in cognitive performance between the active and placebo groups. However, in the participants with the lowest score at baseline, there was some improvement on the Hasegawa's dementia scale and MMSE versus placebo (Kato-Kataoka et al. 2010). This study demonstrates that PS supplementation may only be effective in those who are more cognitively impaired. However, it should be noted that this sub analysis reduced the group numbers to 11, 9 and 14 and there was no indication whether this stratified analysis was adequately powered to draw definitive conclusions.

The current body of evidence exploring the effects of supplemental PS on cognition in older adults does show some promise. However, several studies appear underpowered and have been limited to older adults with subjective memory complaints. PS preparations are often enriched with DHA (Vakhapova et al. 2010). Supplemental PS will be subject to partial or complete hydrolysis during digestion, therefore the beneficial effects of PS on cognition, particularly

from krill or bovine sources are possibly produced by DHA released from the PS rather than the intact PS itself (Cohn et al. 2010). However, it must also be noted that the effect of DHA on signalling pathways related to neuroprotection have been shown to be dependent on DHA being enriched in PS (Akbar et al. 2005). It is assumed that positive effects on cognition by supplemental PS will be as a result of the incorporation of PS into neuronal membranes. There is preliminary evidence from animal models that orally administered PS does alter the structure of neuronal membranes (Nunzi et al. 1989). However, there is currently no evidence in humans that supplemental PS crosses the blood-brain barrier, if it crosses intact and if it results in an increase in PS in neurons or glial cells.

Overall from a mechanistic standpoint, there is strong evidence for the critical role PS plays in the function of the human brain, and the interaction between PS and DHA. Early studies on supplemental PS have yielded some positive results, however, it remains unclear whether this is due to PS supplementation or its prior enrichment with DHA. Furthermore there is a lack of evidence in humans demonstrating that supplementing with PS can increase tissue levels, particularly in the brain where it is most likely to exert any positive effect.

1.7 Vitamin E

1.7.1 Dietary Sources and Nutrient Status in Older Adults

Nuts, seeds, dark green leafy vegetables and cereals are all good dietary sources of vitamin E. There is currently not a defined reference nutrient intake for vitamin E in the UK, however in the United States of America, the recommended daily allowance is 15 mg. Data from the Hertfordshire Cohort Study including 1,414 women aged 59 to 73 found median dietary intake of vitamin E, assessed via a 129 item food frequency questionnaire, to be 9.8 mg per day (Robinson et al. 2008).

1.7.2 Metabolism and Key Roles

Vitamin E actually refers to eight different compounds, including four tocopherols and four tocotrienols. The different tocopherols and tocotrienols are identified by the prefixes alpha (α), beta (β), gamma (γ) and delta (δ). α-tocopherol is the most biologically active form of vitamin E and is the second most abundant form in the diet after γ-tocopherol (Brigelius-Flohe 2006). ***α***-tocopherol accumulates in cellular membranes of tissues, whereas the other isoforms are rapidly metabolised and excreted (Raederstorff et al. 2015). α-tocopherol has been studied for its role in protecting cell membranes from oxidative damage caused by free radicals (Krajcovicova-Kudlackova et al. 2004). The fatty acids in cell membranes are particularly

susceptible to oxidative damage, for two key reasons. Firstly oxygen and free radicals are more soluble in the fluid lipid bilayer than in the aqueous solution (Codorniu-Hernandez and Kusalik 2012). Membranes contain an interior organic phase, in which oxygen tends to concentrate, therefore membrane lipids become primary targets of oxidative damage (Cordeiro 2014). The second property is related to the fact that PUFAs enriched in phospholipids are extremely sensitive to oxidation. The presence of a methylene group between two double bonds renders PUFAs sensitive to free radical damage (Bielski et al. 1983). The sensitivity to peroxidation of a specific PUFA increases exponentially as a function of the number of double bonds per fatty acid molecule, which makes DHA particularly susceptible (Bielski et al. 1983). Free radicals react with a hydrogen atom from PUFA side chains, removing it from the carbon backbone of the fatty acid, creating a lipid radical (Ayala et al. 2014). This lipid radical rapidly reacts with oxygen, producing a lipid peroxyl radical. Next, the lipid peroxyl radical reacts with another unsaturated lipid to form a lipid hydroperoxide and a new lipid radical, leading to a chain reaction (Pratt et al. 2011). Lipid hydroperoxides are more hydrophilic than unperoxidised fatty acid side chains (Pamplona 2008). They try to migrate to the membrane surface to interact with water, thus disrupting the membrane structure, altering fluidity and other functional properties (Wong-ekkabut et al. 2007). α-tocopherol acts as a free radical scavenger reacting with lipid peroxyl groups inside membrane bilayers, reducing them to hydroperoxides, thus inhibiting the propagation of the previously mentioned peroxidative chain reaction (Esterbauer et al. 1991) (*Figure 10*).

Vitamin E has a higher affinity for accumulation into domains of cell membranes that are enriched with DHA, where it exerts its main functions: to stabilise the membrane and protect the highly unstable omega-3 PUFA from lipid peroxidation by free radicals (Atkinson et al. 2010). This affinity with DHA was demonstrated in a mouse model specifically designed to induce α-tocopherol deficiency. The retina, an area that is normally highly enriched with DHA, became depleted of DHA which was also replaced by AA, suggesting that synthesis of AA to replace DHA was occurring, which provides evidence that vitamin E deficiency allowed DHA depletion (Tanito et al. 2007).

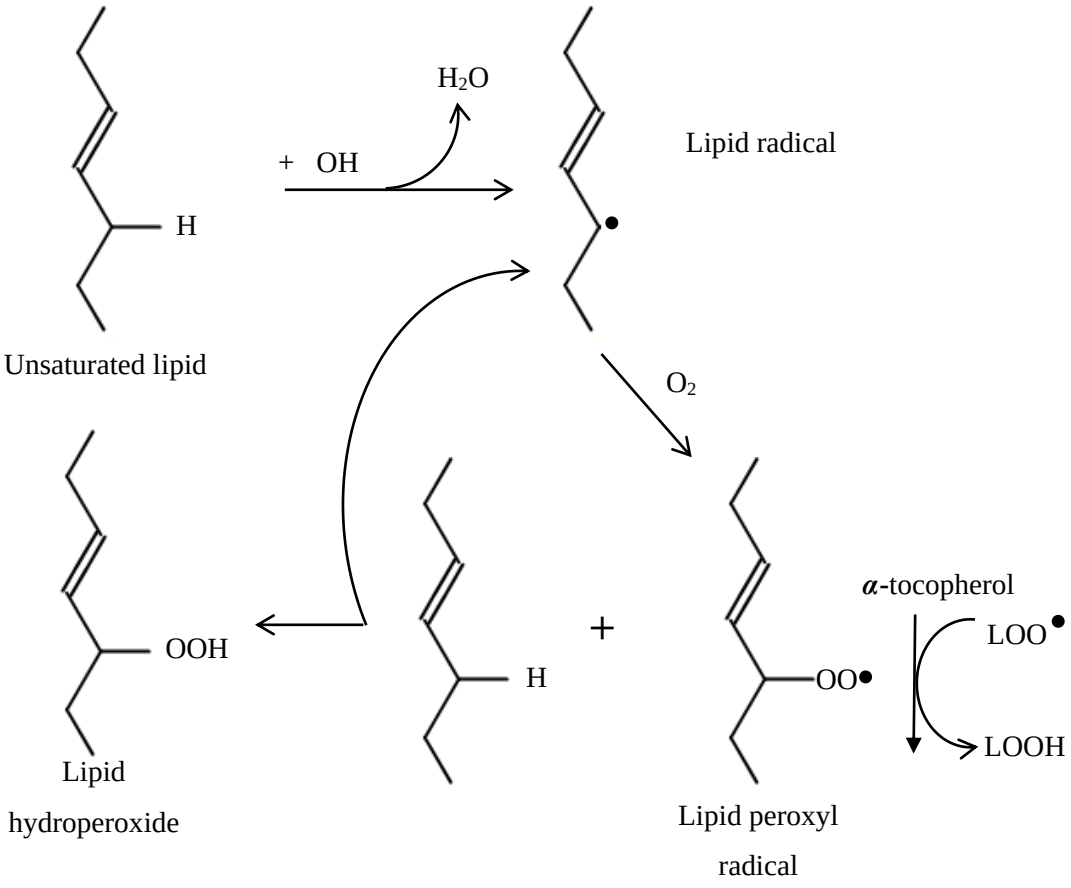


Figure 10 *Mechanism of lipid peroxidation in an unsaturated fatty acid molecule. Free radicals react with a hydrogen atom from PUFA side chains, creating a lipid radical. Lipid radicals rapidly react with oxygen, creating a lipid peroxyl radical. Next, the lipid peroxyl radical reacts with another unsaturated lipid to form a lipid hydroperoxide as well as a new lipid radical, which initiates a chain reaction. α-tocopherol acts by donating a single electron to a lipid peroxyls unpaired electron (•) reducing them to hydroperoxides, thus inhibiting the propagation of the previously mentioned peroxidative chain reaction. Amended from Ayala et al. (2014).*

1.7.3 The Effects of Vitamin E on Mobility and Cognition in Older Adults

There is limited evidence on how vitamin E may impact mobility in older adults, however greater dietary intake of vitamin E was associated with higher grip strength in 2,983 older men and women (Robinson et al. 2008). Epidemiological studies have shown consistent associations between serum α-tocopherol and cognitive performance in older adults (Perkins et al. 1999; Ortega et al. 2002; Mangialasche et al. 2013). Considering the link between oxidative stress and brain pathology (Agostinho et al. 2010), and α-tocopherol's role as an antioxidant this has led to investigations of the effects of supplemental vitamin E on cognition in older adults.

A three year study supplemented 769 older adults with MCI, with 2,000 IU (1800 mg) α-tocopherol per day. Vitamin E supplementation did not influence progression towards AD versus placebo (Petersen et al. 2005). A follow up study in 131 of the participants from the Petersen et al. (2005) study used magnetic resonance imaging and found no significant effect of vitamin E supplementation on brain atrophy (Jack et al. 2008). One study in 613 patients with mild to moderate AD found that supplementation with 2,000 IU (1800 mg) α-tocopherol per day for six months to four years slowed down functional decline assessed via the Alzheimer's disease Cooperative Study/Activities of Daily Living Inventory (Dysken et al. 2014). 4

One important factor to consider in regards to the literature on vitamin E supplementation is the baseline nutrient status of the participants. Following a supplementation period of 9.8 years with 600 iu (540 mg) of α-tocopherol on alternate days within the Women's Health Study, including data from 39, 876 healthy women no effect on cognition was observed. However when participants were stratified by baseline intake of vitamin E, those with habitual intake of less than 6.1 mg per day had improved global cognition versus the placebo group (Kang et al. 2006). Within the aforementioned RCTs conducted by Dysken et al. (2014) and Petersen et al. (2005) there is a lack on consideration for the baseline nutrient status and how this may have impacted response to supplementation. Neither study limited participation based on dietary intake and whilst Dysken et al., (2014) did measure vitamin E status via serum samples baseline status was not reported as such it is unclear as to whether baseline intake of vitamin E could have been a factor in the incongruence of the results between these trials.

Overall the current evidence would suggest that vitamin E supplementation may slow down the decline in functional capacity in those with mild symptoms. There is little evidence exploring the effect vitamin E has on specific cognitive domains and no evidence of effects on mobility. However, there is a clear interaction between DHA and vitamin E, whereby vitamin E protects membrane bound DHA from peroxidation, therefore the role vitamin E may play in preventing functional decline is likely defined by this role (Atkinson et al. 2010). Within the aforementioned RCTs conducted by Dysken et al. (2014) and Petersen et al. (2005) there is a lack on consideration for the baseline nutrient status and how this may have impacted response to supplementation. In the study by

1.8 B Vitamins

1.8.1 Dietary Sources and Nutrient Status in Older Adults

Fish, molluscs, meats, eggs and fortified cereals are all considered good sources of vitamin B12 and folic acid is contained in dark green leafy vegetables, cruciferous vegetables, beans and pulses (Mendonca et al. 2016). Current UK reference nutrient intake for B12 and folic acid in

women aged ≥50 are 1.5 μg and 200 μg respectively (Committee on Medical Aspects of Food Policy 1991). Older adults are particularly susceptible to B12 deficiency due to lower food intake and reduced gastrointestinal absorption, and despite often meeting UK recommendations, they often have low plasma B12 levels (Mendonca et al. 2016). Folate deficiency is less common than B12 deficiency in older adults, however, still occurred in 12.4% of women aged ≥65 according to data from the NDNS (NDNS, 2015) . Older adults often have deficiencies in B12 and folic acid which is thought to be the predominant reason why serum homocysteine levels are found to be significantly higher in older versus younger adults (Bates et al. 2010).

1.8.2 B Vitamin Metabolism and Key Roles in Relation to Ageing

To become metabolically active, folic acid must first be converted to dihydrofolate and then tetrahydrofolate through enzymatic reduction (Shane 2008). Thereafter, tetrahydrofolate can be converted to the biologically active 5-methyltetrahydrofolate by the enzyme methylenetetrahydrofolate reductase. 5-methyltetrahydrofolate is then used for methyl donations for nucleic acids, neurotransmitters, phospholipids, and hormones (Greenberg et al. 2011). Folate acts as a cofactor for enzymes involved in DNA and RNA biosynthesis (Crider et al. 2012).

Malabsorption of vitamin B12 from food is the predominant cause of low circulating vitamin B12 concentrations in older adults, with 60% of cases of deficiency being caused by malabsorption (Andres et al. 2004). Absorption of vitamin B12 from dietary sources is complex and requires several steps that involve the stomach, pancreas and small intestine (Watanabe 2007). Malabsorption of vitamin B12 due to dysfunction at any of these steps can potentially lead to vitamin B12 deficiency (Allen 2008). A deficiency in vitamin B12 can also result in a functional folate deficiency, as folate becomes trapped in the form of methyltetrahydrofolate (Reynolds 2006).

The functions of these two vitamins are strongly linked due to their complementary roles in the methylation of homocysteine to form methionine (the methionine cycle) (Shane 2008). Homocysteine is a non-protein-forming, sulphur amino acid formed as a by-product of methyl-transfer reactions in methionine metabolism, and is not a normal dietary constituent (Selhub 2008). In the methionine cycle the folate cofactor, 5-methyltetrahydrofolate, donates its methyl group to a vitamin B12 dependent enzyme, methionine synthase, which recycles homocysteine back to methionine (Miller 2003). Homocysteine can also be metabolised through the transsulfuration pathway where vitamin B6 is required as a cofactor, (*Figure 11*).

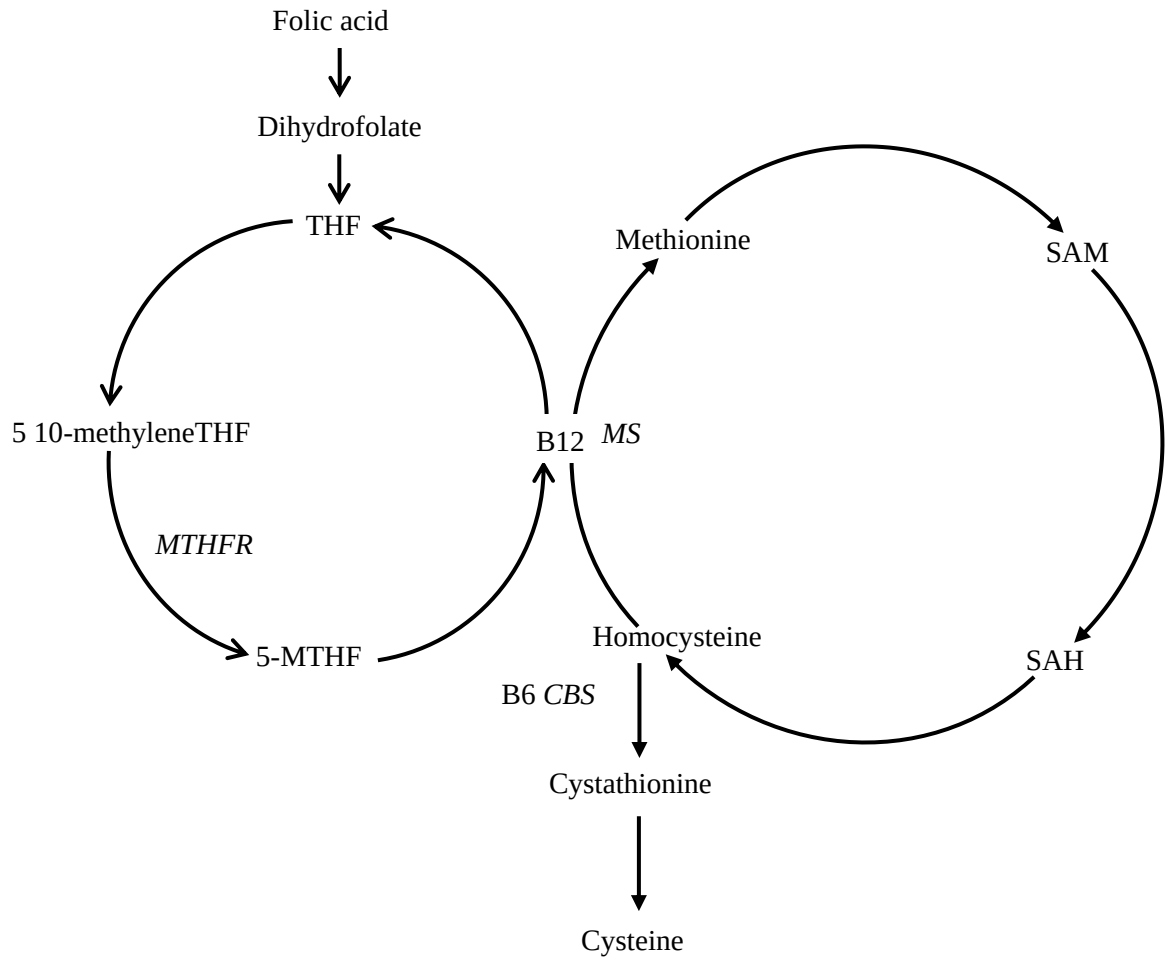


Figure 11 *The methionine homocysteine cycle contains re-methylation and transsulfuration components. In the methylation component dietary folic acid must be first converted to 5-methyltetrahydrofolate. 5-methyltetrahydrofolate donates a methyl group using vitamin B12 as a cofactor to convert homocysteine into methionine. In the transsulfuration pathway homocysteine is converted in cysteine by a series of reactions using vitamin B6 as a cofactor. Abbreviations tetrahydrofolate (THF), 5 10-methylenetetrahydrofolate (5 10-methyleneTHF), methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate (5-MTHF), methionine synthase (MSy), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cystathionine beta synthase (CBS). Amended from Selhub et al. (2000).*

The potential role B12 and folate vitamins can play in the health of older adults have largely focused on their effects on cardiovascular and cognitive outcomes, under the hypothesis that by lowering homocysteine this would exert a positive effect on these outcomes (McCully 1969; McCaddon and Kelly 1992)**.**

There are some potential mechanisms by which homocysteine might influence the health of older adults. However, the underlying studies were largely performed in animal or *in vitro* models and there is a lack of current evidence in humans. Mechanisms include increasing the

vulnerability of hippocampal neurons to excitotoxic and oxidative injury by causing DNA damage which leads to activation of poly (ADP-ribose) polymerase and mitochondrial dysfunction (Kruman et al. 2000), potentiation of amyloid neurotoxicity (Ho et al. 2001), promotion of tau phosphorylation (Zhang et al. 2008) and reducing glutathione production, resulting in a reduced capacity to handle oxidative stress (Toroser and Sohal 2007). Interestingly in the study by Ho et al. (2001) when vitamin E and N-acetyl-L-cysteine were added to a cell culture model of amyloid β toxicity, they blocked the homocysteine induced cell apoptosis suggesting that the negative effect of homocysteine was a result of oxidative stress, and that vitamin E along with and N-acetyl-L-cysteine could play an important role in negating this effect.

1.8.3 The Effects of B vitamins on Mobility and Cognition in Older Adults

1.8.3.1 Observational Evidence

In perhaps one of the most comprehensive reviews of the observational literature, elevated homocysteine along with low education attainment and low physical activity were identified as having the strongest associations with cognition and dementia. Overall, 16 of 19 cohort studies and 12 of 14 cross-sectional studies confirmed an association between high homocysteine and impaired cognition. Considering the incidence of AD as an outcome, the pooled relative risk associated with high blood homocysteine concentrations from 5 prospective studies was 1.93 (CI95%: 1.50–2.49). The population attributable risk percentage for high compared with low homocysteine was 21.7% (CI95%: 12.8%–30.6%). This is in agreement with another recent meta-analysis that estimated the risk reduction for dementia achievable with folic acid and vitamin B12 treatment to be 22% (CI95%: 7%–34%) for a 3 -µmol/L decrease in serum homocysteine, if the relationship between homocysteine and dementia is indeed causal (Wald et al. 2011).

In human studies within cognitively healthy participants, lower levels of plasma B12 (<308 pmol/L) have been found to be associated with a six fold increase in rates of brain atrophy over 5 years (Vogiatzoglou et al. 2008). Brain atrophy was also positively associated with plasma homocysteine at baseline but not over the 5 year follow up period. Furthermore, a cross-section evaluation of 156 older adults with no cognitive impairment found an inverse relationship between plasma homocysteine and the size of the hippocampus, a region of the brain which has been demonstrated to atrophy during healthy and pathological ageing (O'Shea et al. 2016).

These studies indicate that even in cognitively healthy older adults homocysteine could be a factor in brain atrophy (Williams et al. 2002). A similar trend has also been observed in the studies that have explored the relationship between homocysteine and cognitive function. The Hordaland Homocysteine Study found that participants with a lower episodic memory score

(<25 on the Kendrick Object Learning Test) had significantly higher plasma homocysteine levels (12.6 μmol/L versus 11.5 nmol/L) and lower plasma folate levels (6.7 μmol/L versus 7.6 nmol/L) compared with those with a score ≥25 (Nurk et al. 2005). The observational literature does indicate a concentration-response relationship for homocysteine and cognition. An example of a potential exposure threshold was demonstrated in an Italian cohort consisting of 816 older adults without cognitive impairment followed up over four years. Overall baseline plasma hyperhomocysteinemia defined as >15 μmol/L was associated with an increased risk in dementia diagnosis (HR:2.08, CI95%: 1.31, 3.30), but cumulative incidence of dementia in those with homocysteine levels <10.1 μmol/L was 5 fold lower than those with hyperhomocysteinemia (Ravaglia et al. 2005). As a result several prospective cohorts have used cut off points to define elevated homocysteine and in a meta-analysis elevated plasma homocysteine was associated with an increased risk (RR:1.53, CI95%: 1.23, 1.91) for cognitive decline in 15, 908 healthy older adults compared to non-elevated levels (Nie et al. 2014). Although this does provide further evidence for a negative relationship between homocysteine and cognition in the older adults as well as perhaps an exposure threshold, the cut offs for elevated homocysteine for the included cohorts varied between 10.8 and 27.5 μmol/L so a precise cut off for elevated risk cannot be established based on this evidence alone.

The impact of homocysteine is likely not limited to cognition. Plasma homocysteine levels of above 15μmol/L were positively associated with the prevalence of physical frailty in 4,248 men aged 70-80 years (OR: 1.49, CI95% 1.22, 1.81) (Wong et al. 2013). In a study of 499 healthy older adults aged 74±3 years with mean plasma homocysteine of 11.6 ±4.3 μmol/L, every SD increase of homocysteine was associated with a greater risk for being in the worst quintile of physical health assessed via a battery of tests of balance, gait, lower body strength and coordination, and manual dexterity (Kado et al. 2002). In a cohort of 1,667 adults aged 70±7 plasma homocysteine concentrations of 11.25 μmol/L were associated with increased odds for inability to perform activities of daily living (OR 2.18, CI95% 1.32, 3.59), as well as lower HGS when compared to those with levels <7.42 μmol/L. (Kuo et al. 2007). Serum homocysteine was shown to be positively associated with slower gait speed at baseline in 574 older adults with no cognitive impairment, and those in the highest quartile of homocysteine (>15 μmol/L) had a faster rate of gait speed decline over 1.4 years versus those with levels of ≤10.8 μmol/L (Rolita et al. 2010).

Together with the mechanistic data the observational research provides evidence for a negative association between homocysteine and outcomes related to healthy ageing in older adults. Given the role that B vitamins play in the lowering of homocysteine, these lines of evidence support the plausibility of B vitamin intake being able to promote healthy ageing, in part through the preservation of cognitive function. However, the question still remains as to whether elevated

homocysteine actually plays a causal role in the development of age related disease thus the efficacy of B vitamin supplementation requires testing in randomised placebo controlled trials.

1.8.3.2 Human Intervention Trials

Despite the strong observational evidence and mechanistic underpinnings for the importance of B vitamins in older adults, and this effect being mediated by a reduction in homocysteine, RCTs using B vitamin supplements have yielded mixed results**.**

There have been some RCTs that have shown benefits of B vitamin supplements. Two years of supplementation with 0.8 mg folic acid, 0.5 mg B12 and 20 mg B6 in a double blind placebo controlled study in 266 older adults with MCI improved MMSE, episodic and semantic memory versus the placebo. However, it is important to note that this was observed in the supplemented participants, who at baseline had higher plasma homocysteine levels (de Jager et al. 2012). Three year treatment with folic acid supplements in 818 adults aged 50-70 years with elevated plasma homocysteine at baseline (≥13 μmol/L) also led to improvements in memory, processing speed and sensorimotor speed versus a placebo (Durga et al. 2007). Most recently, two years of high B vitamin supplementation (folic acid 0.8 mg, B12 0.5 mg, vitamin B6 20 mg) slowed the rate of brain atrophy, measured via magnetic resonance imaging, by 32% versus placebo in 187 older adults with MCI (Smith et al. 2010a). This result was significant for the entire cohort but similarly to the aforementioned study by de Jager et al. (2012), baseline levels of homocysteine played a role, as those with levels above ≥13 μmol/L at baseline had a 53% reduction in brain atrophy versus placebo. These results showed practical implications as the rate of brain atrophy was associated with lower final cognitive scores. Together these results suggest that baseline homocysteine may be a considerable factor in the effectiveness of B vitamin supplementation and that the relationship between homocysteine and cognition may not be linear. Participants with baseline levels below 11.1 μmol/L do not appear to benefit from supplementation. This is consistent with the observational literature that suggested an exposure threshold effect for homocysteine on cognitive function. However, the effect of B vitamin supplementation in those with elevated homocysteine has not been consistent across all trials. A two year study supplementing daily with 500μg B12 and 400μg folic acid found no effect on episodic memory, attention and working memory, information processing speed and executive function in a sample of 720 adults aged 74±6.5 with homocysteine levels above 12 μmol/L (van der Zwaluw et al. 2014). Twelve months of daily supplementation with 1mg vitamin B12 in 191 in older adults with mild B12 deficiency (serum concentrations: 107–210 pmol/L) and serum homocysteine ≥13 μmol/L had no effect on verbal memory, reaction time or verbal fluency versus placebo (Dangour et al. 2015). The only RCT to date investigating the effects of B vitamins on physical performance supplemented 2,919 adults aged 74±6 with 500 μg vitamin

B12, 400 μg folic acid, and 600 IU vitamin D for two years (Swart et al. 2016). The authors found no effects on physical performance decline, defined using gait outcomes and ability to stand from a chair, hand grip strength or risk of falls. An important factor in this study and the studies by Dangour et al. (2015) is that despite lowering homocysteine levels significantly in the active groups, levels reached 14.2 μmol/L and 12.1 μmol/L. The aforementioned observational data from Wong et al. (2013) and Nurk et al. (2005) suggest that at these levels homocysteine would still be associated with negative cognitive and mobility outcomes (Nurk et al. 2005; Kuo et al. 2007). Furthermore, these levels are notably higher than the studies by de Jager et al. (2012), Durga et al. (2007) and Smith et al. (2010a) which all achieved levels ≤10.1 μmol/L in the active groups.

Overall, there is some supporting evidence from observational and pre-clinical studies for the role of B vitamins in preventing age related decline, and that this effect is likely mediated by lowering homocysteine. However, clinical trials have thus far yielded mixed results. This could be due to the differing levels of homocysteine at baseline and the magnitude of change in homocysteine during the trial. There is a need for further work to better establish a more precise threshold upon which benefits to healthy ageing outcomes could be expected from lowering homocysteine. The current evidence suggests that B vitamins may only be effective in those with elevated levels of homocysteine at baseline and that homocysteine may need to be lowered to ≤10.1 μmol/L to see an effect.

1.9 Ginkgo Biloba

1.9.1 History of Traditional Use

Ginkgo Biloba is derived from the leaf of the Maidenhair tree and is one of the most widely used and studied herbal extracts for cognitive impairment and dementia in older adults. The interest in the potential medicinal properties of *Ginkgo Biloba* can be traced back 5,000 years to ancient China (McKenna et al. 2001). In recent years most research into *Ginkgo Biloba* has focused on its effects on cognitive disorders and vascular conditions (Zhang et al. 2016a). In the UK *Ginkgo Biloba* supplements have been given traditional herbal registration. However, this is to relieve the symptoms of Raynaud's syndrome and tinnitus and is based on traditional use only (Medicines & Healthcare products Regulatory Agency 2016).

1.9.2 Metabolism and Key Roles

The active components of *Ginkgo Biloba* are flavonoids, terpenoids, and terpene lactones (ginkgolides and bilobalide) (Ude et al. 2013). With the exception of ginkgolides and bilobalide,

these compounds are found extensively in plants, including a wide range of fruits and vegetables (Liu 2013). A standardised extract, EGb761, is produced from the ground leaves and is used for dietary supplements (Birks et al. 2002).

The mechanisms by which *Ginkgo Biloba* extract is proposed to modulate circulatory and cognitive function are increasing blood supply by dilating peripheral blood vessels, increasing cerebral blood flow, modifying neurotransmitter systems and reducing the levels of oxidative damage (Birks et al. 2002).

1.9.3 The Effects of Gingko Biloba on Mobility and Cognition in Older Adults

There is some evidence supporting the use of *Ginkgo Biloba* extract in people with dementia, as supplementation was shown to improve performance on clinical cognitive outcomes in three separate meta-analyses versus placebo, with 240mg of the standardised extract being shown to be the most effective dose (Weinmann et al. 2010; Hashiguchi et al. 2015; Tan et al. 2015). It should be noted that a number of the early RCTs on *Ginkgo Biloba* have been criticised due to their relatively small sample sizes and poorly designed randomisation processes (Birks et al. 2002). Furthermore, it cannot be ruled out that publication bias may impact some of these meta-analyses with null or negative results potentially not being published before the implementation of the trials registry (Birks et al. 2002).

RCTs exploring the preventive effects of *Ginkgo Biloba* in cognitively healthy older adults are limited and have not yielded positive results so far. A multicentre double blind RCT in 3,069 adults aged ≥75 years with no cognitive impairment (n=2587) and MCI (n=482), supplemented with 240mg of standardised Ginkgo Biloba extract per day over a median follow up of 6.1 years, saw no effect on incident of dementia or AD (DeKosky et al. 2008).

One study did look at the effects of 240 mg of *Ginkgo Biloba* supplementation for six months on habitual and dual-task gait in 50 older adults with MCI. The authors reported significant increases in dual-task cadence, with no effects on HGS. This increase in cadence did not translate to any changes in the clinically relevant dual-task gait speed or stride variability (Springer et al. 2006; Hausdorff et al. 2008) and DTE was not measured (Gschwind et al. 2017).

Ginkgo Biloba is potentially beneficial for the improvement of cognitive function in individuals with MCI or AD. However, due to limited sample size, inconsistent findings and methodological quality of previous studies, more research is required to confirm the effectiveness of *Ginkgo Biloba* in preventing cognitive decline and healthy older adults.

1.11 The Role of Exercise in Mobility and Cognition in Older Adults

Both regular physical activity and exercise are promoted by the World Health Organisation (WHO) to improve functional health and reduce the risk of non-communicable disease (WHO, 2010). The hypothesis that physical activity promotes health and longevity is not a recent trend. The earliest records of health promotion through organised physical activity date back to 2500 BC, in ancient China (MacAuley 1994). As well as having a profound effect on the physical domain exercise has also been shown to influence cognitive function (Northey et al. 2017). This link between exercise and cognition may even have roots within human evolution. When early man shifted towards foraging for food this required them to combine aerobic activity with motor control, memory, spatial navigation and EFs, thus it was important to simultaneously develop both aerobic capacity and cognitive function to survive (Raichlen and Alexander 2017).

There are multiple underlying mechanisms and pathways by which exercise may promote healthy ageing and prevent frailty (*Figure 12*). Exercise promotes adaptations to physiological systems that can in turn influence factors associated with healthy ageing. This includes neuromuscular adaptations that influence strength and the ability to coordinate movements (Cadore et al. 2013a), improvements to cardiorespiratory fitness (Sui et al. 2007) and preservation of brain volume (Colcombe et al. 2006). These responses to exercise demonstrate clear pathways by which exercise could influence the health trajectory of older adults. Furthermore, exercise can influence psychological processes such as stress and depressive symptoms (Blake 2012), which in turn, can impact cognition and physical functioning (Callahan et al. 2005; Leritz et al. 2011). With regards to cognition, aerobic exercise has been shown to enhance angiogenesis and hippocampal neurogenesis in aged rats, primarily through the upregulation of neurotrophins and growth factors, including BDNF and IGF-1 (Black et al. 1990; Vaynman et al. 2004; Praag et al. 2005; Cotman et al. 2007; Trejo et al. 2008). These animal models identify some key mechanisms by which exercise may preserve mobility and cognitive function, however it is important to investigate if exercise actually induces clinically relevant changes in functional outcomes in humans.

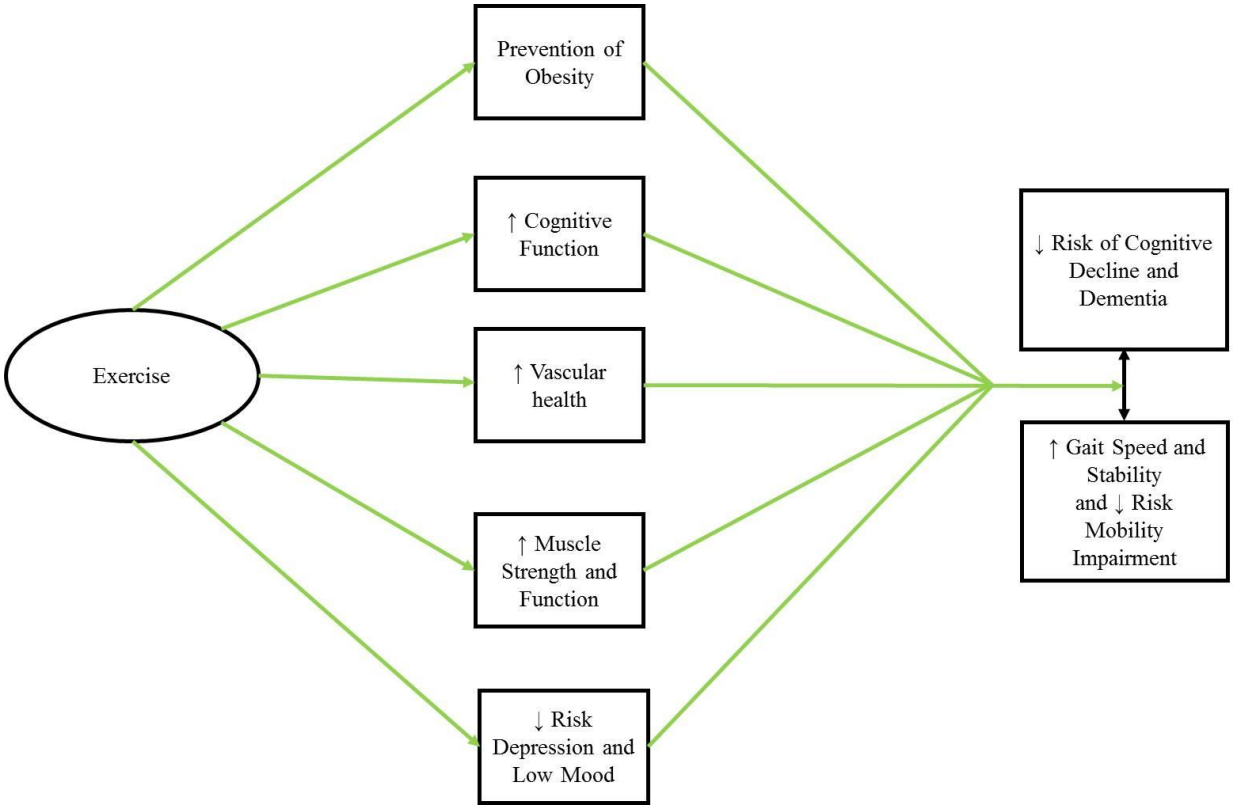


Figure 12 *Summary of the beneficial effects of exercise on mobility and cognitive function in older adults. Exercise has been shown to be beneficial for weight loss, cognitive function, vascular effects, muscle strength and preventing low mood and depression (Sui et al. 2007; Harber et al. 2009; Beavers et al. 2014; Jonasson et al. 2016; Schuch et al. 2016). Effects on these outcomes can all translate into downstream influences on cognitive function and gait outcomes, which themselves are linked (Montero-Odasso et al. 2012b).*

1.11.1 Observational Evidence on the Associations between Exercise and Healthy Ageing Outcomes

The observational evidence has largely focused on the benefits of physical activity, rather than exercise specifically, in older adults (Sofi et al. 2011; Blondell et al. 2014). Moderate and vigorous activity at least once per week is associated with healthier ageing, defined as not developing a chronic disease, depressive symptoms, physical or cognitive impairment, over eight years in 3,454 adults with a mean age 63.7±8.9 years (Hamer et al. 2014b). A meta-analysis including data from 47 cohort studies found that those with the highest levels of physical activity had reduced risk for both cognitive decline (RR 0.65; CI95% 0.55-0.76) and dementia diagnosis (RR 0.82; CI95% 0.73-0.91). An earlier meta-analysis that included a number of the same cohorts, found similar results to Blondell et al. (2014). This analysis differed in that it ran comparisons across three activity levels based on the amount of exercise that was reported; low, low to moderate and high(Sofi et al. 2011). When compared to low, low

to moderate levels of physical activity were associated with a risk for cognitive decline (HR 0.65, CI95% 0.57-0.75). Higher levels of activity were only associated with a further reduced risk of 3% versus low to moderate levels (HR 0.62, CI95% 0.54-0.70), possibly indicating that avoiding low levels of activity or sedentary behaviour may be more impactful than promoting high levels of activity. Furthermore, activity of both moderate and vigorous intensity was associated with a reduced likelihood of the presence of frailty in a cross-sectional analysis on 622 older adults aged 60-96 years (Tribess et al. 2012). An interesting finding from the observational data is that starting an exercise regimen in middle or old age is positively associated with healthy ageing across both physical and cognitive function (Berk et al. 2006; Sabia et al. 2012; Hamer et al. 2014b), thus providing further evidence that exercise interventions in later life could still be effective to promote healthy ageing.

The epidemiological evidence relies heavily on physical activity diaries or questionnaires which can be influenced by recall bias. A meta-analysis that compared direct and self-reported measurements of physical activity in adults found that self-reported measures had only a low to moderate correlation with direct measures, with R values ranging between -0.71 to 0.91(Prince et al. 2008). The epidemiological evidence does demonstrate the promise of exercise as an intervention to prevent age related degenerative health conditions. However, the lack of reliability of self-reported methods for quantifying physical activity coupled with the inability to infer causation from these studies means there is a strong requirement for RCTs using structured exercise interventions, as well as studies aiming to uncover the mechanistic underpinnings behind the effects of exercise. Use of exercise interventions allows researchers to quantify adherence and standardise the activity of participants to gain a more valid insight into the effects of the intervention (Opdenacker et al. 2011).

1.11.2 Does Exercise Modality Impact Results on Healthy Ageing?

There are several modalities of exercise that are used in clinical trials including, resistance training, balance training, aerobic training, coordination training and multi-component exercises (i.e. simultaneous strength, endurance, and balance training) (Cadore et al. 2013b). There have been studies, comparing different exercise interventions, mostly focusing on aerobic versus resistance training in older adults (Roma et al. 2013; Canuto Wanderley et al. 2015; Hortobágyi et al. 2015). These have shown that both types of exercise have beneficial outcomes on gait speed and walking ability assessed via the six-minute walk test, but with no significant difference between the two. This would indicate that the type of exercise is of less importance and positive adaptations can occur through either type of exercise. RCTs using exercise interventions have found increases in leg strength in older adults (Latham et al. 2004), as well as improvements of 0.08m/s in gait speed (Liu and Latham 2009). There are several

inconsistencies in the design of RCTs investigating the effects of exercise on functional measures in older adults. There is a broad range of training protocols, exercise intensity and duration, intervention lengths and sample specific characteristics across the body of literature, making it difficult and perhaps inefficacious to pool these studies together. In particular variances in exercise intensity could influence results across studies that use similar exercise types and durations. Although low levels of exercise intensity have shown to be beneficial for cognitive and physical health in older adults (Tse et al. 2015), higher intensity activities may offer additional benefits for the older adult. Analysis from the English Longitudinal Study of Ageing, which includes data from 10, 426 participates with a mean follow up of 7.8 years, found a dose dependant response for exercise with those who spent more time exercising vigorously having the lowest risk of all-cause mortality (Hazard Ratio (HR) 0.44 CI95% 0.39, 0.50 however, light activity was still associated with a lower risk (HR 0.76 CI95% 0.69, 0.83) (Hamer et al. 2014a). Twelve weeks of high intensity resistance training, conducted at 80% of the participants one repetition maximum provided significantly greater benefits to muscle strength versus a lower intensity intervention (40% of one repetition maximum) in a sample of 39 older adults (Onambélé-Pearson et al. 2010). Furthermore a meta-analysis comprising of 29 trials including 1313 older adults found that higher intensities of resistance training (60-85% of one repetition max) had greater effects on muscle strength versus lower intensities; however there was no difference between the two on functional tests which included gait speed and ability to rise from a chair (Steib et al. 2010). There are a limited number of studies comparing exercise intensities of aerobic exercise. There was no difference between ten weeks of low (40% heart rate reserve) or high intensity (70% heart rate reserve) aerobic exercise on a cycle ergometer in older osteoarthritis patients on chair rise time or six minute walk test with both intensities showing a significant benefit, however this may not be consistent for those without osteoarthritis (Mangione et al. 1999). Together this indicates that although lower intensity activities can be of benefit encouraging older adults to take part in moderate or high intensity activities, as is stated in the WHO guidelines (World Health Organization 2010), could provide additional benefits.

1.11.3 Effects of Aerobic Exercise on Mobility and Cognition in Older Adults

Aerobic exercise is defined as any type of activity that uses large muscle groups and can be maintained over a period of time including activities such as brisk walking, cycling swimming or dancing (Chodzko-Zajko et al. 2009). Aerobic training has shown promise with regards to healthy ageing due to its ability to act across a broad range of health related factors including both the physical and cognitive domains (Barnett et al. 2003; Denison et al. 2013; Jonasson et al. 2016). Of particular importance to older adults, aerobic exercise interventions were shown to influence processing speed and executive function in a meta-analysis including data from 2,049

participants (Smith et al. 2010c), which are two key cognitive domains that have been associated with gait speed and stability (Coppin et al. 2006; Holtzer et al. 2006; Springer et al. 2006; Bruce-Keller et al. 2012; Kearney et al. 2013; Martin et al. 2013a). However, it should be noted that this analysis only found small effect sizes of the aerobic exercise interventions, so it is unclear whether these would be adequate to produce clinically relevant downstream effects on gait. Furthermore, although the sample populations were predominantly from older adults, six of the included 29 RCTs were conducted in younger adults or those with a chronic health condition equating to 284 of the included participants, so it is unclear whether this result would be maintained in just healthy older adults.

Cycling is a form of aerobic exercise that can benefit muscle strength, cardiopulmonary fitness, balance and proprioception in older adults (Harber et al. 2009; Rissel et al. 2013). Furthermore, cycling may be preferential for older adults as it is non-weight bearing, has a low impact on joints and has been found to be suitable and beneficial for those with joint pain (Wainwright et al. 2016). In spite of the apparent benefits, the effect of cycling interventions on functional outcomes is less consistent. In an RCT of 105 older adults with mild strength and balance deficits, there was no significant improvement in gait and balance tests, after 24 weeks of aerobic training on a cycle ergometer (Buchner et al. 1997). Another RCT did report a significant decrease in the time taken to complete the six-meter timed up and go in the cycle ergometer group versus the control, after 12 weeks of sessions. However, there was no difference in the three meter walk test (Denison et al. 2013). In this study the control group did not experience any notable decline, possibly due to the short follow up period, therefore this does not fully encapsulate how exercise could be used as a preventative for functional decline. In contrast, 10 weeks of cycle ergometer training led to a significant improvement in chair rise time and in the 6-minute walk test among older adults with knee osteoarthritis (Mangione et al. 1999).

Overall there is an abundance of evidence supporting the positive impact of exercise in older adults with effects being demonstrated across a variety of outcomes related to the HAP, with mechanistic underpinnings demonstrating broad range of effects across multiple systems (Figure 12). The greatest benefits of exercise appears to occur by moving an individual from a sedentary to a light to moderately active state in line with WHO guidelines. The precise type of exercise appears to be of less importance with benefits being demonstrated for both aerobic or resistance type training with no notable differences between the two in regards to mobility outcomes (Roma et al. 2013; Canuto Wanderley et al. 2015; Hortobágyi et al. 2015). As such greater importance should be placed on factors that may enhance the uptake and long term adherence to exercise such as enjoyment, social support and social connection (Farrance et al. 2016).

1.12 The Interaction between Exercise and Omega-3 PUFA in Healthy Ageing

Given that diet and exercise are concurrent components of daily living and have separately been proposed to improve cognitive and physical functioning, it is important to examine their combined actions in an effort to better understand the role lifestyle interventions can play in preserving the health of older adults. There is now some promising evidence that an interaction may occur between omega-3 PUFAs and exercise in older adults on both mobility and cognition.

Rodacki et al. (2012) found that supplementation with 400 mg EPA and 300 mg DHA combined with 12 weeks of resistance training increased maximum voluntary contraction and rate of torque development in leg muscles of older women versus resistance training alone. A major limitation of this study was the lack of a placebo capsule, which is important considering there is a wealth of evidence suggesting the placebo effect can have a significant impact on strength and power outcomes (Kalasountas et al. 2007; Rawdon et al. 2012). A study in 50 healthy adults, male(n=27) and female (n=23) aged 70.6±4 years found that combined daily supplementation with 2100 mg EPA and 600 mg DHA and resistance training for 18 weeks, which involved 4 sets of 9 repetitions at 70% of their one-repetition maximum on leg extension, leg press, leg curl, and calf raise exercise, provided an additional benefit to maximal isometric torque in knee extensor muscles versus resistance training alone in female participants only (Da Boit et al. 2016b). This measure of peak torque was calculated relative to muscle anatomical cross-sectional area demonstrating an improvement in muscle strength and quality. The authors found no additional benefit of omega-3 PUFA supplementation to resistance training on muscle mass, balance, gait speed or chair rise time. Although there was no change in the functional outcomes, the positive effect on muscle strength and quality is promising considering these are important factors that contribute towards progression to sarcopenia (Cruz-Jentoft et al. 2019). Besides the lack of placebo capsule there is another notable difference between the studies by Rodacki et al. (2012) and Da Boit et al. (2017). The changes in plasma DHA are comparable between the studies, changes in EPA from baseline in female participants were 3.3% in the study by Da Boit and colleagues, whereas changes in the study by Rodacki et al. (2012) were ≈0.7%. EPA may be the more important omega-3 PUFA in terms of muscle health (Kamolrat and Gray 2013), this could suggest that even small changes in EPA could elicit benefits on muscle strength or could further call into question the validity of the findings by Rodacki et al. (2012), given the lack of placebo capsules. Nonetheless future work should seek to develop a better understanding of a possible dose response of omega-3 PUFAs on muscle function and quality in older adults.

The potential interaction between exercise and omega-3 PUFAs may not just be limited to muscle function. A study in 22 older adults with MCI compared the effects of daily

supplementation with omega-3 PUFA (1320mg EPA and 880mg DHA) alone and in combination with twice-weekly cycle ergometer training and a programme of cognitive stimulation (active cognitive stimulation prevention in the elderly) for eight months. The combined intervention led to an enhanced reduction of brain atrophy. Furthermore, the rates of brain atrophy were positively and strongly correlated with the decrease in serum homocysteine ($r = -0.706$) that was observed in the combined intervention group only, indicating that changes in homocysteine could be a potential mechanism underlying the effects of the combined intervention. There was no additional benefit to combining omega-3 PUFA and aerobic exercise on executive function, memory, sensorimotor speed and attention, thus the preservation in brain volume did not appear to result in functional improvements (Kobe et al. 2016).

The largest study to date within this area is the Multidomain Alzheimer Preventive Trial, a multicentre trial in 1680 older adults with subjective memory complaints or mild mobility impairment defined as having gait speed of <0.8 m/s or a limitation in one instrumental activity of daily living (Andrieu et al. 2017). The study was designed the test the effectiveness of omega-3 PUFA supplementation alone (800 mg DHA and 225 mg EPA) or in combination with a multidomain intervention that consisted of groups sessions that consisted of cognitive training as well as encouragement to adopt a healthier overall diet and to increase physical activity. Participants were randomly assigned to one of four groups: multidomain intervention combined with omega-3 PUFA supplementation, multidomain intervention and placebo supplement, omega-3 PUFA supplementation only or placebo supplement only with an intervention period of three years. Following the intervention period, there was no effect of any of the active intervention groups versus the placebo on cognitive function expressed as composite Z score of five cognitive tests or on individual cognitive domains that comprised of tests of episodic memory, executive function, verbal fluency and processing speed. Furthermore, secondary analysis from this study found no effects of any of the intervention groups on muscle strength assessed using repeated chair stand test and hand grip strength (Rolland et al. 2019). Whilst this study has many strengths, in particular the long term follow up and the factorial style design, a major limitation was that participants in the omega-3 PUFA supplement and placebo supplement only reported reducing their physical activity by 94 and 107 minutes respectively, whilst both groups allocated to the multidomain intervention reported no change in their physical activity habits. This makes it impossible to draw any conclusions about the combination of omega-3 PUFA supplementation and physical activity as physical activity was never increased in any group, and also highlights the importance of structured exercise intervention over only encouraging exercise at home.

Overall there is some promising evidence for an interaction between omega-3 PUFAs and exercise in older adults, which affects both physical and cognitive function, however, there is still a lack of evidence supporting the combination of the two interventions on more functional outcomes including both mobility and cognitive function (Rodacki et al. 2012; Kobe et al. 2016; Da Boit et al. 2017). The studies in humans thus far have taken place in small sample sizes and have not had a placebo only controlled group or have been unable to actually increase physical activity habits. Both interventions have separately shown promise in clinical trials in older adults across a broad range of outcomes applying to the HAP including muscle strength, gait and cognitive function (Latham et al. 2004; Harber et al. 2009; Hutchins-Wiese et al. 2013; Strike et al. 2016). Omega-3 PUFAs and exercise share a number of common mechanisms having both been shown to enhance the production of BDNF mediated synaptic plasticity (Akbar et al. 2005; Erickson et al. 2011), modulate the inflammatory response (Levy et al. 2001; Campbell et al. 2009) and enhance MPS in old age (Smith et al. 2011a; Smith et al. 2012) (*Figure 13*). In support of a possible BDNF mediated mechanism of combined exercise and omega-3 PUFA on cognitive function, a study in Sprague Dawley rats found that combining dietary enrichment with 1.25% DHA and exercise resulted in significantly better spatial working memory, assessed via the Morris water maze, compared with each intervention applied separately (Wu et al. 2008). Expression of downstream signalling targets of BDNF, calmodulin-dependent protein kinase II and protein kinase B, which are shown to be crucial for the effect BDNF has on synaptic plasticity and learning and memory (Elgersma et al. 2004; Yoshii and Constantine-Paton 2007) were also enhanced by the combination**.** Furthermore, exercise has been shown to lower homocysteine (Vincent et al. 2006; Kobe et al. 2016) and omega-3 PUFAs have been shown to increase gene expression of 5-methyltetrahydrofolate reductase (Huang et al. 2013) and interact with B vitamins to lower rates of brain atrophy (Jerneren et al. 2015). This effect on homocysteine metabolism may underpin the effect of combined exercise and omega-3 PUFA supplement intervention on rates of brain atrophy in older adults (Kobe et al. 2016).

Despite animal models showing a potential synergy between omega-3 PUFAs and exercise, mediated by an enhancement of BDNF (Wu et al. 2008), the nature of this interaction is currently unclear in human trials. The question remains whether exercise and omega-3 PUFAs act separately on similar pathways or whether they actually interact to produce additive or synergistic benefits. Despite this apparent shared effect on pathways related to cognitive function, omega-3 PUFA and exercise intervention studies have shown some differing effects on cognitive domains. Omega-3 PUFAs predominantly have been shown to influence verbal memory, immediate recall and attention (Yurko-Mauro et al. 2010; Sinn et al. 2012; Lee et al. 2013; Zhang et al. 2016b; Hooper et al. 2017), whereas aerobic exercise has shown more promise for effects on attention, processing speed and executive function (Smith et al. 2010c).

There is now mounting evidence that combining exercise and omega-3 PUFA interventions could be an effective approach to preventing age related decline. Not only could they interact to provide additive or synergistic benefits to mobility and cognitive function, but they also have the potential to act upon a broad range of cognitive outcomes, which would be favourable to promoting a positive ageing trajectory towards the HAP.1

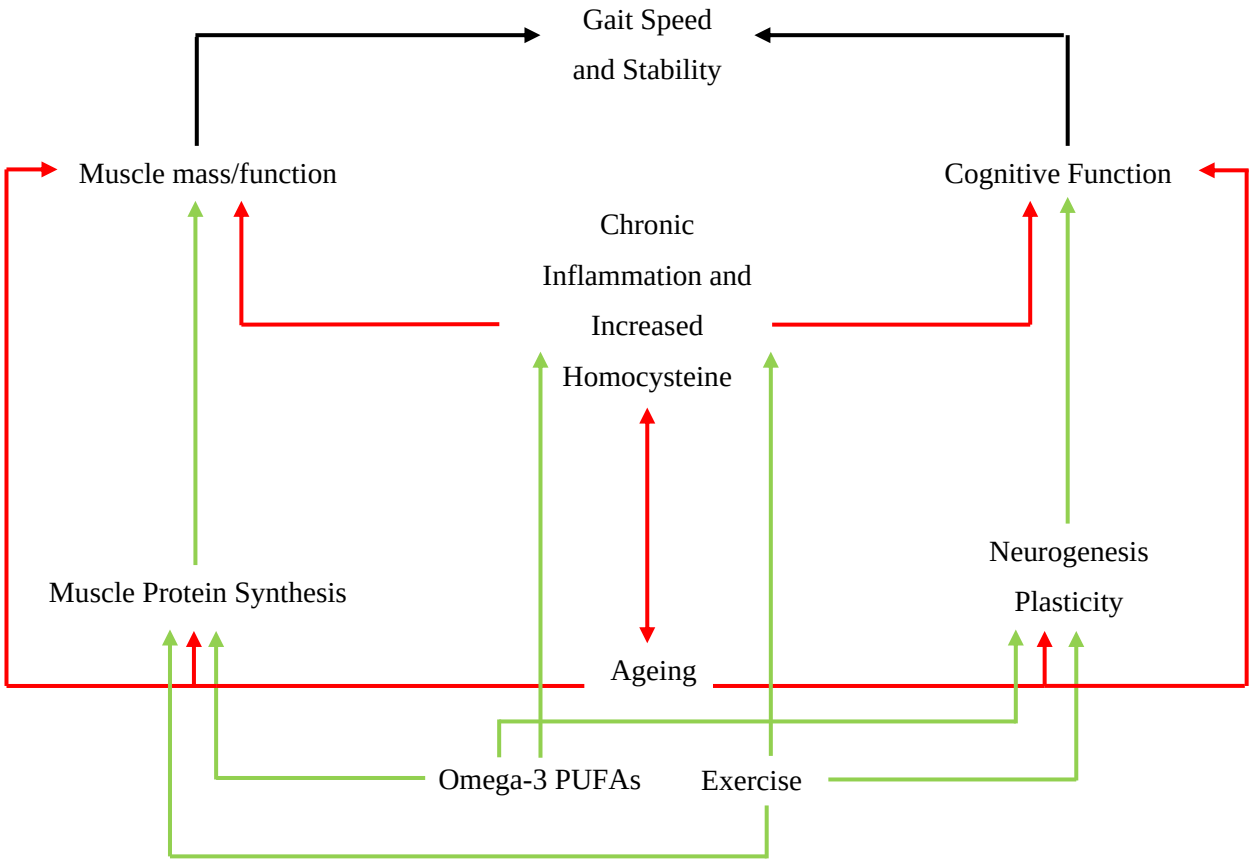


Figure 13 *Summary of the separate and combined roles of omega-3 polyunsaturated fatty acids and exercise on healthy ageing. Red lines indicate a negative effect or relationship and green lines indicate a positive effect or relationship. Both exercise and omega-3 polyunsaturated fatty acids have been shown to share a number of similar effects including increasing neurogenesis and plasticity, muscle protein synthesis and influencing inflammation and reducing homocysteine (Short et al. 2004; Nicklas et al. 2008; Wu et al. 2008; Serhan et al. 2011; Smith et al. 2011a; Kobe et al. 2016). Furthermore there is now evidence to suggest an additive or even synergistic effect of these interventions on muscle strength and cognition (Kobe et al. 2016; Da Boit et al. 2017), with the cognitive benefits possibly being mediated by an enhancement in the production of brain derived neurotrophic factor and dependent on homocysteine lowering (Wu et al. 2008; Kobe et al. 2016). There is a relationship between gait outcomes with cognition and muscle mass/function in older adults (Montero-Odasso et al.*

2012a; Layne et al. 2017). Therefore, positive effects on these health domains could lead to downstream influences on gait, improving the ageing trajectory towards the healthy ageing phenotype.

Chapter 2 - The combined effects of Omega-3 Polyunsaturated Fatty Acids and B vitamins on Cognition in the older adult: A Systematic Review and Meta-analysis 5

This thesis will explore the effects of a multi-nutrient intervention on mobility and cognition within the older adult. Key ingredients within the intervention will include omega-3 PUFAs and B vitamins. As such the following chapter presents a systematic review with a meta-analysis on the effects of omega-3 PUFAs and B vitamins on cognition within older adults. This systemic review with a meta-analysis is currently under review for publication in the British Journal of Nutrition.

2.1 Introduction

In Europe, the proportion of adults aged 65 years and over is expected to rise from 16.1 to 22% by 2031 (Soong et al. 2015). It has been estimated that in the year 2015, 688, 300 people in England had dementia with 6.7% of older adults having the condition (Wittenberg et al. 2019). Due to the insidious onset, cognitive impairment often goes unnoticed for several years, with clinical diagnosis being made late into the disease progression, thus it is likely that a much higher number of older adults are currently living with some form of cognitive impairment (Bradford et al. 2009). Cognition is critical for functional independence as people age, including whether someone can live independently, manage finances, take medications correctly, and drive safely. In addition, intact cognition is vital for humans to communicate effectively, including processing and integrating sensory information and responding appropriately to others (Murman 2015).

Observational evidence has consistently demonstrated a positive association between adherence to a healthy dietary patterns such as the Mediterranean or Dietary Approaches to Stop Hypertension diets and cognition in the older adult (Lourida et al. 2013; Tangney et al. 2014). Within this body of observational literature some individual key nutrients have been identified as being protective of brain health in ageing populations, with omega-3 polyunsaturated fatty acids (omega-3 PUFAs) and B vitamins showing particular promise (Xu et al. 2015; Zhang et al. 2015). These observations are supported by mechanistic work that has demonstrated that omega-3 PUFAs can reduce markers of inflammation and influence membrane properties, which in turn affects cell signalling, increases neurogenesis and promotes neuronal survival (Akbar et al. 2005; Dyall 2015a; Dyall 2017), with vitamins B12, B6 and folic acid playing essential roles in the metabolism of homocysteine preventing hyperhomocysteinemia, which has been strongly linked to cognitive decline in older adults (Xu et al. 2015).

Despite the consistent findings within the epidemiological literature, randomised control trials focusing on supplementation with omega-3 PUFAs or B vitamins in isolation have produced inconsistent and inconclusive results (Dangour et al. 2010; Quinn et al. 2010; Smith et al. 2010a; de Jager et al. 2012; van der Zwaluw et al. 2014; Witte et al. 2014; Dangour et al. 2015). Whilst there is a great degree of heterogeneity in the way randomised control trials have been designed within this area including the dosage of supplementation, length of follow-up, population characteristics and chosen outcomes one characteristic that is a common choice is to supplement with omega-3 PUFAs or B vitamins alone. Whilst this approach allows us to draw causal inferences for specific nutrients, a major limiting factor of this approach is that single nutrients often have very small effect sizes that could be influenced by bias and could lack clinical significance (Siontis and Ioannidis 2011). Indeed, whilst the observational literature has found positive relationships between single nutrients and cognitive outcomes, it must be acknowledged that the intake of these nutrients has predominately come from whole food sources which are of course sources of many other essential nutrients which may have also contributed towards such associations.

It is now clear that there are complex interactions between several nutrients that make up a balanced diet and that it is important to explore potential synergistic or additive effects to elicit how whole dietary interventions can impact a specific outcome. For instance, some evidence suggests that there may be an interaction between B vitamins and omega-3 PUFAs, and optimising intake of both nutrients may be key to eliciting beneficial effects on cognition (Jerneren et al. 2015; Oulhaj et al. 2016).

The mechanisms underpinning this potential interaction between omega-3 PUFAs and B vitamins is currently not well understood, however, homocysteine has been shown to impact phospholipid and docosahexaenoic acid (DHA) metabolism by inhibiting methylation reactions that convert phosphatidylethanolamine enriched with DHA to phosphatidylserine (PS), which in turn would influence PS synthesis by PS synthase-1 and PS synthase-2 (Selley 2007). Furthermore, lower intakes of DHA could result in there being reduced availability of phospholipids for methylation. In particular reduced phosphatidylethanolamine availability increases the S-adenosylmethionine, S-adenosylhomocysteine ratio, leading to the hypermethylation of histones *in vitro*. This leads to downstream effects on gene expression that increases stress related responses and decreasing protein translation and mitochondrial function (Ye et al. 2017). Furthermore, *in vitro* DHA has been shown to increase gene expression of 5-methyltetrahydrofolate reductase, the enzyme required to convert 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is an important factor in the re-methylation of homocysteine (Huang et al. 2012).

A lack of consideration for this potential interaction between omega-3 PUFAs and B vitamins could contribute somewhat to the variance in results from single nutrient supplementation trials. It is therefore important to examine the current available literature that has investigated the combined effects of omega-3 PUFA and B vitamins. As such, the aim of this systematic review was to firstly investigate whether supplementation with a combination of omega-3 PUFAs and B vitamins can prevent cognitive decline in older adults. Secondly, the review sought to determine whether the effects of a single nutrient intervention with either omega-3 PUFAs or B vitamins could be modified by the status of the other nutrient. This meant investigating whether dietary omega-3 PUFA intake modulated the effects of B vitamin supplementation and likewise whether dietary B vitamin intake impacted the effects of omega-3 PUFA supplementation.

2.2 Methods

This systematic review was registered and made available to the public through The International Prospective Registration of Systematic Reviews (https://www.crd.york.ac.uk/PROSPERO)(CRD42020210361).

2.2.1 Search Strategy

The following databases were searched in December 2020 for articles published between January 2010 and December 2020: the Cochrane Library, EMBASE, CINAHL, Scopus, and MEDLINE. The search strategy used terms related to the combination of omega-3 PUFA (Omega-3 polyunsaturated fatty acid, omega-3 PUFA, N-3 PUFA, eicosapentaenoic acid, EPA, docosahexaenoic acid, DHA, fish oil) and B vitamins (B vitamin, vitamin B, B12, folic acid, folate, homocysteine, cobalamin, multivitamin, multi-nutrient). The key words were then combined by the EBSCO host operator AND/OR. Supplementary literature searches included examining the reference lists of all relevant studies, pertinent review articles, and meta-analyses.

2.2.2 Study Selection Criteria

Articles were included if they met the following criteria: (1) the study type was a randomised control trial and was available in the English language; (2) The mean age of the participants in the study is 60 years or greater; (3) the study intervention provided a combination of omega-3 PUFAs and B vitamins alone or in combination with other nutrients; (4) tested for interactions between omega-3 PUFAs and B vitamins by providing omega-3 PUFA alone but also including an objective measure of B vitamin status including measuring B vitamins directly or through assessment of homocysteine, provided B vitamin supplementation alone but also measured an objective

measurement of omega-3 PUFA status; (4) assessed cognitive function through a change in a composite score on neuropsychological testing or change in single cognitive test score. The articles were excluded if they met one of the following criteria: (1) duplicated publications; (2) Non-randomised control trials including letters, case reports, position statements, conference proceedings, prevalence surveys, reviews, in vitro studies and studies in animals; (3) studies that did not report specific dosages for individual nutrients provided in supplements. The initial screening was performed by the lead researcher (PF) and included a review of all titles and/or abstracts compared to eligibility criteria, with consensus sought from a second researcher (FT) where there was ambiguity. Full-text publications of any studies not eliminated within the initial screening were retrieved for complete review.

2.2.3 Data Extraction

Data extraction and coding stages of the review were completed by the first reviewer (PF) using structured data extraction forms. The following information was extracted from the manuscripts: first author, year of publication, location, number of participants, duration of intervention, age, the intervention (dose and formulation of dietary supplements), method used to assess cognitive testing, the cognitive domains that were tested and any additional relevant biomarkers that were measured (Omega-3 PUFA, B vitamin or homocysteine levels). A proportion of the extracted data (30%) was checked for accuracy by second reviewer (FT).

2.2.4 Risk of Bias in Individual Studies

Risk of bias was assessed using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) which comprises the respective RoB2 domains: (1) risk of bias arising from the randomisation process; (2) risk of bias due to deviations from the intended interventions (effect of assignment to intervention); (3) risk of bias due to missing outcome data (4) risk of bias in the measurement of the outcome and (5) risk of bias in the selection of the reported result (Sterne et al. 2019). These domains were used to inform the overall risk of bias judgement for the selected studies. PF independently assessed the risk of bias for all included studies. FT performed an independent check on the risk of bias scores to ensure the accuracy of scoring. Disagreements were resolved by discussion and a third opinion was sought from an independent researcher where there was a discrepancy.

2.2.5 Data Synthesis

Effect sizes were based on group mean differences (post-study minus pre-study test scores) and corresponding standard deviations (SDs) between the combined omega-3 PUFA and B vitamin intervention group and the control (placebo) group. When SDs were not reported, methods described in the Cochrane Handbook for Systematic Reviews of Interventions were relied upon to calculate or estimate SDs from other statistics provided in the published paper. For each effect size the Hedges G statistic was calculated. This approach permits combining multiple methods of cognitive testing for which different scales were used to determine scores. This effect size is a variation on Cohen's d, which corrects for biases due to small sample sizes (Brydges 2019). The scales of cognitive tests were made to have a consistent direction of effect across all included studies, with positive estimates favouring intervention groups and negative estimates favouring placebo groups. Random-effects meta-analysis models were used to generate between group effect sizes.

A priori analyses were defined for between groups. Macro-level models included data on all subjects, regardless of baseline cognitive status or age, at all dose levels using the longest duration of exposure for each cognitive domain. The primary analysis included studies which had used a composite score for cognition using a neuropsychological test battery to assess overall cognitive status. It has become evident that commonly used tests to assess global cognition such as the mini-mental state examination and Alzheimer's Disease Assessment Scale-cognitive subscale are not adequate to detect changes particularly in healthy older adults or those with mild cognitive impairment (Vellas et al. 2008). As such, the use of a neuropsychological test batteries is increasingly seen as a promising method to detect changes in cognition at an early stage of cognitive decline (Vellas et al. 2008). Secondary analyses were performed on single tests of global cognition as well as episodic memory and executive function. Episodic memory and executive function are commonly assed cognitive domains within the literature and have been demonstrated to be sensitive indicators of pathological cognitive decline (Rajan et al. 2015; Mortamais et al. 2017). Where multiple single tests of global cognition were used the primary outcome was selected for use in the meta-analysis. The weight of each study in the meta-analysis was based on the inverse of the variance, a measure that accounts for the sample size within each group. The statistical heterogeneity among studies was determined according to the Chi^2 test and I^2 statistic, with $P>0.1$ and $I^2<50\%$ was considered as low heterogeneity, and otherwise it indicates high heterogeneity when the I^2 value was higher than 50%.

The exploratory post-hoc analyses of previous studies providing single nutrient interventions aimed to detect interactions between supplementation and background diet thus were conducted and analysed in a different manner to the trials that provided combined omega-3 PUFA and B vitamin interventions. As such, it would not be appropriate to include these studies within the meta-analysis alongside the interventions trials that have provided a combination of nutrients. These studies have been descriptively summarised.

2.3 Results

The databases identified 4,715 results with three additional sources coming from forward citation searching (Figure 14). After the removal of duplicates 4,378 articles remained. The titles and abstracts of these articles were screened, 4, 359 articles were excluded, with the 18 remaining articles undergoing a full-text screening. A further 5 articles were excluded at this stage as they did not meet the criteria for inclusion. One study had a mean age of below 60 years, two studies had no measurement of cognitive testing through neuropsychological testing, one study was not available in the English language and a translation was not possible and one study was a proof of concept study thus was not designed for hypothesis testing.

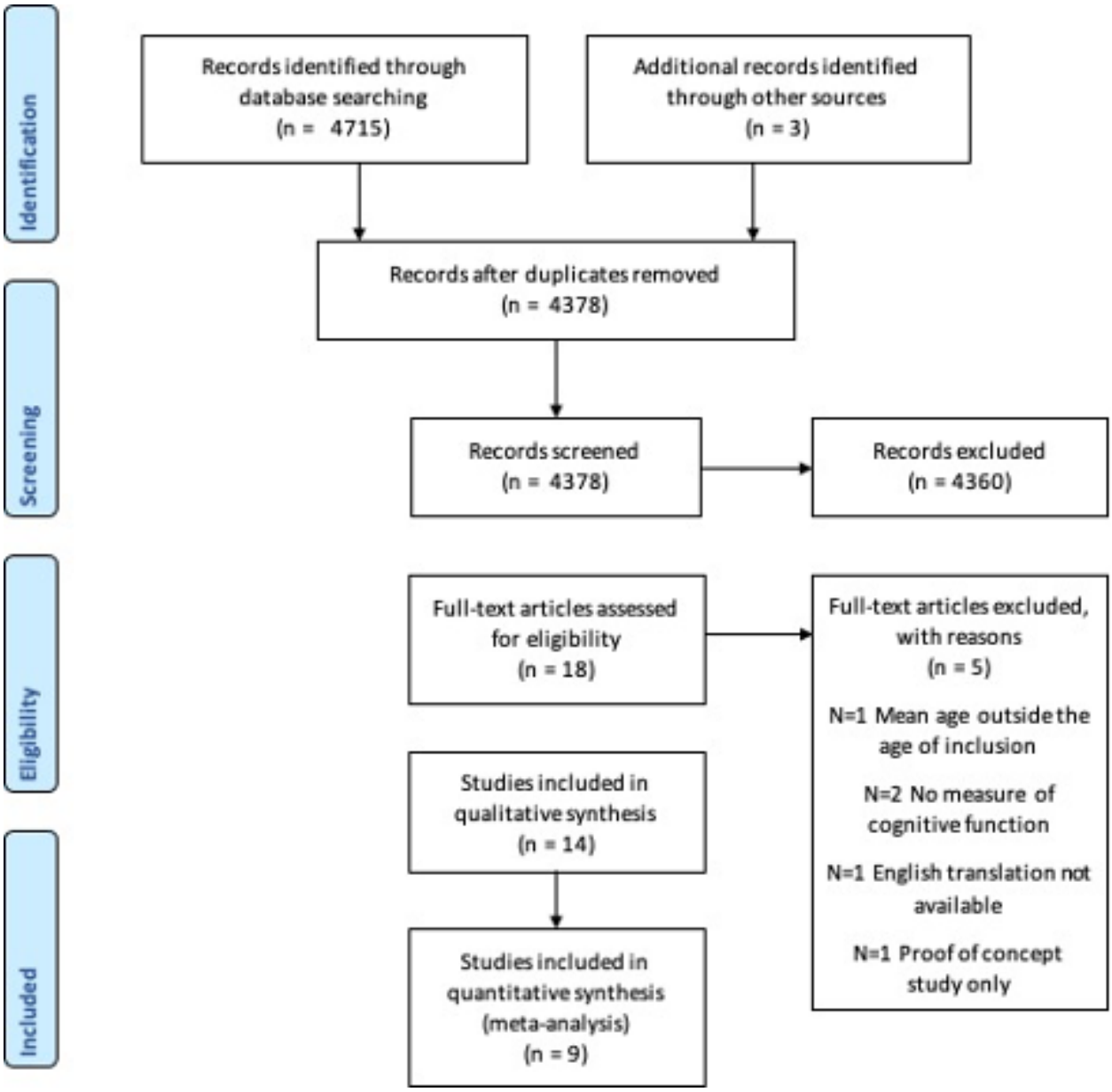


Figure 14 PRISMA flow chart showing the literature screening process and study selection

2.3.1 Characteristics of Included Studies

A full summary of the characteristics of each included study can be found in Table 2 Characteristics extracted from fourteen included studies. All of the included studies were randomised placebo-controlled studies with follow up lengths varying between 24 weeks and 4 years.

2.3.2 Nutrient Interventions and Formulas

Eleven studies were RCTs analysing the effects of a combination of omega-3 PUFAs and B vitamins. Of these eleven studies nine provided multi-nutrient supplement formulas that included additional active ingredients beyond just omega-3 PUFAs and B vitamins (Scheltens et al. 2010; Scheltens et al. 2012; Shah et al. 2013; Jackson et al. 2016a; Strike et al. 2016; Soininen et al. 2017; Baleztena et al. 2018; Fairbairn et al. 2020; Soininen et al. 2021). Two studies provided only a combination of B vitamins and omega-3 PUFAs with no additional active ingredients (Andreeva et al. 2011a; Li et al. 2020). Three of the included studies included additional intervention arms that provided single nutrient interventions of either omega-3 PUFAs or B vitamins which allowed for comparison between the single nutrient and multi-nutrient formulas (Andreeva et al. 2011a; Jackson et al. 2016a; Li et al. 2020). Three included papers were post-hoc analyses of RCTs, one was a study that provided omega-3 PUFA supplements and proceeded to measure homocysteine (Jernerén et al. 2019), with two providing B vitamin supplements and proceeding to measure omega-3 PUFA status (Oulhaj et al. 2016; van Soest et al. 2021). All of the post-hoc studies analysed the interaction between omega-3 PUFAs and B vitamins on cognitive outcomes.

2.3.3 Participant Health Status at Baseline

Participant's health status at baseline varied between the studies particularly in regards to cognitive health status. Three of the studies included participants who were cognitively healthy at baseline (Strike et al. 2016; Baleztena et al. 2018; Fairbairn et al. 2020). One study included participants with a subjective memory complaint (Jackson et al. 2016a). Four studies included participants who were at the prodromal stage of AD or had MCI (Oulhaj et al. 2016; Soininen et al. 2017; Li et al. 2020; Soininen et al. 2021), with four studies having participants with diagnosed AD (Scheltens et al. 2010; Scheltens et al. 2012; Shah et al. 2013; Jernerén et al. 2019). Of the studies that included participants with AD two were in drug naive patients (Scheltens et al. 2010; Scheltens et al. 2012) with the other two being in patients taking medications with taking either acetylcholinesterase inhibitors, an N-methyl-D-aspartate receptor antagonist or a combination of the two (Shah et al.

2013; Jernerén et al. 2019). The final study included participants who were cognitively healthy at baseline but had suffered a cardiovascular event (myocardial infarction, unstable angina, or ischemic stroke) (Andreeva et al. 2011a).

2.3.4 Measurement of Cognitive Function

A variety of methods were used to quantify cognitive function across all studies. Two studies used measures of global cognition only (Andreeva et al. 2011a; Jernerén et al. 2019), with eleven studies electing to use some domain specific testing (Scheltens et al. 2010; Scheltens et al. 2012; Shah et al. 2013; Jackson et al. 2016a; Strike et al. 2016; Soininen et al. 2017; Baleztena et al. 2018; Fairbairn et al. 2020; Li et al. 2020; Soininen et al. 2021). Four studies used a composite score of multiple cognitive testing methods to quantify overall global cognition (Scheltens et al. 2012; Shah et al. 2013; Soininen et al. 2017; Soininen et al. 2021). The majority of the studies (n=11) included a measurement of global cognition. With regards to domain specific cognitive testing there was a notable variance in the selected domains that were selected for assessment. The domains that were tested were executive function (n=9), episodic memory (n=7), processing speed (n=3), semantic memory (n=3), working memory (n=3), attention and concentration (n=2), interference control (n=1) and mental fatigue (n=1).

Table 2 Characteristics extracted from fourteen included studies

Author	Age and Number of Participants	Participants in Study	Nutrient Interventions/Post-Hoc Nutrient Measurements	Intervention Duration	Cognitive Domains Te
			Primary Studies		
Fairbairn et al., 2020	Mean age 67±7 y N = 25	Healthy older adults	Active: 1 g DHA, 160 mg EPA, 240 mg *Ginkgo biloba*, 60 mg phosphatidylserine, 20 mg D-α tocopherol, 1 mg folic acid and 20 µg vitamin B12 Placebo: Fatty Acid blend typical of U.K. Diet	24 weeks	Episodic Memory Executive Function Interference Control Working Memory
Strike et al., 2016	Mean age 66±5 y N = 29	Healthy older adults	Active: 1 g DHA, 160 mg EPA, 240 mg *Ginkgo biloba*, 60 mg phosphatidylserine, 20 mg D-α tocopherol, 1 mg folic acid and 20 µg vitamin B12 Placebo: Fatty Acid blend typical of U.K. Diet	24 weeks	Episodic Memory Executive Function Processing Speed

Jackson et al., 2016	Mean age 60±5 y N = 100	Older adults with subjective memory deficits in the absence of cognitive impairment (MMSE scores ≥ 26)	Active: 1 g DHA, 160 mg EPA, 240 mg *Ginkgo biloba*, 60 mg phosphatidylserine, 20 mg D-α tocopherol, 1 mg folic acid and 20 µg vitamin B_{12} Active: 896 mg DHA, 128 mg EPA Placebo: Fatty Acid blend typical of U.K. Diet	6 months	Working Memory Processing Speed Mental Fatigue
Baleztena et al., 2018	N = 99 Mean age 87±6 y	Healthy older adults	Active: 750 mg DHA, 120 mg EPA, 15 mg vitamin E, 45 mg phosphatidylserine, 285 mg tryptophan, 15 µg vitamin B12, 750 µg folate and 180 mg *Ginkgo Biloba* Placebo: Gelatin capsule		Global Cognition Semantic Memory Executive function
Soininen et al ., 2020	N = 162 Mean age 71±7 y	Prodromal Alzheimer's disease, defined according to the IWG-1 criteria	Active: Fortasyn Blend: 300 mg EPA, 1200 mg DHA, 106mg phospholipids, 400 mg choline, 625 mg uridine monophosphate, 40 mg vitamin E, 80 mg vitamin C, 60 µg selenium, 3 µg vitamin B12, 1 mg vitamin B6, and 400 µg folic acid. Placebo: Isocaloric drink	36 months	Global Cognition Episodic Memory Executive Function

Soininen et al ., 2017	Mean age 71±7 y N = 311	Prodromal Alzheimer's disease, defined according to the IWG-1 criteria	Active: Fortasyn Blend: 300 mg EPA, 1200 mg DHA, 106mg phospholipids, 400 mg choline, 625 mg uridine monophosphate, 40 mg vitamin E, 80 mg vitamin C, 60 µg selenium, 3 µg vitamin B12, 1 mg vitamin B6, and 400 µg folic acid. Placebo: Isocaloric drink	24 months	Global Cognition Episodic Memory Executive Function
Shah et al., 2013	N = 527 Mean age: 77±8 y	Older adults with mild to moderate Alzheimer's Disease receiving Alzheimer's medications (Acetylcholinesterase inhibitor, Memantine or combination)	Active: Fortasyn Blend: 300 mg EPA, 1200 mg DHA, 106mg phospholipids, 400 mg choline, 625 mg uridine monophosphate, 40 mg vitamin E, 80 mg vitamin C, 60 µg selenium, 3 µg vitamin B12, 1 mg vitamin B6, and 400 µg folic acid. Placebo: Isocaloric drink	24 weeks	Global Cognition Attention and Concentration Executive Function Processing Speed Semantic Memory

Scheltens et al., 2012	N = 259 Mean age 74±7 y	Mild Alzheimer's patients, MMSE score 20-26, without medications	Active: Fortasyn Blend: 300 mg EPA, 1200 mg DHA, 106mg phospholipids, 400 mg choline, 625 mg uridine monophosphate, 40 mg vitamin E, 80 mg vitamin C, 60 µg selenium, 3 µg vitamin B12, 1 mg vitamin B6, and 400 µg folic acid. Placebo: Isocaloric drink	24 weeks	Global Cognition Memory Executive Function
Scheltens et al., 2010	N = 225 Mean age 74±8 y	Mild Alzheimer's patients, MMSE score 20-26, without medications	Active: Fortasyn Blend: 300 mg EPA, 1200 mg DHA, 106mg phospholipids, 400 mg choline, 625 mg uridine monophosphate, 40 mg vitamin E, 80 mg vitamin C, 60 µg selenium, 3 µg vitamin B12, 1 mg vitamin B6, and 400 µg folic acid. Placebo: Isocaloric drink	12 weeks	Global Cognition Episodic Memory
Li et al., 2020	N = 240 Mean age 70±7 y	Older adults with MCI according to DSM-5	Active: 800 µg Folic acid and 800 mg DHA Active: 800 µg Folic acid Active: 800 mg DHA Placebo: Soybean oil	6 months	Global Cognition Executive Function Working Memory

					Semantic Memory Attention and Concentration
Andreeva et al., 2011	N = 1748 Mean age 61±9 y	Cognitively healthy adults who have had a cardiovascular event (myocardial infarction, unstable angina, or ischemic stroke	Active: 560 µg folate, 3 mg vitamin B6, 20 µg vitamin B12 and 400 mg EPA and 200 mg DHA Active: 560 µg folate, 3 mg vitamin B6 and 20 µg vitamin B12 Active: 400 mg EPA and 200 mg DHA Placebo: Gelatin capsule	4 years	Global Cognition
Post-Hoc Analyses					
Oulhaj et al., 2016	Mean age 77±5 y	Older adults with MCI	Active 800 µg folic acid , 0.5mg vitamin B12 and 20 mg vitamin B6	2 years	Global Cognition

	N = 266		Placebo: Placebo tablet		Episodic Memory
			Post-hoc measurement: plasma omega-3 PUFA		
Jernerén et al., 2019	Mean age 77±4 y N = 171	Older adults with Alzheimer's Disease treated with acetylcholinesterase inhibitors	1.7 g DHA and 600 mg EPA Placebo: isocaloric placebo oil (1 g of corn oil, including 0.6 g of linoleic acid) Post-hoc measurement: Plasma homocysteine	6 months	Global Cognition
Van Soest et al., 2021	Mean age 60.2 ± 5.6 N = 791	Cognitively healthy adults with elevated plasma homocysteine (≥13 μmol/L)	800 μg folic acid Placebo: Placebo Tablet Post-hoc measurement: plasma cholesteryl ester omega-3 PUFA	3 years	Global Cognition Episodic Memory Processing Speed Executive Function

Abbreviations: DHA (docosahexaenoic acid); DSM (The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition); EPA (eicosapentaenoic acid); IWG-1 (International Working Group-1); MCI (mild cognitive impairment); MMSE (mini mental state examination); Omega-3 PUFA (omega-3 polyunsaturated fatty acid); y (year)

2.3.5 Quality Assessment

A summary of the risk of bias assessment from the RoB 2 can be found in Figure 15.

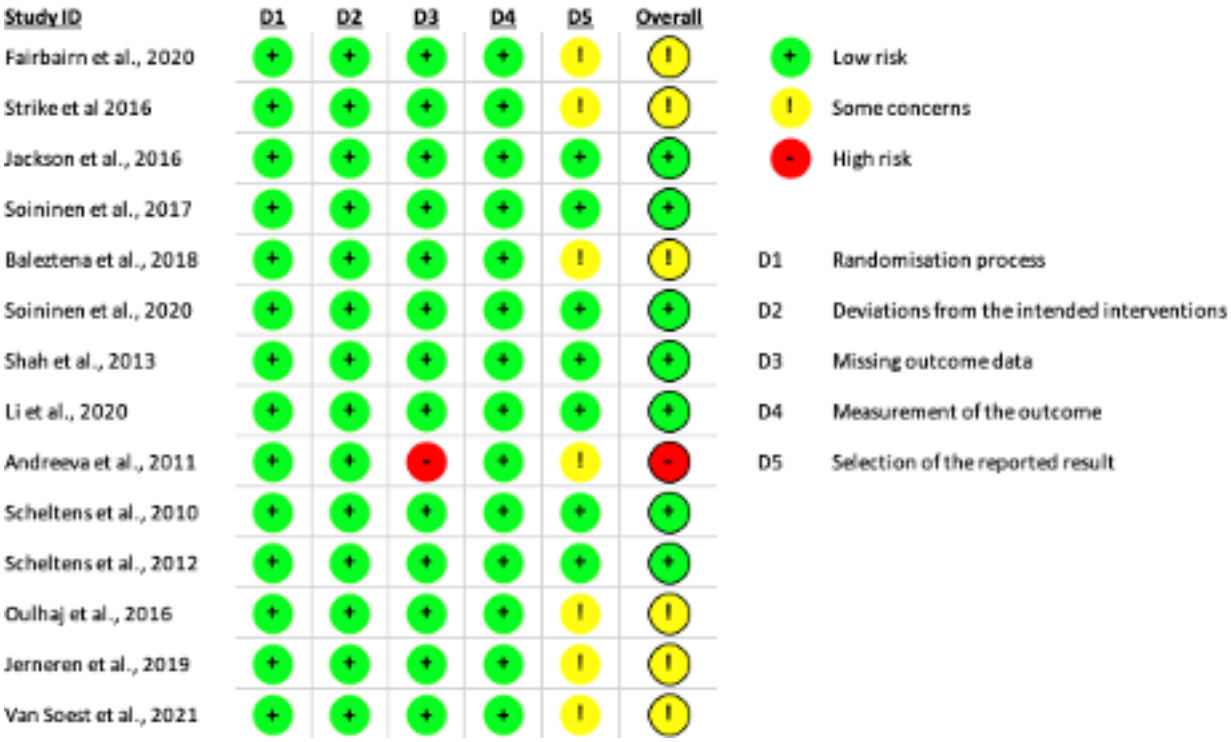


Figure 15 Individual study results from the Cochrane risk-of-bias tool for randomized trials version 2 (RoB 2). In this color-coded ranking, green colour represents low risk of bias, yellow some concerns, and red high risk of bias.

2.3.6 Findings of the Included Studies

Within the studies that used multi-nutrient formulas that included ingredients beyond omega-3 PUFAs and B vitamins, three different nutrient formulas were subject to testing. Five of these publications were derived from testing of Fortasyn Connect nutrient blend. The early human trials of the Fortasyn Connect blend were performed in drug naive mild AD patients (Formal diagnosis of probable AD and MMSE score >20) and yielded positive results (Scheltens et al. 2010; Scheltens et al. 2012). Both of these studies showed positive effects of the nutrient intervention versus placebo on memory; however, there were no significant effects on other cognitive domains and tests which included executive function, Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) and the 12-item Neuropsychiatric Inventory. Following on from these initial human trials in drug naive AD patients a study in 527 participants who were taking AD medications found that 24 weeks of the Fortasyn Connect blend had no effect on ADAS-cog, attention and concentration, executive function, processing

speed or semantic memory (Shah et al. 2013). Within 311 participants at the prodromal stage of AD, following a 24 month intervention with the Fortasyn Connect blend, there were no significant effects on the composite score for the neuropsychological test battery as well as composite scores for episodic memory and executive function in both intention to treat and per-protocol analysis, however a positive effect was observed on the clinical dementia rating score with a difference in the rate of decline of 0.6 versus the placebo (Soininen et al. 2017). This study was subsequently extended by an additional 12 months which included 162 of the original participants. After 36 months of supplementation there was a significant improvement in the composite score for the neuropsychological test battery, composite episodic memory and clinical dementia rating all with small effect sizes based on the Cohens D statistic (Soininen et al. 2021).

Outside of the work conducted on the Fortasyn Connect blend, six other RCTs were identified that providing participants with a blend of omega-3 PUFAs and B vitamins. The SU.FOL.OM3 trial tested the effects of B vitamins and omega-3 PUFA supplementation alone and in combination in a factorial design (Andreeva et al. 2011b). The study was initially designed to detect changes on cardiovascular events however ancillary findings have been published on cognition where sub group analysis was performed to focus on participants aged 65-80 years. The authors found that in the participants aged 65-80 years B vitamin supplementation alone was associated with a lower global cognition with no other effects of supplementation in any other group. In a similar factorial design, which provided DHA and folic acid alone and in a combined formula in a sample of 240 older adults with MCI, the combined intervention led to a significant improvement in global cognition versus placebo with each intervention alone providing no significant benefit to this domain. Improvements in executive function and working memory were also observed for the combined DHA and folic acid intervention (Li et al. 2020).

The effects of a high DHA multi-nutrient supplement containing a daily dosage of 1000 mg DHA, 160 mg EPA, 20 µg vitamin B12, 1 mg folic acid, 124 mg PS, 20 mg vitamin E and 240 mg *Ginkgo Biloba* standardized leaf extract in older adults has been examined in three RCTs. Strike et al. 2016 found that 24 weeks of dietary supplementation resulted in improved episodic memory and processing speed versus placebo in a sample of 27 healthy older adults. A subsequent 24 week RCT from this lab found improvements in episodic memory and executive function within a sample of 25 healthy older adults (Fairbairn et al. 2020). The final study to examine the effects of this nutrient formula was conducted in a sample of 100 older adults with subjective memory complaints for 24 weeks (Jackson et al. 2016a). As well as having the multi-nutrient supplement and placebo interventions, the researchers also added a third group that took a similar dose of fish oil alone (896 mg DHA and 126 mg EPA) to assess whether the

addition of the supporting nutrients influenced the results. The primary outcome of this study was cerebral blood flow in the prefrontal cortex with cognitive tests that are specific to this region being included as secondary outcomes. Following the intervention period there was no effect of the multi-nutrient formula or omega-3 PUFA alone on cerebral blood flow or cognitive outcomes. The final identified RCT examined the effects of a nutrient blend containing a daily dosage of 750 mg DHA, 120 mg EPA, 15 mg vitamin E, 45 mg phosphatidylserine, 285 mg tryptophan, 15 μg vitamin B12, 750 μg folate and 180 mg *Ginkgo Biloba*. The study included 99 older adults who were either cognitively healthy or were showing signs of MCI, assessed via the Global Deterioration Scale. No significant effects of supplementation were detected across the tested cognitive domains which included global cognition, executive function and semantic memory.

Three published articles from post-hoc analyses of previous primary data sets were identified. The first was from The Vitamins in Cognitive Impairment (VITACOG) trial by Smith and co-workers (2010a), a study on B vitamin supplementation, which reanalysed the data on cognitive function based on participants plasma omega-3 PUFA levels. Participants from the study by Smith and co-workers (2010a), which provided B vitamin supplementation (daily dose 0.8 mg folic acid, 20 mg B6 and 0.5 mg B12) to 187 older adults with MCI, were divided into tertiles based on their plasma omega-3 PUFA levels at baseline. The authors found that total EPA+DHA above 590 μmol/L in conjunction with B vitamin supplementation improved episodic memory, global cognition, and clinical dementia rating score whereas participants with omega-3 PUFA levels of less than 390 μmol/L showed no benefits of B vitamin supplementation (Oulhaj et al. 2016). When results from these analyses were stratified by EPA and DHA separately, DHA alone but not EPA led to significant effects on clinical dementia rating and episodic memory. In a similarly designed post-hoc analysis of the Folic Acid and Carotid Intimamedia Thickness (FACIT) trial, where 791 women with elevated plasma homocysteine (≥13 μmol/L) were provided with 800 μg folic acid for a period of three years, participants were stratified by omega-3 PUFA content in cholesterol esters. Positive interactions were detected between folic acid supplementation and omega-3 PUFA levels for global cognition and processing speed, however these interactions were only observed within the lower tertile of omega-3 PUFAs, indicating that folic acid supplementation was only effective whilst accompanied by lower levels of circulating omega-3 PUFAs (van Soest et al. 2021). The final post-hoc analysis reanalysed results from a trial on omega-3 PUFA supplementation, the OmegAD study by Freund-Levi et al. , according to baseline plasma homocysteine levels. The OmegAD trial provided 171 older adults with AD with omega-3 PUFA supplements containing 1720 mg DHA and 60 mg EPA for six months. Overall, no effects of supplementation were observed on cognitive outcomes. In the post-hoc analysis participant's baseline plasma

homocysteine was measured to determine whether this modified the response to omega-3 PUFA supplementation. The results from the analysis showed that those in the active omega-3 PUFA supplementation group who also had homocysteine levels <11.7 mol/L had a 7.1% improvement in their MMSE scores versus those in the same homocysteine tertile assigned to the placebo supplement. A similar result was found for clinical dementia rating sum of boxes score with improvements of 22.3% (Jernerén et al. 2019).

2.3.7 Meta-Analysis Results

A summary of the result of the meta-analysis along with forest plots can be found in Figure 16. Four studies were included within the primary analysis as they assessed cognition using a composite score derived from neuropsychological test battery. The nutrient formulas that combined omega-3 PUFAs and B vitamins demonstrated a significant improvement in cognitive function versus the placebo with no significant heterogeneity between studies (G=0.23, 95% CI 0.09–0.37, P=0.002, I^2=0%).

Five studies were included in the analysis of single measures of global cognition, these measures included the mini mental state examination (MMSE), (ADAS-cog), Clinical Dementia Rating Sum of Boxes and full-scale IQ. Values for the level of variability were not provided for the study by Scheltens et al., 2010, thus this study could not be included. In the study by Andreeva et al., 2011 cognitive function was assessed at the conclusion of the supplementation period and no baseline assessment was conducted. As such this analysis was comparing absolute values for cognition between groups at the end of the study so no conclusions can be drawn as to whether the supplementation slowed the rate of cognitive decline over time so this study was not included within the meta-analysis. The combined omega-3 PUFA and B vitamin interventions demonstrated a significant improvement in single measures of global cognition versus the placebo, with their being significant heterogeneity between studies (G=0.28, 95% CI 0.09–0.47, P=0.004, I^2=56%). Five studies included domain specific measurements of episodic memory. The combined omega-3 PUFA and B vitamin interventions in these studies demonstrated a significant improvement in cognitive function versus the placebo with there being significant heterogeneity between studies (G=0.32, 95% CI 0.13–0.51, P=0.001, I^2=62%). Seven studies included domain specific measurements of executive function, no significant effect was demonstrated in response to combined intervention with omega-3 PUFAs and B vitamins, with there being significant heterogeneity between studies (G=0.29, 95% CI -0.13–0.71, P=0.17, I^2=63%).

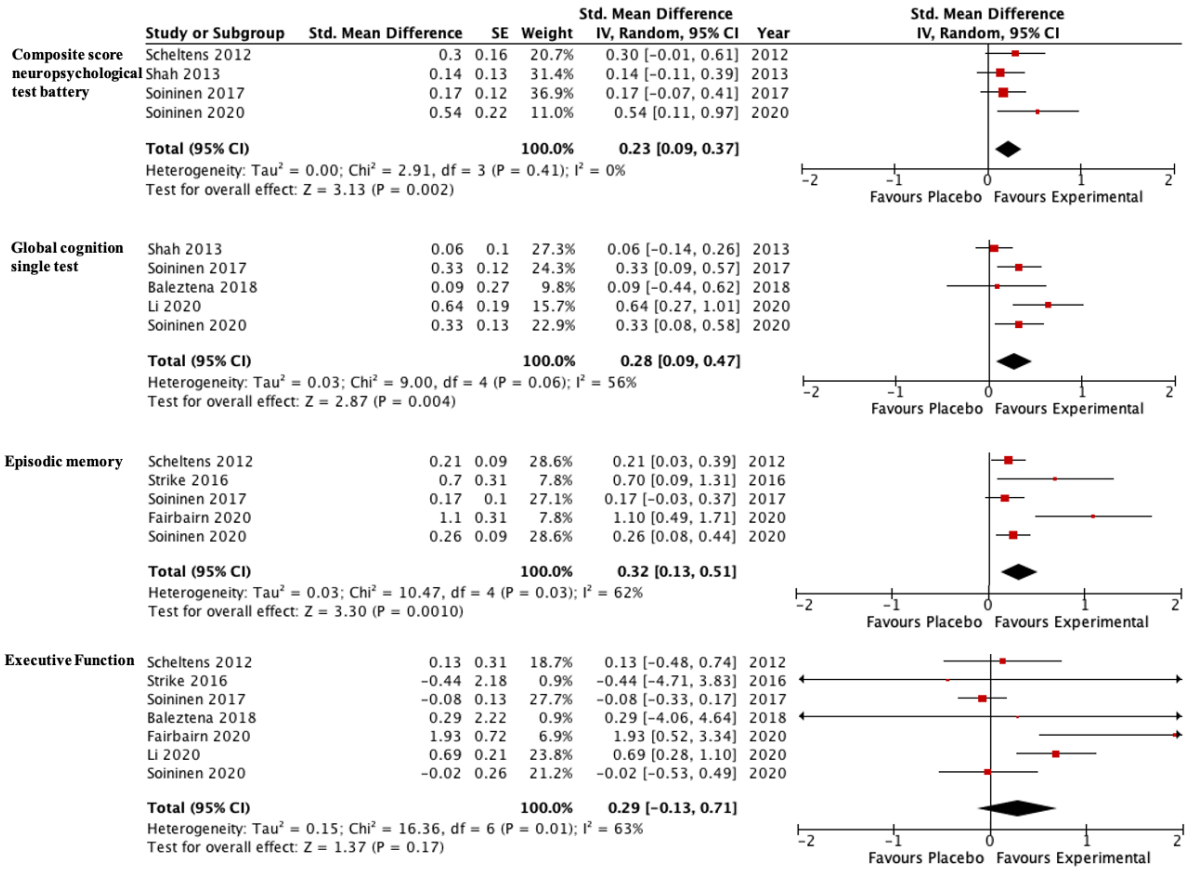


Figure 16 Meta-analysis and forest plots for the effects of multi-nutrient interventions containing both omega-3 polyunsaturated fatty acids and B vitamins on composite scores from neuropsychological test batteries, single measures of global cognition, episodic memory and executive function.

2.4 Discussion

Results from this meta-analysis suggest a benefit of supplementing with nutrient formulas that contain both omega-3 PUFAs and B vitamins on global cognition and episodic memory with small to moderate effect sizes. The positive result from the primary analysis on global cognition assessed using composite scores derived from neuropsychological test battery were all from studies which had used the Fortasyn Connect formula. The Fortasyn Connect blend has shown promise in animal and *in vitro* models enhancing neuronal survival and hippocampal cholinergic neurotransmission by enhancing synaptic membrane formation (Cansev et al. 2015; van Deijk et al. 2017), as well as increases in neurogenesis and spatial memory compared with omega-3 PUFA alone (Jansen et al. 2013; Koivisto et al. 2014). Despite this success at the pre-clinical stage, the Fortasyn Connect formula did not lead to significant improvements versus placebo in overall neuropsychological test battery score in older adults at the prodromal stage of Alzheimer's disease (Soininen et al. 2017). A major limitation of this study was that the

expected decline in cognitive function in the placebo group was 74% lower than expected rendering the primary endpoint inadequately powered to detect any changes. This is an important consideration when evaluating the evidence for the role of interventions on cognitive decline as without adequate decline in the placebo group it is unlikely that any effect will be able to be detected. In the extension of this trial more notable decline was observed within the placebo group and a significant effect was observed for global cognition as well as episodic memory.

Results from the post-hoc analyses of previous studies are in support of the interaction between omega-3 PUFAs and B vitamins. Data from the post-hoc analyses from the VITACOG study indicated that response to intervention with B vitamins are impacted by omega-3 PUFA status with a combination of higher levels of omega-3 PUFAs alongside B vitamin supplementation conferring the greatest benefit to cognition (Oulhaj et al. 2016). Similarly, data from the OmegaAD demonstrated that response to omega-3 PUFA supplementation appears to be diminished in those with elevated homocysteine (Jernerén et al. 2019). There is however some discrepancy amongst the data from post-hoc analyses as results from the FACIT trial suggested that B vitamin supplementation was more effective at lower levels of omega-3 PUFAs. There are some important differences between the methods to note within this analysis. Firstly, in the previously mentioned post-hoc analyses the participants already had some level of diagnosed cognitive impairment either having MCI or AD whereas the FACIT trial was conducted in healthy older women between the ages of 50-70 years. Within healthy participants it can be common to observe lower levels of cognitive decline which will impact the ability to detect changes. Indeed, within the FACIT trial participants did not show any evidence of cognitive decline for global cognition, episodic memory or executive function in the placebo group, with declines in performance only being observed for processing speed. This make it difficult to draw direct comparisons to the VITACOG study where some decline was observed in the placebo group likely due to the increased susceptibility to decline of the participants recruited for the study, given that they were already displaying symptoms of cognitive impairment (de Jager et al. 2012). Another important distinction is that the blood fraction upon which omega-3 PUFAs were quantified in the FACIT trial was in the plasma cholesterol esters, where results were expressed as a percentage of total fatty acids, whereas within the similarly analysed VITCOG study omega-3 PUFAs were measured in total plasma and presented as absolute values. Omega-3 PUFAs are differentially incorporated into different blood fractions with EPA being preferentially incorporated into cholesterol esters and DHA into plasma phospholipids and triglycerides (Vidgren et al. 1997), thus measuring cholesterol esters is a less reliable method for quantifying total omega-3 PUFA status. The different fractions and the way in which the results have been expressed make it difficult to draw comparisons between these two

studies, as omega-3 PUFA levels have been divided into tertiles it is unclear as to whether their tertiles are actually comparable as the respective circulating levels could be quite different. There is a need for a more standardised approach to measuring and reporting fatty acid status within human trials to allow for more direct comparisons between studies (Brenna et al. 2018), however it is understandable that this may be difficult to achieve in such post-hoc analysis where samples have previously be taken and stored. It is also important to note that although these post-hoc analyses do provide some very interesting insight into the potential interaction between these nutrients, these studies were exploratory in nature. As such they were not adequately powered to draw definitive conclusions, thus results should be considered preliminary at this stage. Furthermore, it is important to note that these studies did not provide both omega-3 PUFAs and B vitamins in their supplement formulas thus the post-hoc analysis was looking into nutrient status from background diet. These results do however provide rationale for studies measuring the effects of a single nutrient intervention of omega-3 PUFAs or B vitamins to consider that the status of the other nutrient could impact the responsiveness of participants to supplementation.

2.5 Strengths and Limitations

To the best of the authors knowledge, this is the first time the evidence base on combining omega-3 PUFAs and B vitamins has been systematically reviewed. This provides an update date overview on the current evidence base and highlights the need to consider the complex relationship between these key nutrients in relation to cognition within the older adult. The review has focused on randomised double blinded placebo control trails meaning that causality of interventions can be better established.

Whilst the focus of this study was to evaluate the effects of a combination of omega-3 PUFAs and B vitamins, studies which provided formulas with additional ingredients were also included. As such it is not possible to determine whether these additional ingredients may have modified the response to such formulas. That being said when looking at these formulas, omega-3 PUFAs and B vitamins arguably do stand out as having the strongest evidence base for potential efficacy. There is evidence to suggest that response to omega-3 PUFA or B vitamin supplementation may be modified by baseline cognitive status. Participants who are healthy or at the earlier stages of cognitive decline have been shown to be more likely to respond to nutrients intervention versus those with diagnosed AD or dementia (Freund-Levi et al. 2006; Cederholm et al. 2013). Due to the limited available studies for the meta-analysis participants were not stratified by baseline cognitive status. Furthermore, at this stage there was no stratification performed for the dosage of nutrients or for the specific fatty acid composition of omega-3 PUFA formulas. DHA is highly enriched within the brain and appears to be the more

important bioactive omega-3 PUFA in regards to prevention of cognitive decline (Yurko-Mauro et al. 2015; Zhang et al. 2015). Moreover, there is some data to suggest that combined dosages of 1 g EPA+DHA including a minimum of 580 mg DHA per day are required to elicit positive effects on episodic memory in adults with memory complaints and without (Yurko-Mauro et al. 2015). Within the meta-analyses of the present study all nutrient formulas had a DHA dosage of greater than 580 mg per day, which may have contributed towards the observed positive effects on cognition versus previous reviews of single nutrient interventions, which have included studies which provided lower dosages (Mazereeuw et al. 2012). In regards to B vitamins there is limited dose-response data for nutrient formulas however baseline homocysteine levels do appear to modify response to supplementation with those who have higher levels responding more favourably to supplementation (de Jager et al. 2012). A definitive threshold by which homocysteine needs to be lowered to observe a positive effect on cognition is yet to be established but it has been suggested that attaining levels ≤10.1 μmol/L could be effective (Smith et al. 2010a; de Jager et al. 2012). As we seek to develop a greater understanding of the potential interaction between omega-3 PUFAs and B vitamins we also may need to consider the specific fatty acid composition and dosage provided in any nutrient blends, as well as the baseline homocysteine status of the participants.

There was notable heterogeneity in the methods that were used to assess cognition within the included studies as well as the domains that were tested. The decision was made to focus on studies which had used composite scores derived from neuropsychological tests batteries due as this method may be more sensitive to detecting changes (Vellas et al. 2008), with secondary analyses on single tests of global cognition and domain specific tests of episodic memory and executive function. This meant that not all cognitive data from the included articles were entered into the meta-analysis.

Within the research into cognitive decline there is currently no standardised protocol for cognitive testing. As the field moves forward it is important to have more uniform methods for assessing cognitive status. The use of neuropsychological test batteries that form a composite score for multiple tests would likely be a good choice to use as a primary outcome for future research, with domain specific tests being used as secondary outcomes. This approach would allow for the assessment of global cognition as well as allowing labs to detect any potential domain specific effects.

2.7 Conclusion

Taking into consideration the literature described above, there is now evidence to suggest that providing nutrient formulas that contain both omega-3 PUFAs and B vitamins could be efficacious for preserving cognition in the older adults. A possible positive interaction between

B vitamins and omega-3 PUFAs is promising and has largely not been considered in previous human trials. This may be of particular importance when we consider that vitamin B12 inadequacies are common in older adults (Allen 2009), and omega-3 PUFA status has been demonstrated to be sub-optimal across multiple population groups (Stark et al. 2016b). More experimental work providing a combination of nutrients including both omega-3 PUFAs and B vitamins, in healthy older adults or those showing early signs of cognitive decline, is clearly warranted to better explore how nutrition as a whole can impact the trajectory of cognition in older adults.

2.8 Conclusion to Literature Review and Aims and Objectives of the Thesis

Ageing is complex and involves declines in several domains including mobility and cognitive function and quality of life (Lara et al. 2013). With the expected rise in the prevalence of age related disease, and the healthcare costs related to this, there is a strong need for the development of lifestyle interventions to prevent age related decline and encourage healthy ageing. It has become apparent that there is a need to consider multiple outcomes in studies into healthy ageing including mobility, cognitive function, quality of life and biochemical markers to fully assess the effectiveness of lifestyle interventions in older adults (Lara et al. 2015).

Aerobic exercise and the dietary components of omega-3 PUFA, B vitamins, vitamin E, PS and *Ginkgo Biloba* have separately been identified and shown promise in preventing age related declines in mobility and cognitive function in older adults (Harber et al. 2009; Reay et al. 2013; Dysken et al. 2014; Tan et al. 2015; Strike et al. 2016). A combination of an omega- 3 PUFA based supplement and exercise may have a greater benefit to mobility and cognition than each separately (Wu et al. 2008; Rodacki et al. 2012; Da Boit et al. 2016b; Kobe et al. 2016), however, there is currently limited data in humans which has investigated this combination. Despite providing some promising results for the efficacy of combining an omega-3 PUFA supplement and exercise, the studies in humans have had small sample sizes and do not include a true placebo controlled control group.

Not only is the combination of diet and exercise a novel concept within research into healthy ageing, nutrient interactions have become a vital consideration within the field. The multi-nutrient supplement being tested is a unique blend of nutrients containing EPA, DHA, B12, folic acid, PS, vitamin E and *Ginkgo Biloba*. Results from the systematic review with a meta-analysis in chapter 2 on the combination of omega-3 PUFAs and B vitamins has highlighted the potential for beneficial effects of multi-nutrient formulas containing both of these key ingredients on cognition within the older adult. Furthermore results from our lab has indicated this formula may be effective in slowing the decline in gait speed within this population group (Strike et al. 2016). Whilst the mechanisms that underpin this potential interaction are yet to be

fully elucidated it has been suggested that elevated levels of homocysteine could disrupt DHA and phospholipid metabolism whilst low DHA intakes could lead to lower availability of phospholipids for methylation leading to the hypermethylation of histones, which in turns alters gene expression (*Figure 17*). Moving forward the potential for an interaction between omega-3 PUFAs and B vitamins should be considered when designing RCTs into healthy ageing, especially when cognitive outcomes are being measured.

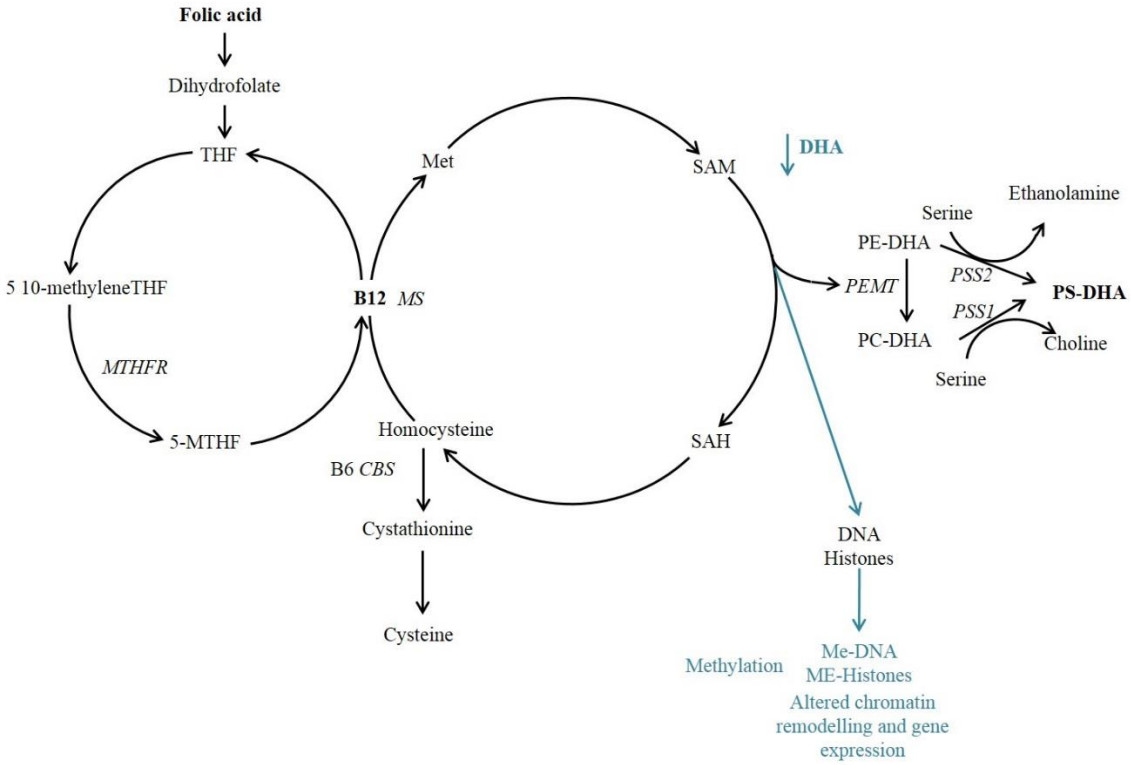


Figure 17 *Proposed mechanisms for the interaction between B vitamins, DHA and PS. Folic acid is converted to 5-MTHF which then donates a methyl group to resynthesize methionine from homocysteine using B12 as a cofactor. A build-up of homocysteine increases SAH which inhibits SAM methyltransferase reactions, which disrupts DHA enriched phospholipid metabolism and conversion to PS enriched with DHA by inhibiting PE methyltransferase (Selley 2007). Low DHA intakes could lead to lower availability of phospholipids for methylation leading to the hypermethylation of histones, which in turns alters gene expression (Ye et al. 2017).*

Abbreviations: 5, 10 methylenetetrahydrofolate (5, 10-methylene THF), Methylenetetrahydrofolate reductase (MTHFR), 5-Methyltetrahydrofolate (5-MTHF), Tetrahydrofolate (THF), methionine synthase (MSy), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), Methylenetetrahydrofolate reductase (MTHFR), Cystathionine beta synthase (CBS), PE Methyltransferase (PEMT), PE (PE), Phosphatidylcholine (PC),

Docosahexaenoic Acid (DHA), Phosphatidylserine (PS) Phosphatidylserine-docosahexaenoic acid (PS-DHA), PE- docosahexaenoic acid (PE-DHA), Phosphatidylcholine- docosahexaenoic acid (PC-DHA), methylated (ME).

The overarching aim for the PhD is to investigate the effects omega-3 PUFAs and supporting nutrients along with exercise on a series of outcomes, including mobility cognitive function and quality of life, that encapsulate multiple domains related to healthy ageing in older females (Lara et al. 2015). To meet this aim a randomised semi-blinded placebo controlled trial investigating the effects of a high DHA multi-nutrient supplement containing EPA, DHA, vitamin B12, folic acid, PS, vitamin E and *Ginkgo Biloba* extract and exercise on mobility and cognition in women aged ≥60 years was performed.

The aims for this study were as follows:

- Investigate the effects of a high DHA multi-nutrient supplement and exercise alone and in combination on a series of outcomes related to the HAP including, gait parameters, verbal memory, spatial memory, executive function, interference control and health related quality of life versus placebo in pre-frail and non-frail older women.
- Determine whether the combination of a high DHA multi-nutrient supplement and exercise can have a broader effect on outcomes related to the HAP in pre-frail and non-frail older women by significantly impacting a greater number of outcomes than each separate intervention. 6

This study had several novel aspects to its design

- The use of a multi-nutrient intervention that contains a unique formula, which may exert additive or synergistic benefits to healthy ageing outcomes.
- The combination of the multi-nutrient and aerobic exercise intervention given the promising evidence for the potential positive interaction between omega-3 PUFAs and exercise in older adults.
- The incorporation of a range of outcomes that span across multiple health domains to better encapsulate the HAP.

Chapter 3 - Method Development and Study Protocol 7

The experimental work conducted within this thesis involved measuring several outcome measures across multiple health domains, including mobility, cognitive function and biochemical outcomes. Due to the complex nature of the planned methodology a series of pilot/feasibility studies were conducted to develop and refine the methods. This chapter will outline these preliminary studies, explain how they informed the methodology for the experimental work and conclude with the final published protocol.

The development of the cognitive and gait testing protocols were established with a pilot/feasibility study.

The aims of the feasibility phase of the method development were as follows.

- Assess whether all equipment and tests for assessing cognitive and gait function could be carried out in the specific testing area.
- Ensure that the written and verbal instructions for each test are clear and can be understood by the participants.
- Establish the amount of time that is required to complete measurements so that clear information can be given during study recruitment and that the time taken does not create a burden on the participants.

The aims of the pilot phase of method development were as follows.

- Establish whether there were any floor or ceiling effects of the testing protocols in the specific target demographic for the RCT.
- Assess the difficulty levels of the secondary tasks in the dual task gait testing to narrow down dual task testing to one secondary task.
- Determine which outcomes from individual cognitive testing should be included within the RCT.

Quantification of whole blood fatty acids is an essential component of the planned experimental research. As such, a protocol for the measurement of whole blood fatty acids was also established. The aims for this pilot study are outlined below.

- Chief investigator to practice a previously validated technique for the extraction and quantification fatty acids from whole blood (Marangoni et al. 2004).
- Ensure this method can be performed consistently to ensure accurate and reliable and valid reporting of whole blood fatty acids, by demonstrating a coefficient of variation of <10% for DHA and <15% for all other measured fatty acids.

3.1 Pilot and Feasibility study on Cognitive and Gait Measurements

3.1.1 Methods

Ethical Approval

Ethical approval for the study procedure was granted by the Bournemouth University Science Technology and Health research ethics panel (Ethics ID 11502, 13137 and 13138). Recruitment and data collection took place at Bournemouth University within the SportBU facilities from June 2016 to February 2017.

Participants

The first phase of this study was conducted in younger adults. The feasibility data derived from these young adults was subsequently used to help inform the testing scheduling for the second phase of the study which was limited to older adults. Whilst the data from the younger adults did provide some useful insight into the approximate time required to complete the testing protocols, it was anticipated that conducting these tasks within older adults would take longer and participants may need additional breaks, especially during mobility testing. As such, feasibility was considered whilst testing both younger and older adults. Furthermore it was expected that younger adults will perform more favourably on the cognitive and mobility tasks than older adults, thus if there was any indication of floor effects within the younger participants the difficulty of the tasks could be adjusted prior to testing within older adults. 8

Young adults were recruited according to the following inclusion criteria: (1) able to walk unaided for at least 50m, (2) aged 18-59 years. Exclusion criteria were (1) received lower limb surgery, (2) diagnosed neurological conditions (3) vestibular impairments.

The second phase was conducted in older adults. Older women were recruited according to the following inclusion criteria: (1) female, (2) aged 60 years or greater (3) able to walk unaided for at least 50m. Exclusion criteria were (1) received lower limb surgery, (2) diagnosed neurological conditions (3) vestibular impairments.

Cognitive and Gait Analysis

Cognitive and gait analysis was performed in both young and older adults according to the methodology stated below.

The time taken to set up testing equipment in the SportBU studios and carry out the testing itself was recorded for each participant. Furthermore, participants were asked to provide their feedback on the clarity of both the verbal and written instructions for each task.

Gait Analysis

Gait speed, stride length variability, stride length, cadence, and double support phase percentage were measured using wearable inertial measurement units each fitted with 3-axis accelerometer, 3-axis gyroscope and 3-axis magnetometer (APDM Inc., Portland, OR, USA) (Each sensor mass: 22 g, dimensions: 48.4 × 36.5 × 13.4 mm) and analysed using Mobility Lab™ software version 3.1 (APDM Inc, http://apdm.com).

The inertial measurement units have previously been shown to have excellent test-retest reliability across HGS, stride length, cadence and stride time measurements with a Lin's concordance correlation coefficient of 0.93 (Washabaugh et al. 2017), as well as showing a strong positive correlation with the gold standard Vicon system for cadence and stride length (Simoes 2011). On an individual basis, minimal detectable change for gait speed using the Opal inertial sensors and Mobility Lab™ software version 3.1, has been demonstrated to be 0.12 m/s, however this value must be modified to factor in sample sizes to determine minimal detectable change for an intervention study using the following formula (Washabaugh et al. 2017):

$$MDC_{group} = MDC_{individual} / \sqrt{n}$$

MDC: minimal detectable change; n: sample size 9

Based on the sample size projections of 13 per group for the intervention study (see sample size calculation on p.161) this would result in a minimal detectable change of 0.033 m/s using this gait analysis equipment. Together with the data indicating excellent validity and test-re-rest reliability (Washabaugh et al. 2017) and an ability to detect changes that are in line with the minimal clinically meaningful difference for HGS in older adults (Kwon et al. 2009), the gait assessment equipment that will be used within the RCT has shown strong indications of robustness.

Placement of sensors on the feet has shown to have a higher degree of accuracy, versus placing them on the ankles, with a Lin's concordance correlation coefficient of 0.95 for measuring HGS versus a Lin's concordance correlation coefficient 0.86 for ankle placement. Therefore, Sensors were placed on the feet over the shoes, and participants were asked to walk 13 m for the test. Acceleration and deceleration phases of the gait cycle were removed from the analysis. Before the recorded gait analysis participants were asked to complete three lengths of the walkway to ensure they felt comfortable wearing the sensors.

Participants were asked to complete three different task conditions: habitual single task walking (ST), walking whilst counting backwards in integers of three (3DT) and walking whilst counting back in integers of seven (7DT). Although there is currently no standardised secondary task for dual task gait protocols, a backwards counting task has been used in several prior studies in similar demographics (van Iersel et al. 2007; Hausdorff et al. 2008; Hall et al. 2011;

Ullmann and Williams 2011). During the dual task protocols, participants counted backwards from a randomly generated three digit number given to them three seconds before commencement of the task. For all tasks, participants were asked to walk at a comfortable pace and during dual task conditions no instruction on prioritisation of task was given. Each tested condition was repeated five successful times to obtain representative samples and the means of the five successful trials are used for data analysis.

Cognitive Testing

Domain specific testing was used to assess cognitive function. There is evidence to suggest domain specific effects of both nutrient and exercise interventions (Smith et al. 2010c; Yurko-Mauro et al. 2015). The cognitive domains that were selected were verbal memory, executive function, spatial working memory and interference control. These cognitive domains fall under the category of fluid intelligence and have all been demonstrated to decline during healthy and pathological ageing (Harada et al. 2013) and have shown some promise to be modulated by either the ingredients in the multi-nutrient formula or exercise (Smith et al. 2010c; Strike et al. 2016).

Verbal Memory

The RAVLT was selected to assess episodic verbal memory, a domain that has been demonstrated to be one of the most affected domains during the ageing process (Mortamais et al. 2017). The RAVLT is an established test for assessing verbal episodic memory (Rey 1941), and has been shown to be sensitive enough to predict future memory decline and progression to AD (Estévez-González et al. 2003). The test consists of a total of seven trials. Initially, participants were read a list of 15 pre-defined words. Immediately after each reading, they were required to recall as many words as possible, this was repeated five times with the same list of words. Next, an interference list is read containing fifteen different words, after which participants were asked to recall the original list. During the last trial participants were read the original list a final time and asked to recall the words after a twenty minute delay. Results from the RAVLT are divided into subcategories. Total recall is the number of words recalled across the first five trials, interference recall is the number of words recalled under the inference condition and delayed recall is the number of words recalled after the twenty minute delay.

Spatial Working Memory

Spatial working memory is defined as the ability to maintain spatial information active in working memory over a short period of time and appears to be predominantly reliant upon the hippocampus, a region of the brain that has shown to be impacted during healthy and pathological ageing (Broadbent et al. 2004; Raz et al. 2005). Spatial working memory allows older adults to be able to travel from one place to another, remember the locations of objects

within a specific environment, recall the spatial arrangements of objects, and to recognise their own location in the environment (Korman et al. 2019).

Spatial working memory was assessed using a computerised task, run on Open Sesame version 3.1.1. software, and was developed at Bournemouth University based upon work conducted by Nagamatsu, L. S. et al. (2013). The task required participants to recall the spatial location of dots presented on a laptop screen. Each trial comprised of a presentation, a retention interval and a test phase. In the presentation phase, three dots appeared at randomly allocated locations for 500 m, this was followed by a fixation cross which appeared for 3 s. After the retention interval the test phase comprised of the presentation of a single red test dot on the screen, this could either be in the same location as one of the previous black dots (match) or in a different location (non-match). Participants were asked to identify if the red test dot was a match or a non-match to any of the prior black dots by pressing an assigned key on the keyboard ("y" = match; "n" = non-match). There was no time limit for the participants to respond as the focus of the task was on response accuracy. The task consisted of ten practice trials, followed by sixty recorded trials. Thirty of the trials were matched and thirty were non-matched. The thirty non-matched were evenly split in three degrees of difficulty, whereby they were placed at two (near), four (medium) and eight (far) degrees visual angle (Figure 18). These angles were calculated based on the participant sitting 50 cm from the screen. Accuracy for the task was recorded as the percentage of correct answers provided, and reaction time was calculated using the mean time to answer each trial.

Executive Function

Similarly to episodic memory, executive function has been highlighted as a key domain to assess healthy ageing, as the decline in this domain is common and can be used to predict transition to pathological cognitive decline (Rajan et al. 2015). Furthermore, it is the domain that has demonstrated the strongest and most consistent association with gait speed and stability (Coppin et al. 2006; Holtzer et al. 2006; Springer et al. 2006; Kearney et al. 2013; Martin et al. 2013a), thus making it particularly important for assessing the link between cognition and physical function in the older adult.

A trail making task was selected to assess executive function (Salthouse et al. 2000). Trail making tasks have been used extensively in neuropsychological assessment to assess executive function (Salthouse 2011a). Participants were asked to draw lines between targets as rapidly as possible, in a grid of seven by seven circles on a piece of paper. There were four different conditions for the task: (1) a numbers condition where targets went from one to 49 (numbers), (2) a letters condition where the targets went from A to Z (letters), (3) a condition where participants alternated between numbers (1-25) and letters (A-X), (numbers-letters), and finally, (4) a condition alternating between letters (A-Y) and numbers (1-24) (letters-numbers) (Figure

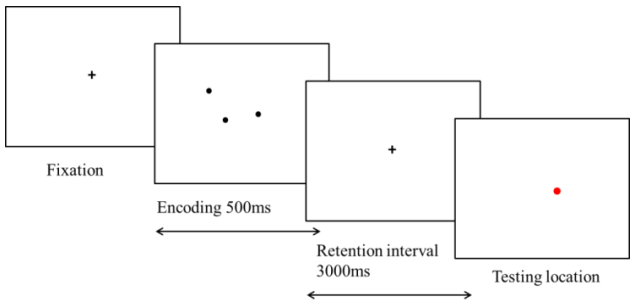
19). Successive targets were always in one of eight adjacent circles located above, below, to the left, to the right, or in one of the four diagonals adjacent to the target. This arrangement lessens the motor skill requirements that would typically be required for a standard trail making task.

Participants were first given a three by three practice trial of each condition to familiarise themselves with the task. Then, participants were then given 20 s to make as many connections as they could on each task condition on the full seven by seven grid. Scores were recorded as the total number of correct connections within the time limit, with the number of errors also being recorded.

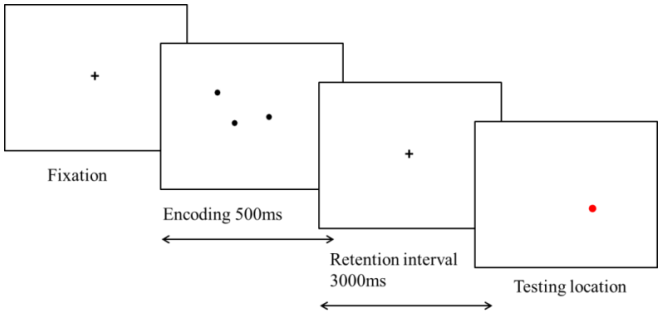
Interference Control

Interference-control is the ability to exclude distractions and focus on a specific task or stimulus. Interference control falls within the domain of executive function and decline in this domain has been demonstrated within healthy ageing (Aschenbrenner and Balota 2015).

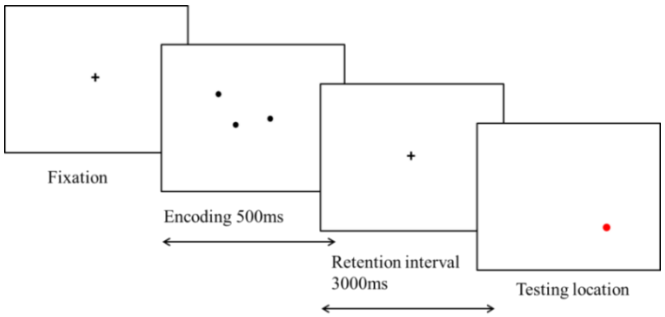
A Stroop test was developed at Bournemouth University and was used to assess interference control (Davidson et al. 2003a) using Open Sesame version 3.1.1. software (Mathôt et al. 2012). During this task, a fixation point appeared on screen for 500 ms followed by the presentation of the names of one of four colours: blue, red, green, and white. These words were presented in four different font colours varying between blue, red, green, and white. Participants were instructed to identify, as quickly as possible without sacrificing accuracy, the colour of the text rather than the word displayed on screen and press a designated key on the keyboard, highlighted using coloured stickers. The test comprised of 144 recorded trials with half of the trials having the text and colour match (congruent trials) and half being a non-match (non-congruent trials). Interference control was defined as the difference between the mean time taken to respond to the congruent and non-congruent trials with the number of errors also being recorded. Reaction times that were plus or minus 2.5 times the median absolute deviation were excluded as anomalous results (Leys et al. 2013).



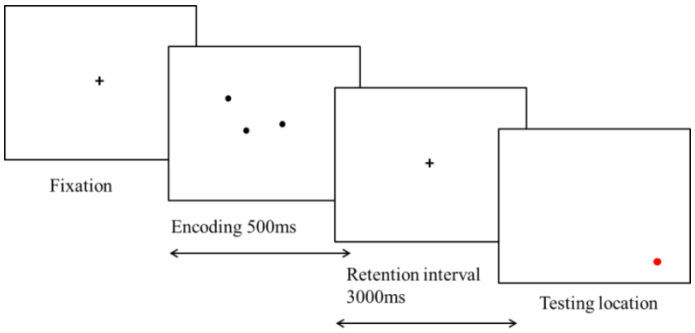
(a) Hit



(b) Lure: Close 2°



(c) Lure: Medium 4°



(d) Lure: Far 8°

Figure 18 *Spatial working memory task examples (a) a match, (b) near, (c) medium, (d) far.*

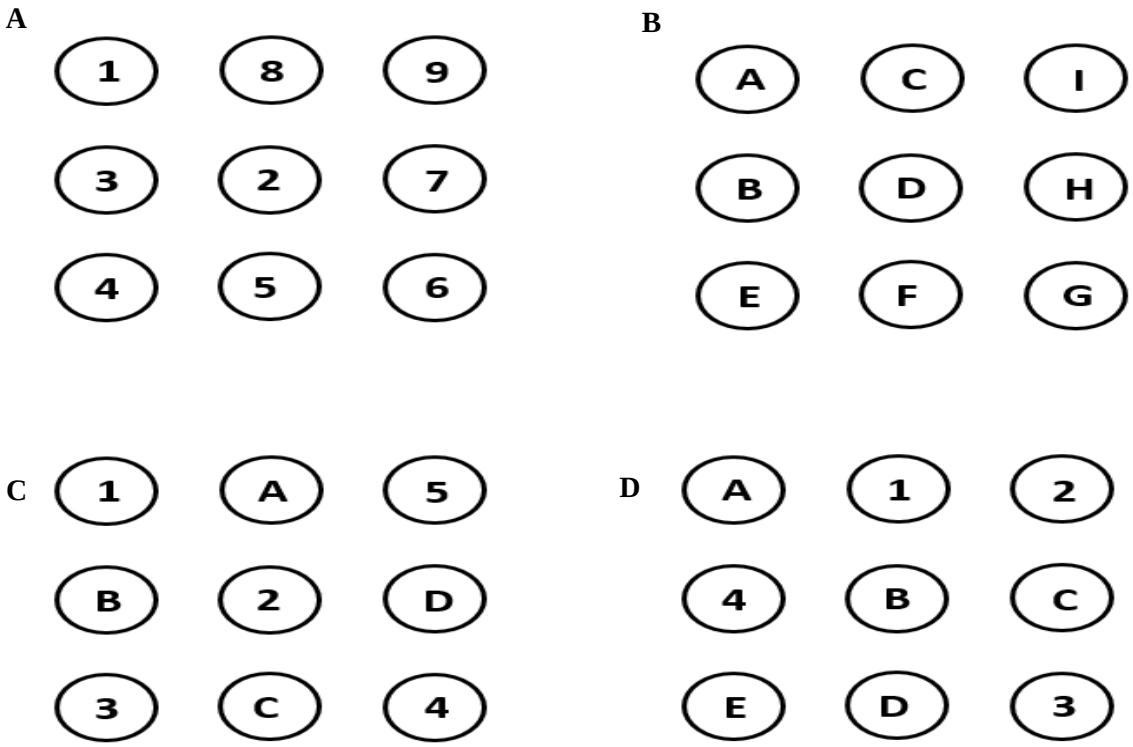


Figure 19 *Examples of the different trail making conditions A: numbers condition, B: letters condition, C: numbers-letters condition D: letters-numbers condition. The actual test comprises of a 7x7 grid rather than 3x3.*

Statistics

Mean values and standard deviations for each outcome were recorded. Floor effects were not considered for the Stroop or trail making task. Floor effects in timed tasks do not normally occur and would normally be a result of a participant not understanding how to perform the task (Rasmussen et al. 2001). A mean score of 50% or less for the spatial working memory was considered a floor effect as this test has a yes/no response, thus 50% would indicate regression towards the mean. An individual participant score of 10% or less would be considered a floor effect in the RAVLT. For the spatial working memory, RAVLT and trail making tasks, any single participant obtaining a maximal score was considered. A ceiling effect of the Stroop test would be considered if the mean value of the incongruent trials was quicker than the congruent trials in any participant.

For dual task gait assessment floor effects were defined as having a single participant stop walking at any point during the test or not being able to complete any one of the dual task gait assessments. Ceiling effects were defined as any participant walking at a speed equal to or greater than their habitual pace whilst under dual task conditions.

3.1.2 Results

Feasibility Testing

Four females and one male aged 29.2±2.7 years gave their written informed consent to take part in the study. All participants completed cognitive testing and gait testing.

In total it took 30 minutes to set up all the equipment required for testing. The cognitive test battery took 75 minutes and the gait testing 45 minutes. These timings were inclusive of providing participants with the opportunity to take short breaks between tests.

Participants expressed that they found the written and verbal instructions to be clear with one exception on the spatial working memory task. One of the written instructions read as follows: *"Your task is to state if the test dot is in the same spatial location as one of the preceding 3 dots or in a different spatial location"*, three of the participants expressed that the term spatial location was ambiguous and questioned whether it meant the same exact location or just a similar location. This was subsequently changed to *"Your task is to state if the test dot is in the exact same spatial location as one of the preceding 3 dots or in a different spatial location".*

All testing equipment was able to be set up and measurements were taken within the SportBU studios, demonstrating that the studios were an appropriate site to carry out data collection and that all equipment was functional.

Pilot Testing

Five females mean age 70±7.7 years gave their written informed consent to take part in the study. All participants completed cognitive testing and gait testing, with the exception of one older female participant who did not complete the 7DT gait condition due to feeling unconformable with the difficulty of the test.

All participants expressed that the written and verbal instructions were clear and that they understood what was expected of them, This included the spatial working memory task where the instructions had been amended based on the aforementioned feedback from the feasibility study.

Cognitive testing results for the older women are summarised in **Error! Reference source not found.**.

Gait speed, stride length variability and dual task effect on gait speed results under ST, 3DT and 7DT conditions for the older females are shown below in **Error! Reference source not found.**, **Error! Reference source not found.** and **Error! Reference source not found.**.

Table 3 Summary of cognitive test scores for older female participants.

Participant Number	Verbal Memory Total Score (%)	Verbal Memory Total Recall (total words recalled)	Verbal Memory Interference Recall (total words recalled)	Verbal Memory Delayed Recall (total words recalled)	Spatial Working Memory (%)	Spatial Working Memory Reaction time (ms)	Interference Control (ms)	Interference Control Errors	Executive Function (Correct Connections)	Executive Function (Errors)
1	50	37	8	8	70	1702	516.7	1	70	3
2	55	38	10	10	86	1338	482.13	5	37	0
3	63	43	12	11	83	2145	418.3	4	74	0
4	59	42	11	10	75	2026	482.16	3	93	1
5	65	44	12	12	71	1593	325	1	65	0
Mean (SD)	57.1 (6.1)	40.8 (3.1)	10.6 (1.7)	10.2 (1.5)	79 (7.5)	1760 (327)	475 (76)	3 (1.8)	69 (20)	1 (1.3)

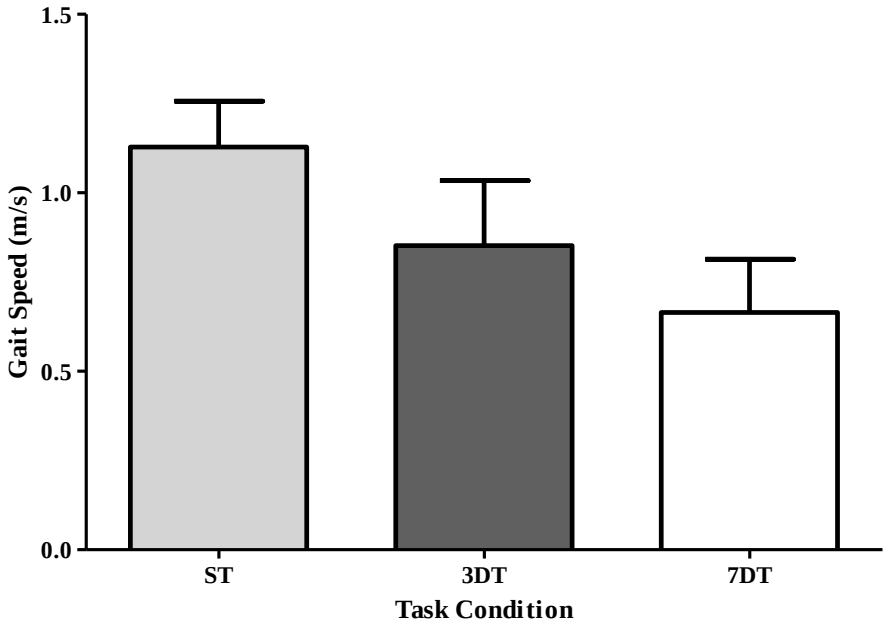


Figure 20 *Gait speed results for older female participants walking under single task (ST), counting backwards in 3s (3DT) and counting backwards in 7s (7DT)*

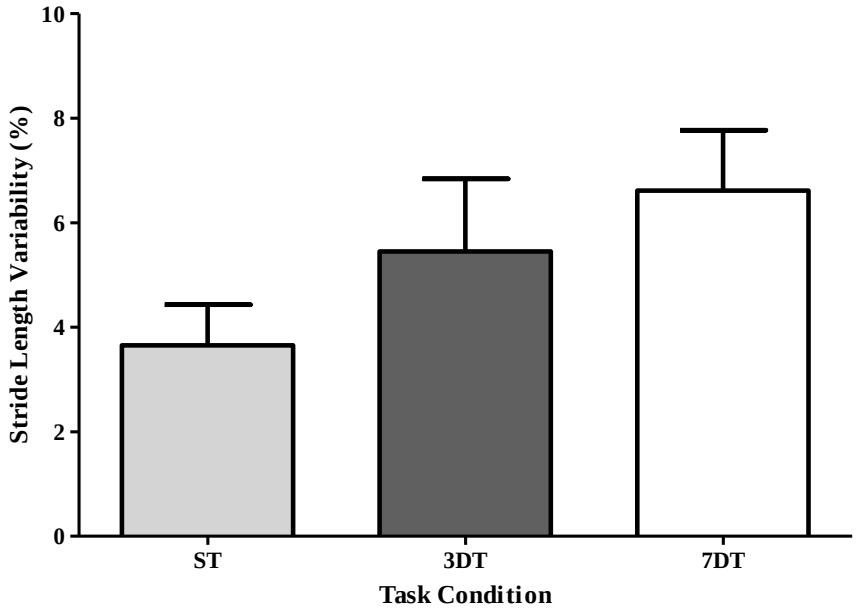


Figure 21 *Stride length variability results for older female participants walking under single task (ST), counting backwards in 3s (3DT) and counting backwards in 7s (7DT)*

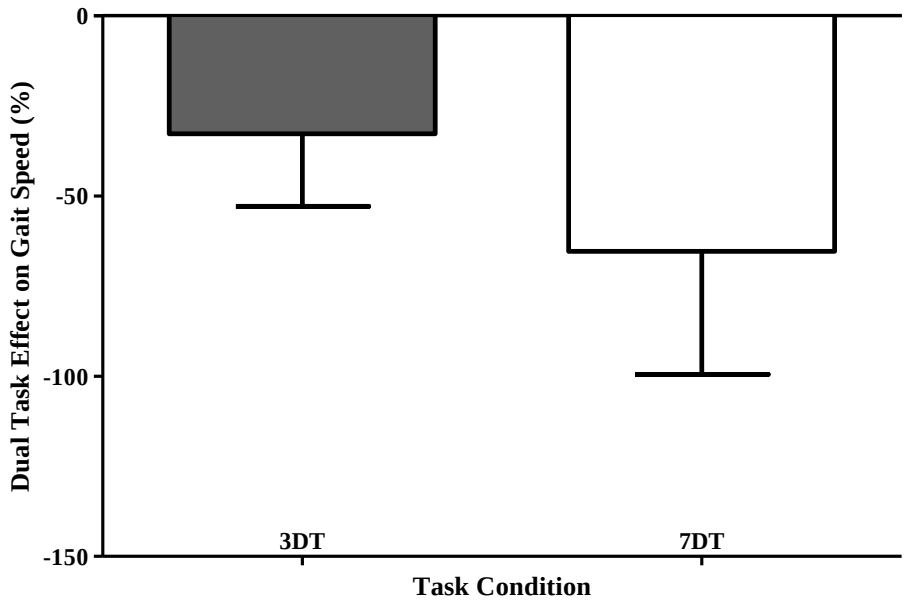


Figure 22 *Dual task effect on gait speed results for older female participants walking whilst counting backwards in 3s (3DT) and counting backwards in 7s (7DT).*

3.1.3 Discussion

Cognitive and gait testing will form an essential part of the experimental work to be undertaken within this thesis. This feasibility and pilot study has provided important information regarding the proposed methods and allowed the chief investigator to gain valuable experience in administering the tests.

The feasibility part of the method development established that all of the necessary equipment could be transported, set up, and was functional within the specific testing area. Overall, the testing took two hours per participant. Being able to establish this timing was important in order to clearly communicate the time requirement to potential participants and to effectively plan how much total time would be required to run a testing phase within SportBU. As the main RCT involved a group based exercise intervention, it was important that participants were able to complete the baseline assessment start the intervention period at a similar time so that these classes could start at the same time for everyone. Furthermore, the chief investigator would be conducting all data collection and participant recruitment so their time management and schedule needed to be factored into the planning of the study. In addition, the cost and availability of the facilities had to be factored into the planning. Based on the time required to test each participant, factoring in that participants would also have to attend separate session to perform blood sampling, the schedule of the chief investigator and the availability of the facilities required to conduct the testing and the exercise intervention the decision was made to run the study in three separate cohorts.

During the feasibility phase, participants were able to complete all tests as instructed. One change was made to the written instructions on the spatial working memory task to enhance clarity. The instruction originally read *"Your task is to state if the test dot is in the same spatial location as one of the preceding 3 dots or in a different spatial location"*, three of the participants expressed that the term spatial location was ambiguous and questioned whether it meant the same exact location or just a similar location. This was subsequently changed to *"Your task is to state if the test dot is in the exact same spatial location as one of the preceding 3 dots or in a different spatial location".* Following this change the older female participants did not raise this same issue indicated that this was a clearer instruction.

In the pilot testing phase the performance on the cognitive testing showed no indication of floor or ceiling effects. Again, tasks were well understood by the participants indicating that the written and verbal instructions provided were adequate and that the difficulty level was appropriate. In the trail making task and interference control task the number of errors across all task conditions was low at a level of 3% of total responses. For the trail making task specifically, this was largely a reflection that most errors were noticed and corrected while

performing the task. The low number of errors is consistent with reported similar cognitive tasks (Salthouse et al. 2000; Mutter et al. 2005; Ashendorf et al. 2008). Trail making errors, in particular, have been shown to be less sensitive to subtle changes associated with ageing leading to the hypothesis that errors reflect impairment across the lifespan rather than being directly impacted by age (Ashendorf et al. 2008). The planned RCT will have a six month intervention phase. The low error rate across both tasks, coupled with the evidence that error rates may not be sensitive enough to detect changes due to ageing has led to the decision not to include these variables within the experimental work. For the spatial working memory task both accuracy and reaction were measured. Although a focus on reaction would provide some insight into the processing speed of the participants, this task was primarily designed to assess spatial working memory. Furthermore, it has previously been observed that older adults are more likely to sacrifice the speed of conducting a cognitive task in order to improve accuracy versus younger adults (Forstmann et al. 2011). Taking into consideration the focus of the cognitive test being to assess spatial working memory and the preference of older adults to focus on accuracy over speed, the decision was made to omit reaction time from the study outcomes and remove any instructions prompting participants to focus on the speed of conducting the task. Whilst previous studies have performed separate analysis on the immediate, delayed and interference conditions of the RAVLT (Nagamatsu et al. 2013a), the decision was made to only include a total score across all trials for the main study. This decision was made to focus on the overall task performance and to reduce the number of outcomes for the main study.

Comparisons of the dual-task scenarios revealed that both the 3DT and 7DT conditions resulted in a reduction in gait speed of 0.27 m/s and 0.46 m/s respectively in older women. This has provided some evidence that both tasks are challenging enough to elicit a dual task effect on gait speed, especially when we consider that the observed dual task effect on gait speed was notably higher than previous research in healthy older adults using similar arithmetic based scenarios, where a DTE on gait speed of 13% was observed (Hausdorff et al. 2008). One of the participants elected not to complete the 7DT as she did not feel comfortable with the difficulty of the task. As the 3DT task resulted in a reduction in gait speed within the present study and was well tolerated by all participants, showing no indications of floor effects, this protocol will be used in the planned RCT.

In conclusion, the results of the feasibility study confirmed that all equipment was functional in the specific testing area, helped to establish timings and booking of facilities for data collection and confirmed that written and verbal instructions were clear. Furthermore, it provided the chief investigator with the opportunity to practice giving the instructions and familiarise himself with performing the tests and extracting the data. This experience was valuable as it gave the chief investigator confidence in conducting these tests with precision and consistency. The pilot study

helped to narrow down the cognitive and gait testing to the most appropriate outcomes and gave a strong indication that floor or ceiling effects were unlikely to occur and that instructions were clear within the specific demographic. Together these studies have provided valuable information that was crucial for the planning and implementation of the main RCT.

3.2 Method Development Study: Assessment of the Precision of Blood Fatty Acid Analysis

3.2.1 Methods

All laboratory chemicals and reagents were of analytical grade and purchased from Sigma–Aldrich and Cell Biolabs Inc. Fatty acid standards were also from Sigma–Aldrich. Prior to starting this work the chief investigator completed a Gas Safe certification to ensure proper and safe handling of gas cylinders.

Blood Sampling

Five blood samples were taken from the fingertip of a volunteer from a single puncture using an automated lancet. Blood spots were collected on filter paper which had been pre-treated with 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene, BHT) diluted in ethanol at 2 mg/ml. This method of pre-treatment had been shown to be efficient in preventing the oxidation of fatty acids in blood spots (Liu et al. 2014).

Lipid Extraction

Each blood sample was placed in a 5 ml Reacti-Vial^{TM} (Thermo Fisher) before adding 1 ml of 1.25 molar hydrogen chloride methanol solution, being sealed under zero-grade nitrogen (99.998% purity, BOC U.K.) and incubated at 90°C for 60 minutes in a Pierce® Reacti-Therm dry bath (Thermo Scientific). Next, 2 ml each of distilled water and saturated potassium chloride were sequentially added and samples homogenised for 30 seconds at 2000 revolutions per minute (Heidolph Instruments Vibramax 110). Two ml of hexane/BHT (0.05% w/v) was added and samples were centrifuged at 1250 x g (Eppendorf 5430R) 5 min at room temperature, creating two distinct layers. The top layer of the sample containing the fatty acid methyl esters (FAMEs) was removed using a Pasteur pipette, placed into 2 ml amber screw-top chromatograph vials (Agilent), and concentrated under a gentle stream of nitrogen (zero-grade) using a Techne® SC-3 Sample Concentrator (Techne). Whilst the FAMEs underwent concentration, 2 ml of hexane was added to the original sample to re-extract remaining FAMEs. The centrifugation and top layer removal process was repeated and both extracts were combined. Samples were concentrated under nitrogen until they reached 1ml in volume and were stored at -20°C under zero-grade nitrogen until analysis.

Fatty Acid Methyl Ester Analysis

Gas chromatography is a separation technique in which the components of a sample partition between two phases (1) the stationary phase and (2) the mobile phase. Samples are first injected into an inlet with a syringe where they are immediately vaporised and transported via a carrier gas (mobile phase) to a column which contains the stationary phase. As the sample moves

through the column, individual components interact with the stationary phase for different times, which results in the separation of each individual fatty acid, and leads to different times of elution from the column (retention time). Individual FAME affinity towards the stationary phase is dictated by their chain length and degree of saturation. The temperature of the column is precisely maintained by the GC oven, a higher temperature moves the sample through the column at a quicker rate and vice versa for a lower temperature, thus influencing retention times. It is essential to have the correct oven temperature settings to ensure individual fatty acids are separated and no co-elusion occurs. Levels of each FAME are quantified by a flame ionisation detector (FID) fitted to the GC which responds to the physicochemical property of each fatty acid when eluting from the column, which it amplifies, generating an electronic signal which the software uses to produce a chromatogram (**Error! Reference source not found.**).

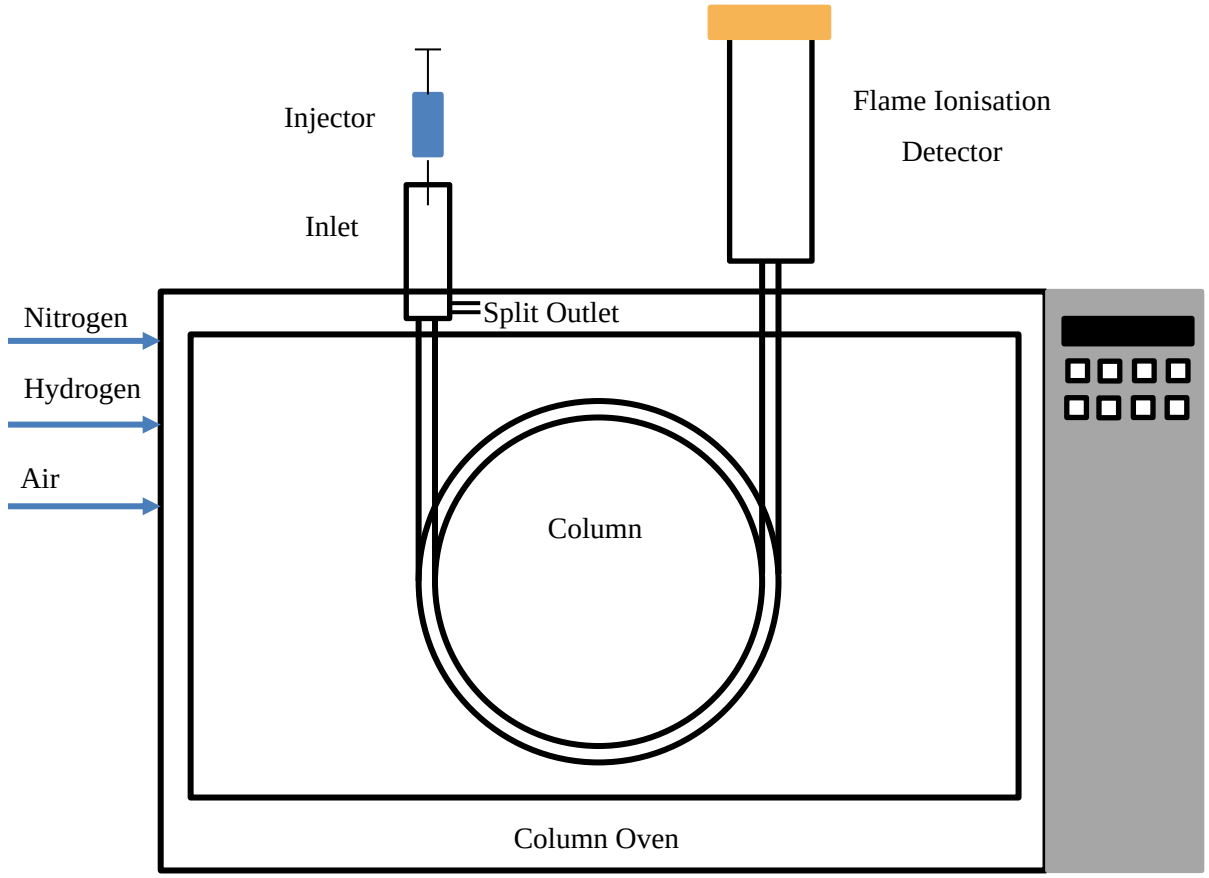


Figure 23 *Gas chromatograph system. Samples are injected, vaporised and transported via a carrier gas (mobile phase) through the column. As the sample moves through the column individual components interact with the stationary phase, which results in the separation each individual fatty acid.*

FAMES were quantified using an Agilent Technologies 7820A gas chromatograph with flame ionisation detector (GC-FID) fitted with an Omega wax 100 fused silica capillary column (15 m x 0.1 mm x 0.1 µm film thickness, Supelco) and analysed using the Open LAB CDS

Chemstation vC.01.04 software (Agilent Technologies). One µl of each sample was injected into the split inlet, set to a split ratio of 20:1, constant flow rate of 1.5 ml/min and 39.45 psi pressure. Inlet temperature was set to 250°C and research-grade nitrogen (99.9995% purity, BOC U.K.) was used as a carrier gas. The column temperature was programmed to rise from 140 °C, with a 40°C/min temperature ramp, to a peak temperature of 265°C. Peak temperature was maintained for 9 minutes. Detector temperature was set at 260°C with 40 ml/min flow of hydrogen, air flow rate was set at 450 ml/min and30 ml/min of make-up gas (research-grade nitrogen). Airflow was maintained using a Chromalytic Bambi HT15 Air Compressor.

Prior to analysing prepared samples, retention times for desired FAME peaks and peak resolution were established by injecting 0.5 µl of standards PUFA-1 (Marine source, analytical standard, Sigma Aldrich), PUFA-2 (Animal source, analytical standard, Sigma Aldrich) and Supelco 37 component FAME mix at a 50:1 split ratio. Fatty acids peaks were integrated using the Chemstation software and results were expressed as % weight of total fatty acids according to the following formula.10

$$\frac{FAME\ peak\ area}{total\ peak\ area\ of\ all\ FAMEs} \times 100\% = Fatty\ acid\ \%\ weight$$

Statistics

The coefficient of variation for each fatty acid was calculated and expressed as a percentage. To be deemed reliable a coefficient of variation of <10% for the main fatty acids of interest, EPA, DHA and AA and <15% for all other measured fatty acids would need to be achieved.

3.2.2 Results

Method development

Initial chromatograph outputs could not be reliably integrated as the peak areas were too small. It was determined that this was caused by a poor quality extraction as fatty acid standards PUFA-1 and PUFA-2 were both analysed and produced good quality chromatographs.

To determine which element of the extraction phase was causing the issues, further extractions were performed, with some alterations to the methodology. Firstly four extractions were performed using different paper treatment methods: (1) untreated paper, (2) treated with BHT diluted in ethanol at 2 mg/ml, (3) treated with ascorbic acid 5 mg/ml diluted in distilled water and (4) treated with a combination of BHT diluted in ethanol at 2 mg/ml and ascorbic acid 5 mg/ml diluted in distilled water. After extraction results were still not quantifiable for any paper treatment method, this element of the extraction was ruled out as being problematic, thus the previously established method of combining BHT and ascorbic acid was used moving forward (Liu et al. 2014). The next step in the method optimisation was to test a fresh blood spot versus a dried blood spot. The fresh blood spot was processed as previously described

whereas the dried spot sample was stored at 4^0C in a sealed plastic bag lined with aluminium foil until it had dried and was analysed within one week of collection to comply with Human Tissue Authority guidelines (Human Tissue Authority 2004). Following extraction of FAMEs, the dried blood spot yielded clear and quantifiable peaks as shown in **Error! Reference source not found.**. As such the original method was repeated on dried blood spots to yield the coefficient of variation.

Coefficient of Variation Results

PUFA-1 and PUFA-2 standards were used to determine individual fatty acid retention times and representative chromatograms are shown in figure 25 and 26. A summary of the fatty acids percentage weight of total fatty acids of the samples as well as the coefficient of variation analysis is shown in table 4. A coefficient of variation of <10% was achieved for EPA, DHA and AA with all other fatty acids being <15%.

Table 4 Summary of the coefficient of variation for fatty acid extraction of whole blood. All fatty acids had a coefficient of variation percentage of below 15% with eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid all having values below 10%.

	Fatty Acid (%)										
	16;0	16:1	18:0	18:1	18:2	18:3 n-6	18:3 n-3	22:1	20:5 n-3	20:4 n-6	22:6 n-3
Sample 1	28.58	3.68	10.41	32.51	16.87	0.23	1.51	0.58	0.58	4.56	0.91
Sample 2	29.91	3.64	10.09	31.85	16.55	0.24	1.52	0.58	0.58	4.62	0.83
Sample 3	26.30	3.36	10.75	33.92	17.23	0.22	1.42	0.65	0.65	4.94	0.99
Sample 4	27.92	3.53	10.57	32.03	17.34	0.19	1.94	0.61	0.61	4.88	0.81
Sample 5	26.94	3.37	10.59	32.95	17.08	0.25	1.54	0.50	0.63	5.45	0.99
CoV (%)	5.06	4.21	2.38	2.54	1.83	10.32	12.81	9.28	5.10	9.63	7.19

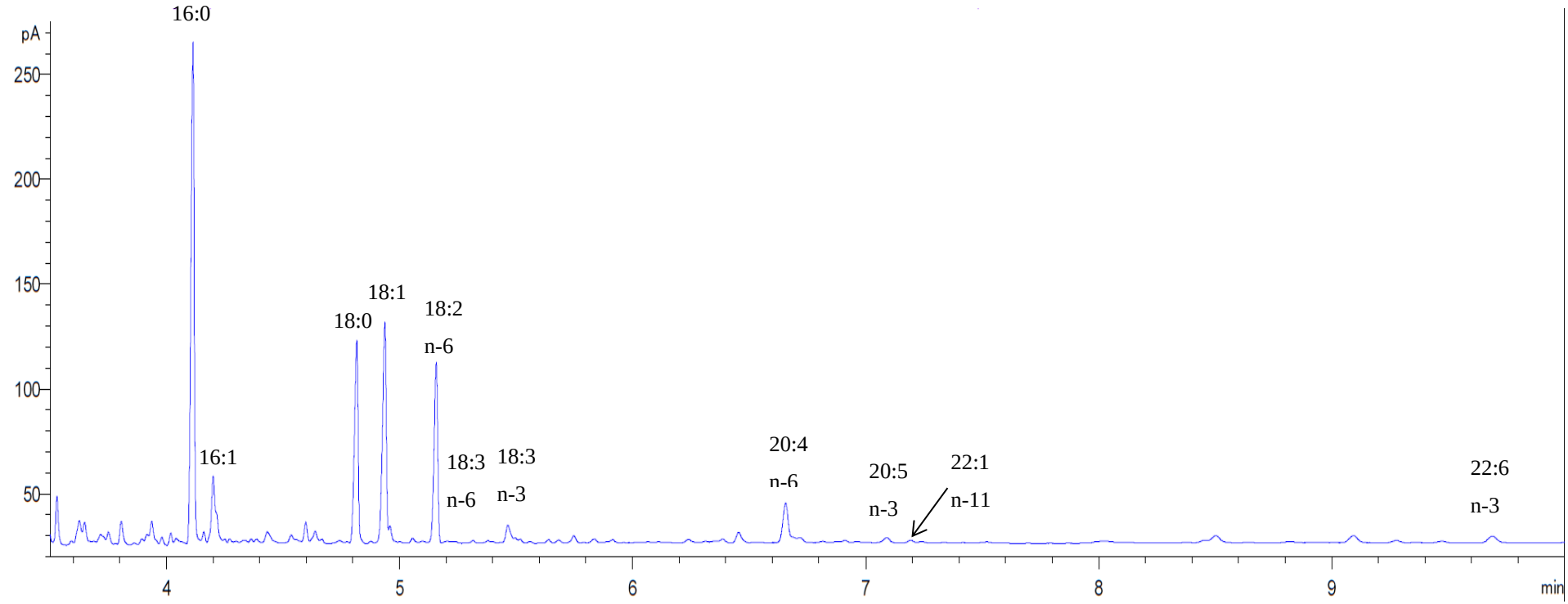


Figure 24 *Example of chromatograph from the extraction of fatty acids from a dried blood spot analysed using gas chromatography fitted with a flame ionisation detector.*

16:0 Palmitic acid, 16:1 Palmitoleic acid, 18:0 Stearic acid, 18:1 Oleic acid, 18:2 Linoleic acid, 18:3 n-6 γ- Linolenic acid, 18:3 n-3 α-Linolenic acid, 20:4 n-6 Arachidonic acid, 20:5 n-3 Eicosapentaenoic acid, 22:1 Erucic acid, 22:6 n-3 Docosahexaenoic acid

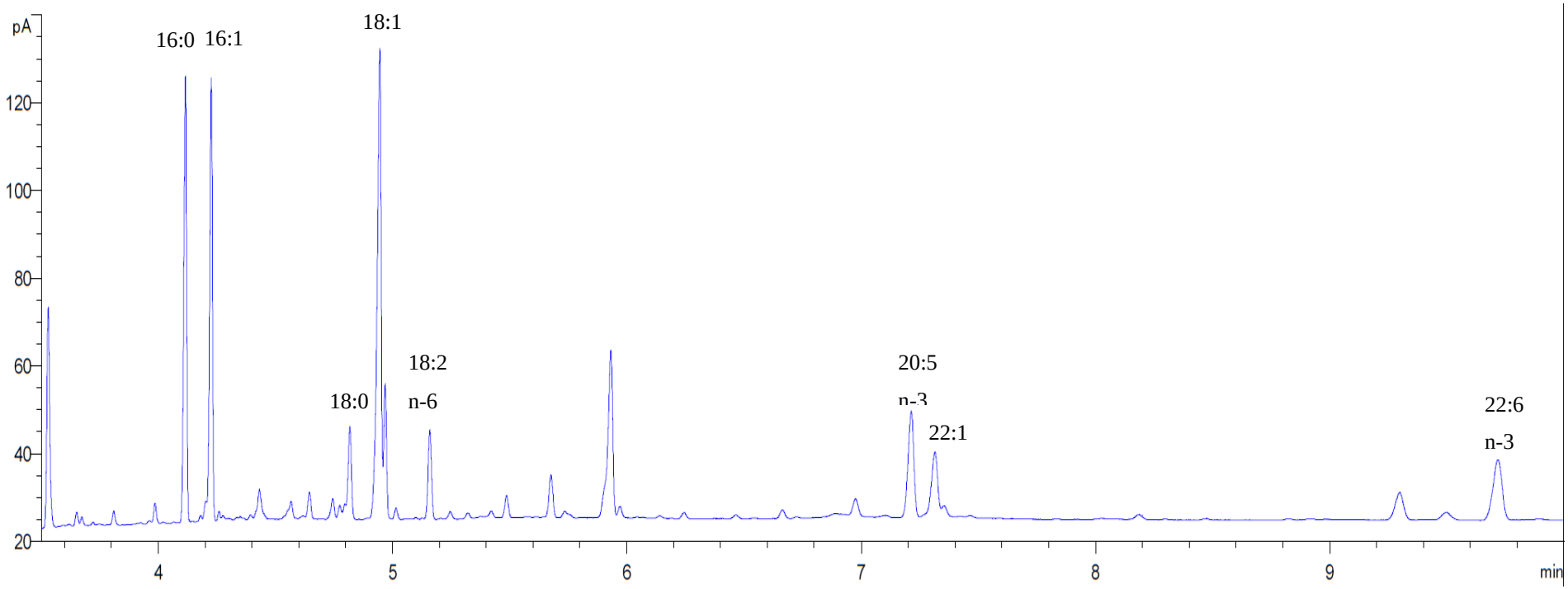


Figure 25 *PUFA-1 Standard*

16:0 Palmitic acid, 16:1 Palmitoleic acid, 18:0 Stearic acid, 18:1 Oleic acid, 18:2 Linoleic acid, 20:5 n-3 Eicosapentaenoic acid, 22:1 Erucic acid, 22:6 n-3 Docosahexaenoic acid

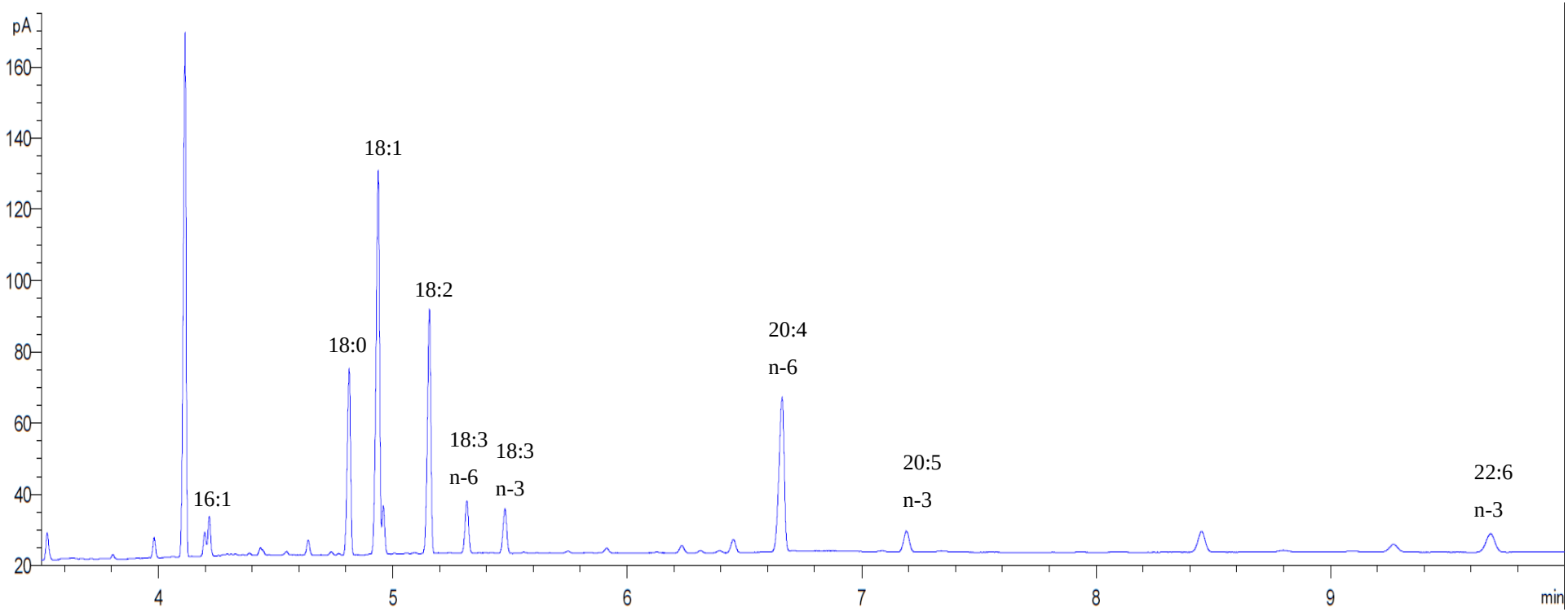


Figure 26 *PUFA-2 Standard*

16:0 Palmitic acid, 16:1 Palmitoleic acid, 18:0 Stearic acid, 18:1 Oleic acid, 18:2 Linoleic acid, 18:3 n-6 γ- Linolenic acid, 18:3 n-3 α-Linolenic acid, 20:4 n-6 Arachidonic acid, 20:5 n-3 Eicosapentaenoic acid, 22:6 n-3 Docosahexaenoic acid

3.2.3 Discussion

Using a dried blood spot from a finger prick sample to quantify fatty acid levels has become a popular method as it negates some of the challenges associated with collection and measurement using larger volumes of blood. Sampling is less invasive, and samples do not require centrifugation or dry ice transportation (Gupta et al. 2009). The research within this thesis requires the accurate measurement of fatty acids to analyse relationships between healthy ageing outcomes and fatty acid levels as well as to measure compliance to a dietary supplement within an RCT. Due to the above described benefits, using dried blood spots was chosen as a method for sample collection for the following RCT. Therefore, it was essential to ensure that this method could produce accurate and reliable results.

A number of factors can influence the precision of fatty acid quantification including user proficiency and handling and storage of the samples before and after lipid extraction. Indeed, inappropriate handling of the sample can result in oxidation of the polyunsaturated fatty acids through air exposure (Zeleniuch-Jacquotte et al. 2000). Therefore, BHT was added to the filter paper, to avoid oxidation, and samples were stored in sealed bags lined with aluminium foil (Liu et al. 2014).The present analysis used a previously validated technique to quantify fatty acid levels in whole blood (Marangoni et al. 2004). All fatty acid measurements had a coefficient of variation of less than 15%, with the PUFAs of interest, EPA, DHA and AA having values below 10%, demonstrating that the chief investigator is proficient in carrying out this protocol.

In conclusion, this widely used method was successfully established in our laboratory to measure fatty acids in finger prick samples from human participants. Results indicate that the method used, storage conditions, sample handling and user proficiency are reliable and can produce consistent results. The results from this study were used to create a standard operating procedure for the research site lab and provide evidence that the method used for fatty acid quantification is reliable and can be used in the planned RCT

3.3 Randomised Control Trial Study Protocol

Following the development of the techniques used to measure mobility, cognitive function and whole blood fatty acids, the study protocol for an RCT investigating the effects of a high DHA multi-nutrient supplement and aerobic exercise on mobility and cognitive function in older women was established. This protocol was subject to peer review and was published prior to the analysis of study results (Fairbairn et al. 2019). The observational and experimental analyses from this study protocol will each have their own separate chapter, which will outline the sample size calculations and statistical methods used.

3.3.1 Design and Setting

The study was a randomised semi-blinded, placebo controlled trial in females aged 60 years and above. The study was restricted to female participants to enable comparison with our previous work (Strike et al. 2016), and it has been suggested that the additive effects of combining exercise and omega-3 PUFA supplementation may be limited to women only (Da Boit et al. 2017). Furthermore, women have a greater life expectancy than men, and there are higher rates of sarcopenia and dementia in women versus men making this group particularly venerable to age related disease (Patel et al. 2013; Prince et al. 2014). Women have also been shown to have greater compliance to exercise interventions (Kelley and Kelley 2013). The study was designed to examine the effects of a high DHA omega-3 PUFA multi-nutrient dietary supplement and aerobic exercise, both on their own and in combination, on outcomes related to mobility and cognitive function. All measurements and data collection, as well as the aerobic exercise intervention took place in the same study site (Bournemouth University, U.K.), with participants being instructed to consume the dietary supplement at home. The study had three separate intakes of participants, starting in February 2017, May 2017 and February 2018. This was done to ensure testing and exercise class facilities did not exceed capacity and so that testing phases were quick and efficient (Figure 27). 11

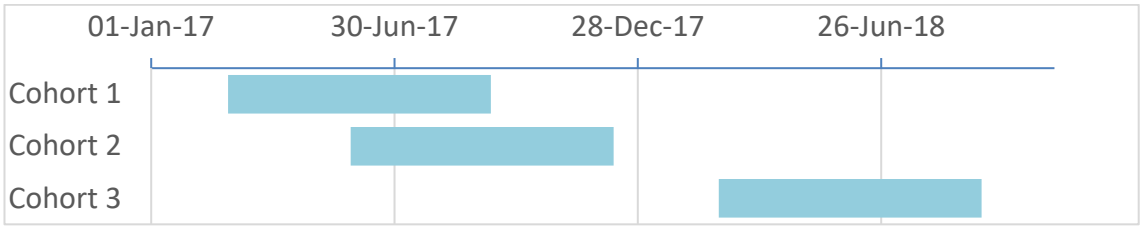


Figure 27 *Timeline for data collection periods for the three cohorts of participants taking part in the study.*

3.3.2 Blinding Randomisation and Allocation

The dietary supplements were packed into identical containers and coded by the Principal Investigator, who has no involvement in the data collection. Omega-3 PUFA capsules have a distinct odour, therefore a small amount of fish oil is added to the placebo capsules to help maintain blinding. Exercise class allocation was communicated through letters which were coded by the Principal Investigator and distributed in sealed envelopes. A stratified block randomization design was followed (Suresh 2011) with stratification based on frailty classification of non-frail or pre-frail, followed by permuted block randomisation. Randomisation was achieved by creating a computer-generated list of numbers consisting of four blocks for each strata referred to without specification of the intervention group (e.g., A, B, C and D). The list was generated and stored by the Principal Investigator, who was not involved in the data collection. Due to the nature of the exercise intervention participants were only blinded to the dietary intervention; however, the experimenters were blinded to the group allocations. In the event of a severe adverse effect being reported by a participant the Principal Investigator was able to gain access to the participant allocation so that appropriate action can be taken, whilst maintaining the blinding of those involved with data collection and analysis.

3.3.3 Participant Recruitment and Eligibility Criteria

Participants were recruited through public advertisements (Appendix 6) and public engagements in Bournemouth, U.K. The public advertisements included a brief study description as well as the contact details for the research team. Publication engagements consisted of a general presentation on nutrition and exercise for healthy ageing, which ranged from 20 minutes to one hour in length. In total thirty seven public engagements were completed. Interested individuals received a participant information document including the design, procedure, benefits, and risks of the trial. Before any data was collected all participants provided signed written informed consent forms.

All participants were screened to assess Frailty Status, according to the criteria developed by Fried and co-workers Fried et al. (2001). The criteria include low muscle strength, self-reported exhaustion, slow gait speed, low levels of physical activity, and unintentional weight loss, as shown in Table 5. A score of zero out of the five indicates non-frail, one or two pre-frail, and three or above frail. As well as a screening procedure non-frail and pre-frail status were used as a prognostic factor in the randomisation.

The MMSE was performed to exclude participants with undiagnosed cognitive impairment (Tombaugh and McIntyre 1992). The test was performed according to British Psychology Society guidelines (2010) and not used for diagnostic purposes, with individual results not being

disclosed. Participants who score ≤ 24 would be told that if they had any concerns regarding their memory that they should visit their general practitioner and that they did not meet the inclusion criteria for the study.

Females aged 60 years and above are recruited according to the following inclusion criteria: (1) able to walk at least 50 m unaided, (2) classified as non-frail or pre-frail and community-dwelling. Exclusion criteria are: (1) vestibular impairments, (2) diagnosed neurological disorder, (3) cognitive impairment (Mini Mental Status Examination score of 24 or below), (4) lower limb surgery, (5) seafood allergy, (6) regular consumption of multivitamin or fish oil supplements within six months prior to baseline measurements, and (6) previously received advice from a health care professional not to undertake strenuous exercise. The full list of inclusion and exclusion criteria can be found in Appendix 7.

Table 5 Frailty Screening assessment methods and defined cut off points (Fried et al. 2001). Cut off points for frailty screening are based on data from 4000 adults mean age 72, where these points were determined to be sensitive for frailty screening (Auyeung et al. 2014).

Frailty Criteria	Assessment Method	Cut-off for Frailty
Unintentional Weight Loss	Self-reported	≥4.5 kg in the last year
Muscle Weakness	Grip Strength (dominant hand) (Jamar® hand grip dynamometer)	≤18 kg
Slow Gait Speed	Gait Speed over 13 meters	≤0.8 m/s
Exhaustion	Two questions from the Centre of Epidemiologic Studies Depression Scale (Radloff 1977)	Answering "much or most of the time" to the questions "I felt that everything I did was an effort" and "I could not get going."
Low Levels of Activity	Physical Activity Scale in the Elderly (Washburn et al. 1993)	A score of ≤56.4

3.3.4 Interventions

The study interventions are described in detail according to the Template for Intervention Description and Replication (TIDieR) guidelines in Table 6.

Table 6 Description of study intervention based on the Template for Intervention Description and Replication (TIDieR) checklist (Hoffmann et al. 2014).

Item	Experimental Group	Experimental Group	Experimental Group	Control Group
1. Group	Multi-nutrient Supplement and Exercise	Placebo Supplement and Exercise	Multi-nutrient Supplement	Placebo Supplement
2. Why?	Multi-nutrient supplement formula was previously been shown to improve habitual gait speed, verbal memory and interference control in older women (Strike et al. 2016). Cycle ergometer training is a form of exercise that can benefit muscle strength and cardiopulmonary fitness in older adults (Harber et al. 2009). There is some evidence for a positive interaction between omega-3 PUFA and exercise in older women on muscle strength and cognitive function (Kobe et al. 2015; Da Boit et al. 2016a)			
3. What materials?	Participants received containers with multi-nutrient supplement capsules and instructions on daily intake. Exercise classes took place in a sport studio on spinning cycle ergometers.			
4. What procedure?	Participants took four capsules per day of their allotted supplement, alongside their main meal of the day. Those allotted to the exercise intervention attended two classes per week for the final 12 weeks of the study. Classes initially lasted 30 min for the first 6 weeks and then increased to 45 min for the final 6 weeks.			
5. Who provides?	Principal Investigator issued participants with their dietary supplements. Exercise classes were carried out by a qualified instructor.			
6. How?	For the dietary supplements, all groups received instructions about intake, duration and dosage by the Principal Investigator. The exercise classes were performed in small groups.			
7. Where?	The participants took the dietary supplements at home. The aerobic exercise classes took place in sports studios at Bournemouth University U.K.			
8. When and how much?	For 24 weeks participants took four capsules per day of their allotted supplement. After 12 weeks the participants started their exercise classes, twice per week for the final 12 weeks of the study.			
9. Tailoring	Participants were asked to maintain a specific revolution per minute on the cycle ergometer. They self-select a resistance to allow them to maintain 12-14 on the Borg scale. This method ensured participants maintained a similar and consistent intensity of exercise despite the likelihood of participants having mixed fitness levels.			

Dietary Supplement

All participants were asked to consume four capsules per day of their respective dietary supplement for the 24 weeks of the study and were instructed to take them with their main meal of the day. The total daily dose from the active capsules contained 1000 mg DHA, 160 mg EPA, 20 µg vitamin B12, 1 mg folic acid, 124 mg PS, 20 mg vitamin E and 240 mg *Ginkgo Biloba* standardized leaf extract. The duration of 24 weeks and dose of supplementation has previously been shown to increase tissue omega-3 PUFA levels and induce improvements in cognition and mobility (Strike et al. 2016). The placebo capsules contained an isocaloric oil blend typical of the U.K. diet including a small amount of fish oil (Appendix 8). Providing an oil blend typical of the U.K. diet is preferable, as providing any other single oil could cause changes to incorporation of fatty acids into the cell membranes, which could in turn influence outcomes (Simopoulos 2002). A small amount of fish oil was added to the placebo capsules to aid with participant blinding, which provided a daily dose of 21.6 mg EPA and 14.4 mg DHA, an amount unlikely to produce any therapeutic benefit. Active and placebo capsules were kindly provided by Efamol Ltd. Compliance to the dietary supplement was measured by changes in DHA levels in whole blood compared to baseline, with a relative change of 15% being the threshold for compliance (Strike et al. 2016), counting returned pills at 12 and 24 weeks, and exit questionnaire. A systematic review concluded that the potential for adverse events with omega-3 PUFA supplementation should be considered mild-moderate at worst and unlikely to be of clinical significance (Villani et al. 2013). Adverse events as defined by Clinicaltrials.gov (2016) were monitored by participant self-reporting and exit questionnaire.12

Exercise Training

The exercise intervention consisted of two group sessions per week for 12 weeks on a Spinner Fit® stationary indoor cycle, led by an instructor who was qualified in studio cycling (Premier Global) as well as personal training and fitness instruction (Active IQ). Exercise has consistently been shown to positively influence health in older adults; however, uptake and adherence to exercise interventions are known to be limiting factor for intervention trials (Chao et al. 2000; Picorelli et al. 2014). Indeed, a survey of >92,000 people in England showed that not only does exercise participation decline progressively throughout the adult lifespan, but the desire to take part in exercise also declines (Department of Culture, 2011). This means that RCTs must not only consider the modality, duration and intensity of exercise interventions in terms of promoting clinically relevant health benefits, but also what is achievable in terms of adherence. Twelve week exercise interventions have previously been demonstrated to elicit positive effects on physical and cognitive outcomes in healthy and frail older adults (Mangione et al. 1999; Todde et al. 2016; Ferreira et al. 2018; McGregor et al. 2018). Given that adherence to the exercise over time was a concern twelve weeks was chosen as a time frame that could

promote health benefits whilst also being achievable from an adherence perspective. This time frame is also in keeping with recommendations that exercise interventions should be a minimum of 3 months to be able to demonstrate significant change in relevant clinical parameters (Cruz-Jentoft et al. 2014).

Supervised exercise classes have shown a higher level of compliance versus self-lead home based programmes (Picorelli et al. 2014), furthermore the group activity can promote of sense of social connectedness, which has also appears to drive engagement with exercise (Farrance et al. 2016). Cycling is a form of aerobic exercise that can benefit muscle strength, cardiopulmonary fitness, balance and proprioception in older adults (Harber et al. 2009; Rissel et al. 2013), with some data suggested improves in function mobility outcomes (Mangione et al. 1999). Furthermore, cycling may be preferential for older adults as it is non-weight bearing, has a low impact on joints and has been found to be suitable and beneficial for those with joint pain (Wainwright et al. 2016). Fear of falling or injury has been identified as a barrier to exercise participation in older adults (Dedeyne et al. 2018), given that some participants were pre-frail, indicating a lower level of physical robustness, cycling also acts as an accessible and safe modality of exercise. Indoor cycling is a non-weight bearing activity and whilst on the bike the participant's upper body was stabilised through the use of the handles. Indeed indoor cycling interventions have been demonstrated to be well tolerated even within frail older adults (Smith et al. 2012).13

For the first six weeks classes lasted 30 min and in the second six weeks session length increased to 45 min. All sessions consisted of a 5 min warm-up and cool down at 7-8 on the Borg scale of rate of perceived exertion (Cadore et al. 2013b) (Appendix 9). During the main body of the sessions, participants were asked to maintain an intensity of 12-14 on the Borg scale. These intensity levels on the Borg scale are considered moderate to vigorous, and similar intensity levels have been shown to produce positive responses in this population (Lepretre et al. 2009; Falck et al. 2017). Older adults are typically heterogeneous in terms of their aerobic fitness (Petrella et al. 2010) therefore using the Borg scale allowed each participant to exercise at their own level, whilst still being encouraged to maintain the moderate-vigorous intensity levels that are desired. Compliance to the exercise intervention was monitored by recording attendances by each participant and calculated as the percentage of classes attended, with 70% being the threshold for compliance (Farrance et al. 2016).

3.3.5 Demographic Information

Information on the age, height, weight, verbal intelligence, and medication use are collected from each participant. Information on medications is self-reported, with both type and number of medications recorded. The national adult reading test (NART) is used to assess verbal

intelligence (Bright et al. 2002). The test requires participants to read aloud 50 pre-prepared words, with a score being calculated based on the number of correct pronunciations. Minor variations from the pronunciations are not penalised as the aim of the test is to assess familiarity with the words rather than exact pronunciation.

3.3.6 Outcomes

All measurements are performed at baseline and at the end of the study.

Gait Analysis

Gait speed, stride length variability, stride length, cadence, and double support phase percentage were measured using Opal inertial sensors and analysed using Mobility Lab™ software version 3.1 (APDM Inc, http://apdm.com).

Participants were assessed under three gait conditions: habitual walking, fast walking, and dual-task walking. Participants walk at a normal comfortable pace for the habitual walking and dual-task protocols and as fast as possible for the fast walking protocol. Habitual gait speed can be influenced by social norms or desirability bias, thus fast walking speed as a test of maximal performance was included as an outcome as it is less impacted by these factors (Dobkin 2006). During the dual-task protocol participants counted backwards in integers of three, as decided from previous pilot studies conducting by the research team, from a randomly generated three digit number given three seconds before the commencement of the task. Participants were not instructed to prioritise either walking or counting backwards during the dual-task condition. The relative dual-task effect as percentage of loss relative to the single-task performance was calculated based on the formula DTE [%] = 100* (single-task score - dual-task score)/ single-task score (McDowd 1986).

Five Times Sit to Stand

The FTSTS is a valid measure of dynamic balance and functional mobility in older adults that is commonly used in studies in geriatric populations (Goldberg et al. 2012). To perform the FTSTS participants started off seated on a standard chair 44 cm in height from the ground, with arms folded across their chest and back against the chair. They were asked to stand up fully from the chair and sit back down again five times, whilst keeping their arms in the same position. This task was assessed by timing participants from the prompt to start until they reached a seated position on the fifth repetition.

Cognitive Function

A Stroop test was used to assess interference control (Davidson et al. 2003a) using Open Sesame version 3.1.1. software (Mathôt et al. 2012). During this task, a fixation point appeared on screen for 500 ms followed by the presentation of the names of one of four colours: blue, red, green, and white. These words were presented in four different font colours varying between blue, red, green, and white. Participants were instructed to identify, as quickly as possible without sacrificing accuracy, the colour of the text rather than the word displayed on screen and press a designated key on the keyboard, highlighted using coloured stickers. The test comprised of 144 trials with half of the trials having the text and colour match (congruent trials) and half being a non-match (non-congruent trials). Interference control was defined as the difference between the mean time taken to respond to the congruent and non-congruent trials. Reaction times that were plus or minus 2.5 times the median absolute deviation were excluded as anomalous results (Leys et al. 2013).

Spatial working memory was assessed using a computerised task, run on Open Sesame version 3.1.1. software, based upon work conducted by Nagamatsu, L. S. et al. (2013). The task required participants to recall the spatial location of dots presented on a laptop screen. Each trial comprised of a presentation and a test phase. In the presentation phase, three dots appeared at randomly allocated locations for 500 m, this was followed by a fixation cross which appeared for 3 s. After the retention interval the test phase comprised of the presentation of a single red test dot on the screen, this could either be in the same location as one of the previous black dots (match) or in a different location (non-match). Participants were asked to identify if the red test dot was a match or a non-match to any of the prior black dots by pressing an assigned key on the keyboard ("y" = match; "n" = non-match). There was no time limit for the participants to respond as the focus of the task was on response accuracy. The task consisted of ten practice trials, followed by sixty recorded trials. Thirty of the trials were matched and thirty were non-matched. The thirty non-matched were evenly split in three degrees of difficulty, whereby they were placed at two (near), four (medium) and eight (far) degrees visual angle. These angles were calculated based on the participant sitting 50 cm from the screen. Accuracy for the task was recorded as the percentage of correct answers.

The RAVLT is an established tool for assessing verbal memory (Rey 1941). The task consists of seven trials. Initially, participants were read a list of 15 pre-defined words (Appendix 10). Immediately after each reading, they were required to recall as many words as possible, this was repeated five times with the same list of words. Next an interference list is read containing fifteen different words (Appendix 10), after which participants were asked to recall the original list. During the last trial participants were read the original list a final time and asked to recall

the words after a twenty minute delay. Results were calculated as the total number of words recalled across the seven trials.

A trail making task was used to assess executive function (Salthouse et al. 2000). In this task, participants were asked to draw lines between targets on a piece of paper, as rapidly as possible, in a grid of seven by seven circles. There were four different conditions for the task: (1) a numbers condition where targets went from one to 49 (numbers), (2) a letters condition where the targets went from A to Z (letters), (3) a condition where participants alternated between numbers (1-25) and letters (A-X), (numbers-letters), and finally, (4) a condition alternating between letters (A-Y) and numbers (1-24) (letters-numbers). Successive targets were always in one of eight adjacent circles located above, below, to the left, to the right, or in one of the four diagonals adjacent to the target. This arrangement lessens the motor skill requirements that would typically be required for a standard trail making task. Participants were given a three by three practice trial of each condition to familiarise themselves with the task. Participants were then given 20 s to make as many connections as they could on each task condition. Scores were recorded as the total number of correct connections within the time limit.

Whole Blood Fatty Acids Analysis

Blood samples were taken from the fingertip of the participant from a single puncture using an automated lancet. Blood spots were collected on filter paper which had been pre-treated with 2,6-di-tert-butyl-p-cresol (butylated hydroxytoluene BHT) diluted in ethanol at 2 mg/ml.

Each blood samples was placed in a 5 ml Reacti-Vial^{tm} (Thermo Fisher) before adding 1 ml of 1.25 molar hydrogen chloride methanol solution, being sealing under zero-grade nitrogen (99.998% purity)(BOC U.K.) and incubated at 90^{0}C for 60 minutes in a Pierce® Reacti-Therm dry bath (Thermo Scientific). Next, 2 ml each of distilled water and saturated potassium chloride were sequentially added and samples homogenised for 30 seconds at 2000 revolutions per minute (Heidolph Instruments Vibramax 110). Two ml of hexane/BHT (0.05%w/v) was added and samples were centrifuged at 1250 x g (Eppendorf 5430R) 5 min at room temperature, creating two distinct layers. The top layer of the sample containing the FAMEs was removed using a Pasteur pipette, placed into 2 ml screw-top chromatograph vials (Agilent), and concentrated under nitrogen using a Techne® SC-3 Sample Concentrator (Techne). Whilst the FAMEs underwent concentration, 2 ml of hexane was added to the original sample. The centrifugation and top layer removal process was repeated. Samples were concentrated under nitrogen until they reached 1ml in volume and were stored at -20^{0}C until analysis.

FAMES were quantified using an Agilent Technologies 7820A gas chromatograph with flame ionisation detector (GC-FID) fitted with an Omega wax 100 fused silica capillary column (15 m x 0.1 mm x 0.1 μm) and analysed using the Open LAB CDS Chemstation vC.01.04 software

(Agilent Technologies). One µl of each sample was injected into the split inlet, set to a split ratio of 20:1, constant flow rate of 1.5 ml/min and 39.45 psi. Inlet temperature was set to 250 °C and research-grade nitrogen (99.9995% purity)(BOC U.K.) was used as a carrier gas. The column temperature was programmed to rise from 140 °C, with 40 °C/min temperature ramp, to a peak temperature of 265 °C. Peak temperature was maintained for 9 minutes. Detector temperature was set at 260 °C. Detector air flow rate was set at 450 ml/min. Airflow consisted of 40 ml/min of hydrogen and 30 ml/min of make-up gas (nitrogen). Airflow was maintained using a Chromalytic Bambi HT15 Air Compressor.

Prior to analysing prepared samples and during analysis, after ten samples had been injected, retention times for desired FAME peaks and peak resolution were established by injecting 0.5 µl of standards (PUFA-1, PUFA-2 and Supelco 37 component FAME mix at a 50:1 split ratio. Fatty acids peaks were integrated using the Chemstation software and results were expressed as % weight of total fatty acids according to the following formula.

$$\frac{FAME\ peak\ area}{total\ peak\ area} \times 100\% = \%\ weight$$

Serum Homocysteine

Before serum homocysteine could be measured a standard operating procedure and risk assessment for venous blood sampling for the research site was created by the CI.This was created in line with WHO guidelines on best practices in phlebotomy (World Health Organisation 2010) (Appendix 11). A non-fasted venous blood sample was drawn to assess serum homocysteine (Fokkema et al. 2003). Samples were collected using a Vacutainer Safety-Lok collection set fitted with a 10 mL serum collection tube (Becton, Dickinson and Company). Each blood sample was allowed to clot and then immediately centrifuged at 2000 x g for 10 minutes at 4^0C and the serum extracted using a Pasteur pipette (Tuck et al. 2009). Serum samples were stored at -80°C and analysed within three months (Hustad et al. 2012).

Serum homocysteine levels were measured using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs Inc.). Firstly the protein binding plate and reagents were prepared as follows. Homocysteine conjugate was diluted in phosphate buffered saline containing 0.1% bovine serum albumin (PBS-BSA) at a ratio of 1:1000 and 100 µL was added to each well of the protein binding plate, before incubating overnight at 4ºC. Following the incubation period, excess conjugate solution was removed by blotting on paper towels and wells washed three times with 200 µL of PBS. Next 200 µl of the assay diluent was added to each well and this was left to block at room temperature for 1 hour. Anti-homocysteine antibody and the secondary antibody, horseradish peroxidase (HRP) conjugate were both diluted in the assay diluent 1:500 and 1:1000 respectively. Wash buffer solution was diluted 1:10 in deionized water. The preparation of the standard curve was conducted according to table 7

Table 7 Concentrations used in the preparation of homocysteine enzyme-linked immunosorbent assay kit standard curve.

Standard Tubes	4 mg/ml Homocysteine-BSA standard (µL)	Assay Diluent (µL)	Homocysteine-BSA (µL/ml)
1	4	369	40
2	100 of tube 1	300	10
3	100 of tube 2	300	2.5
4	100 of tube 3	300	0.625
5	100 of tube 4	300	0.156
6	100 of tube 5	300	0.039
7	100 of tube 6	300	0.010
8	0	300	0

During this 1 hour period serum samples were allowed to defrost and each sample was diluted 5 fold in PBS by adding 24 µl of serum sample to 96 µl PBS-BSA. Assay diluent was removed and 50 µl of samples and standards were added to their allocated wells and incubated for 10 minutes on an orbital shaker (MS1 minishaker 1KA). Next 50 µl of diluted anti homocysteine antibody was added to each well and plate was incubated for 60 min on an orbital shaker. Following incubation all wells were washed three times with wash buffer solution, making sure to completely remove all wash buffer between cycles. After washing 100 µl of the diluted secondary antibody, HRP conjugate was added to each well and the plate incubated for 1 hour on the orbital shaker. During the incubation period, the substrate solution was allowed to reach room temperature. All wells were washed using the wash buffer solution as outlined above and 100 µl of the substrate solution was added to each well. The plate was incubated at room temperature in the orbital shaker, with the manufacturer's guidelines stating that the reaction time could vary between 2 and 30 minutes. A clear change in colour was apparent after 10 minutes at which point 100 µl of the stop solution was added to each well. The plate was immediately read using an absorbance microplate reader set to 450nm (ELx800, BioTek). Results were analysed using the Gen5 software (Version 2.09.2, BioTek) and interpreted using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

Table 7 Concentrations used in the preparation of homocysteine enzyme-linked immunosorbent assay kit standard curve.

Dietary Intake and Physical Activity Levels

Differences in diet and physical activity habits between the groups, as well as changes in these aspects within groups over the course of the study have the potential to influence the outcomes of the study. For example, increasing protein intake has been shown to maintain lean mass and physical function in older adults (Rousseau et al. 2009; Deer and Volpi 2015). Although

participants are asked to maintain their current diet and lifestyle habits, these aspects were also monitored at baseline and completion of the study.

Three day estimated food diaries were used to assess dietary intake (Appendix 12). Written instructions were provided alongside the food diaries and participants were asked to record two weekdays and one weekend day. Participants recorded details of all foods and beverages consumed at the time of consumption. They were asked to include brand names, cooking and preparation methods and an accurate description of the portion size using standard household measures or natural unit sizes. Results were analysed using Nutritics dietary analysis software (www.nutritics.com). A previously validated seventeen item food frequency questionnaire (FFQ) was used to specifically quantify omega-3 PUFA intake (Sublette et al. 2011b) **(**Appendix 13).

The community health activities program for seniors (CHAMPS) questionnaire was used to assess physical activity levels (Stewart et al., 2001) (Appendix 14). The CHAMPS questionnaire is a validated and reliable measure of physical activity in older adults, which covers a broad range of activities and has been shown to be sensitive to change over six months (Stewart et al. 2001).

Health Related Quality of Life

The short form (SF) 36 health questionnaire (Appendix 15) has been shown to be a practical and valid tool for assessing general health status (Walters et al. 2001). The questionnaire was issued at baseline and at the end of the study. Answers are divided into sub-categories: vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning and mental health. Each sub-category was scored on a scale of zero to one hundred with a higher score indicating a more positive health status (Brazier et al. 1992).

3.3.7 Data Management

The chief investigator was responsible for trial registration, participant recruitment, coordination of clinical visits, data collection, administration of nutrition and exercise interventions, handling and analysis of blood samples, as well as data handling and analysis and received training on all collection and analysis techniques.

All data from participants were assigned to a number to prevent results being traced back to an individual. The chief investigator was responsible for all the storing and handling of data. Digital data from the study were stored on a password protected Bournemouth University staff account only accessible by the research team. All paperwork including completed consent forms, lifestyle questionnaires and raw data outputs was locked in a filing system within a

secure building at Bournemouth University (U.K). Results from the study were anonymised with participants being assigned numbers. All data relating to the trial will be archived for 5 years after the conclusion of the study.

3.3.8 Stepwise Procedure

The stages of the study procedure are illustrated in *Figure 28*. Measurements were undertaken at baseline and following the 24 week intervention period. A mid-study appointment was given at 12 weeks to collect unused dietary supplement capsules to monitor compliance, give further instruction on exercise allocation and to issue participants with the dietary supplements required for the remainder of the trial. The baseline measurements consisted of the screening process assessing frailty and cognitive impairment, if eligible this was followed by the main testing battery, which includes the tests of mobility and cognitive function outlined above. Upon completion of the data collection sessions at baseline and 24 weeks, participants were issued with a food diary, FFQ, CHAMPS and SF-36 questionnaires. These were fully explained by a member of the research team and written instructions were provided, Participants were then asked to fill these out at home over the next week and return at a subsequent testing session or via postage.

Participants' began their dietary supplementation intervention on the same day their blood sample was taken. Initially, participants were given 12 weeks supply of their respective supplements, they were asked to bring in remaining capsules at the 12-week point, before issuing them with the second batch of supplements to be taken until the end of the study. The week following the 12-week data collection the aerobic exercise intervention commenced, this took place twice a week for the final 12 weeks of the study.

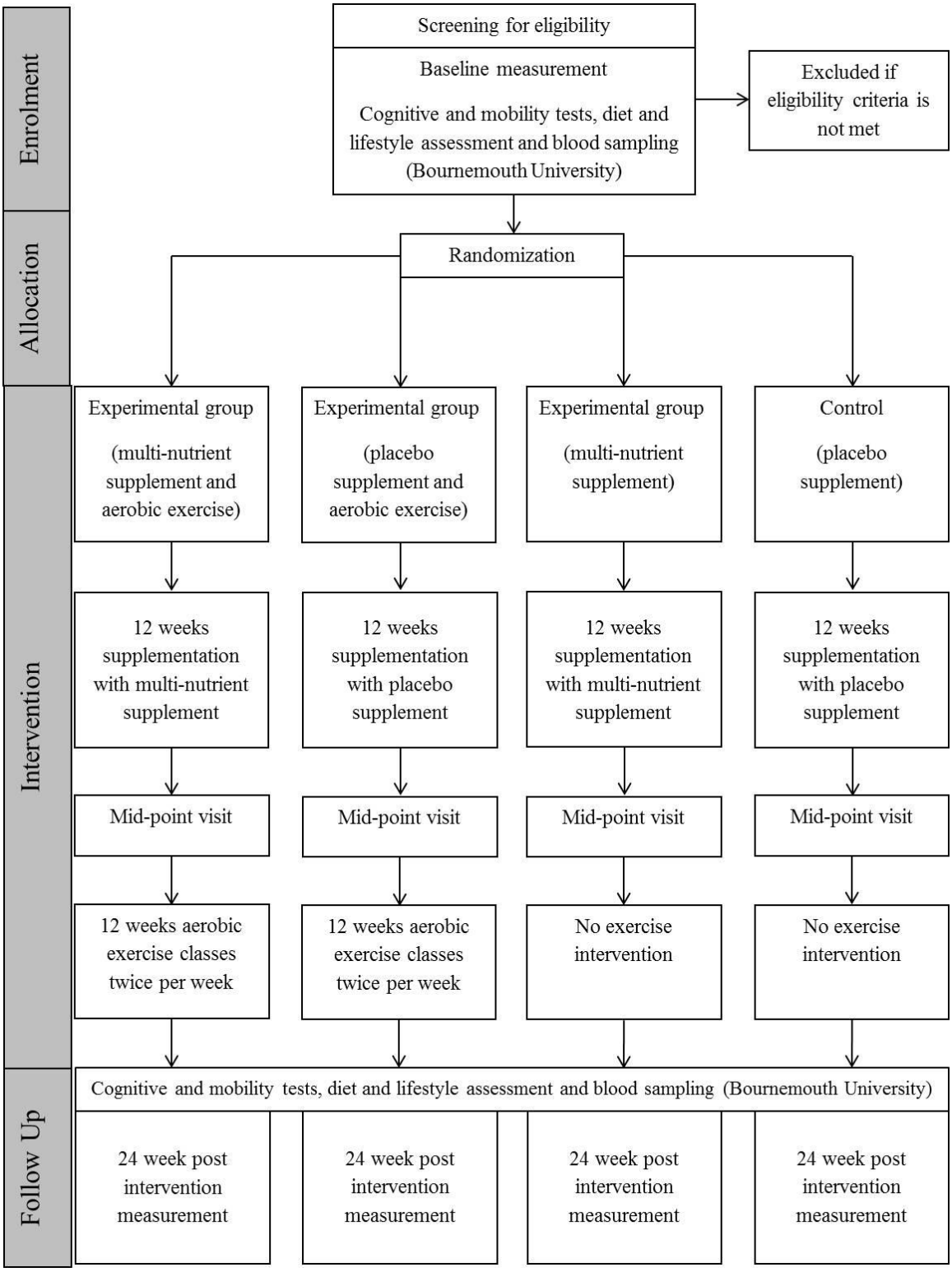


Figure 28 *Flow chart of main study processes.*

3.3.8 Monitoring

The data and safety monitoring was performed by the research team. The team met once per month, to discuss any issue and check on the conduction of the study. Adverse events as defined by Clinicaltrials.gov (2016) were monitored by participant self-reporting and exit questionnaire. Adverse events were reported by the Principal investigator to the institutional research representative and sponsor.

3.3.9 Patient and Public Involvement

The primary and secondary outcomes for the study were chosen based on the latest recommendations for clinically relevant measures in intervention trials on healthy ageing (Lara et al. 2013). In the design phase of the study older women without cognitive or mobility impairment were invited to attend testing sessions, where they were asked to complete the cognitive testing and dual-task gait protocols. These sessions allowed the research team to determine whether there were any floor or ceiling effects of the testing. This meant that changes to the difficulty of the testing could be made to ensure the validity of the testing as well as ensuring the safety and comfort of the participants. Furthermore, participants were invited to give their feedback during these sessions on how the tasks were presented, to ensure that all tests had clear instructions and were well understood. Upon completion of the trial all participants were given a letter providing a full summary of the study and the results.

3.3.10 Ethics and Dissemination

Ethical approval for the study procedure was granted by the Bournemouth University Science Technology and Health research ethics panel (Ethics ID 10788) (Appendix 16) and conforms to the declaration of Helsinki and guidelines for Good Clinical Practice. The trial protocol follows the Consolidated Standards of Reporting Trials (CONSORT) statement on randomised trials of non-pharmacological treatment (Boutron et al. 2008) and the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines (Chan et al. 2013). The results of this study are presented in a PhD thesis, at scientific conferences and peer-reviewed journals. No data was collected until fully informed consent was given by participants (Appendix 17). Interested parties who meet the eligibility criteria were sent a copy of the participant information sheet (Appendix 18), which contains all the necessary information required to take part in the study, participants were given a minimum of 24 hours before being asked to give their consent and were encouraged to contact a member of the research team if they have any questions or concerns regarding participation. Following the completion of the trial, all participants who received the placebo supplement during the study were offered 24 weeks supply of the active supplement.

Chapter 4 – Observational Analysis of the Relationship between Whole Blood Fatty Acids, Homocysteine and Physical Activity with Mobility and Cognitive Function in Older Women 14

This chapter will present an analysis of the baseline data from the RCT. This analysis will aim to establish whether there is a relationship between circulating omega-3 PUFAs and homocysteine with gait outcomes. These dietary factors are key components of the RCT intervention, therefore exploring relationships at baseline could provide an insight into the likelihood of observing a treatment response to either the multi-nutrient supplement intervention.

4.1 Introduction

Ageing is associated with progressive declines in mobility, which over time can lead to ill health and loss of independence (Perera et al. 2016). In regards to assessing mobility, gait speed is established as a clinically relevant marker in older adults, due to strong associations with physical functioning, disability and cognitive function (Montero-Odasso et al. 2005; Montero-Odasso et al. 2012b). An emerging mobility related outcome is that of dual task gait or dual task interference. Dual-task interference occurs when there are competing demands for attentional resources whilst conducting two simultaneous tasks. When the attentional demands of the two tasks exceed the total attentional capacity, performance in one or both of the tasks declines relative to single-task performance (Plummer and Eskes 2015). Dual task interference is associated with risk of falls (Yamada et al. 2011) as well as cognitive decline (Sheridan et al. 2003; MacAulay et al. 2017)**,** making it a novel outcome of interest for two major domains related to healthy ageing, cognition and mobility. Furthermore, there is evidence to suggest that dual-task gait assessments are a more sensitive outcome for predicting falls in cognitively healthy older adults versus single task protocols (Beauchet et al. 2008b; Herman et al. 2010), making it of particular interest for use as an outcome in cognitively robust older adults.

Dietary intake has been proposed as possible modifiable factor for the prevention or delaying of age-associated decline in mobility (Cruz-Jentoft et al. 2014). Within the body of literature on healthy ageing omega-3 PUFAs and B vitamins have shown promise, a large proportion of the literature has focused on cognitive outcomes however recently data has emerged to suggest that these nutrients may positively impact the trajectory of gait speed in older adults (Strike et al. 2016). Omega-3 PUFAs could benefit mobility through multiple pathways including re-sensitising muscle to anabolic stimuli and increasing MPS, improving cognition, reducing joint pain and improving cardiovascular health (Goldberg and Katz 2007; Smith et al. 2011b; Yurko-Mauro et al. 2015). B vitamins have been shown to lower circulating levels of homocysteine.

Higher levels of homocysteine have been positively associated with incidence of physical frailty cognitive decline, cardiovascular disease (Wong et al. 2013; Zhang et al. 2020). Given that omega-3 PUFAs and B vitamins have shown some promise in the prevention of age related declines in mobility, examining the relationship between these lifestyle related factors on mobility, including gait outcomes under single and dual-task conditions could provide a valuable insight their potential role in the promotion of healthy ageing.

Aims:

- Investigate the relationship between whole blood omega-3 PUFAs and mobility outcomes, including gait assessment under single and dual-task conditions, in healthy older women.
- Investigate the relationship between serum homocysteine and mobility outcomes, including gait assessment under single and dual-task conditions, in healthy older women.

Hypotheses

H^1 There will be a positive relationship between omega-3 PUFAs measured in whole blood and mobility outcomes in healthy older women.

H^2 There will be a negative relationship between serum homocysteine and mobility outcomes in healthy older women.

4.2 Methods

All methods related to the participants characterises and data collection have been described previously in chapter 3.

Sample Size

For correlation analysis of gait outcomes, a sample size of 46 was required to achieve a power of 0.80 for a correlation coefficient of 0.40 and an α of 0.05, for a two-tailed test (Algina and Olejnik 2003). This effect size has been selected based on previous work from our lab (Strike et al. 2016) and is based on detecting relationships with the primary outcome of HGS. The multiple regression analysis was of an exploratory nature thus was not designed for hypothesis testing. The purpose of this analysis would be used to aid in the generation of future hypothesises.

Statistical Analysis

The relationships between whole blood fatty acids with gait outcomes were first investigated using correlation analysis. DHA was used as the primary outcome for correlation analysis. As

fatty acids were expressed as percentage weight it was important to consider other fatty acids in the analysis, but these were conducted as secondary outcomes. With evidence now suggesting potentially differential effects and mechanisms of EPA and DHA in humans (Sublette et al. 2011a; Sinn et al. 2012) EPA was included as a separate outcome upon which correlation analysis would be performed. Furthermore, there is evidence to suggest that the ratio of specific fatty acids may be of clinical importance. In particular, the ratio between omega-3 and omega-6 PUFAs may be of relevance for modulation of the inflammatory response (Simopoulos 2002), and given that chronic low-grade inflammation may be a key pathological factor for age related declines in mobility (Soysal et al. 2016) DHA/AA was also included as a secondary outcome. Furthermore, changes in DHA/AA have previously been shown to be associated with habitual gait speed (Hutchins-Wiese et al. 2013).

Age, BMI, NART score and dietary protein intake were collected as potential confounders for this analysis. Correlation analysis was performed on these variables with all mobility and cognitive outcomes and when a significant relationship was identified these variables were included as covariates in a partial correlation. Normally distributed outcomes were analysed using Pearson's correlation. For any variables that did not meet the assumptions of normality for partial correlations, a syntax was used to produce a non-parametric partial correlation. This method consisted of using the NONPAR CORR and /MATRIX OUT commands to produce a Spearman Rho matrix, which could then be read by the /MATRIX IN and PARTIAL CORR command whilst factoring in the appropriate covariates. The Benjamini-Hochberg procedure was used to decrease the false discovery rate and probability of type I errors, with a Q value of <0.25 accepted as significant, and all P values are expressed as raw values (Benjamini and Hochberg 1995).

Spearman rank was used to assess correlations between serum homocysteine and mobility outcomes. There is some evidence to suggest that the relationship between homocysteine and age related decline in health may not be linear with a possible dose-threshold effect existing (Smith et al. 2010a), thus using Spearman rank despite data distribution was the more appropriate statistical method as it can assess the strength and direction of a monotonic relationship.

Where a significant relationship was detected between outcomes in the correlation analysis further testing using multiple linear regression was performed to further explore this relationship. For all regression models DHA, CHAMPS score, verbal memory, spatial working memory, EF, interference control, FTSTS, age, BMI and dietary protein were entered as independent variables. In all multiple regression models a backwards entry stepwise approach was used with the critical P value being set at 0.15. Setting the critical P value at 0.05 for a

stepwise regression model can lead to relevant variables being excluded from model, including important confounding factors (Field 2013). Therefore, a value of 0.15 was selected to reduce the likelihood of incurring type 1 error (Peacock 2006). Collinearity diagnostics and examination of the *ZRESID against *ZPRED plot were used to ensure models met the assumptions of multicollinearity and heteroscedasticity. Furthermore, examination of the normal P-P plots of regression standardised residual were observed to ensure residuals were normally distributed. In all final analyses $P<0.05$ was considered significant.

4.3 Results

Participant Characteristics

60 participants met the study inclusion criteria and gave their written informed consent to take part in the study, participant flow is detailed in Figure 29.

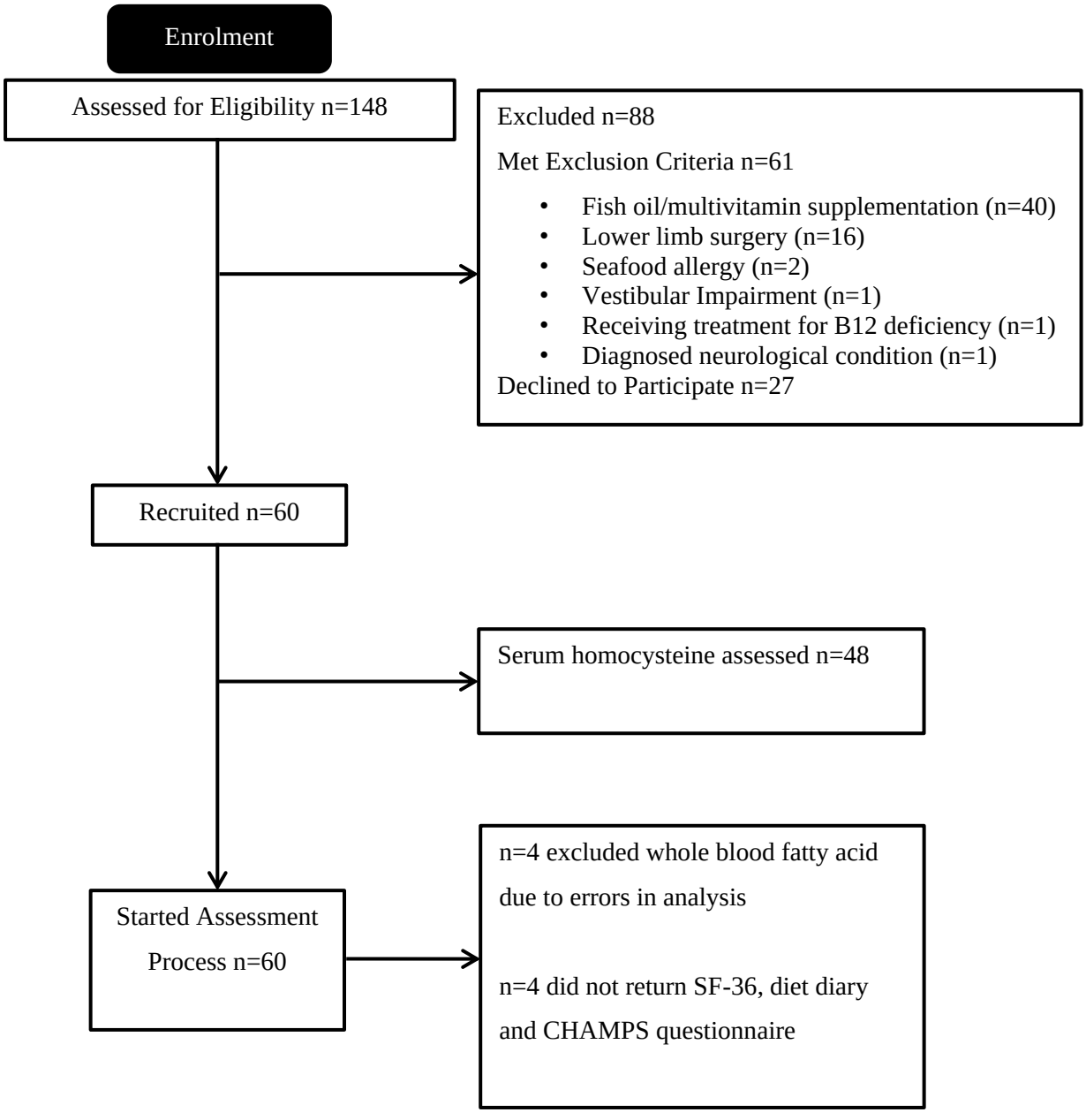


Figure 29 *CONSORT diagram reflecting flow of study participants through the study.*

Descriptive Statistics, Outliers and Transformations

Four outliers were identified in the data for DTE on gait speed. The task was understood by all participants thus results were deemed to be reflective of each individual's performance. For analysis, the outliers for each outcome were corrected by adjusting them to be 1 unit above/below the next nearest value (Kwak and Kim 2017). This meant adjusting values to be 0.1% above or below the nearest non-outlier. Four outliers were identified in the percentage weight of DHA. Two of these outliers were considerably above the normative values reported by Stark et al. (2016b) of 1.95-3.11% with values of 9.26% and 7.28%. The other outliers were 5.37% and 4.86%, despite being above normative values these results could be achieved through regular seafood consumption (Stark et al. 2016b). The omega-3 PUFA FFQ and three day food diary for all four of these participants revealed habitual intakes of EPA and DHA <250 mg/day EPA+DHA. This level of intake would likely not result in such elevated levels as notably higher intakes of above 1g per day have been demonstrated to result in whole blood DHA levels of 2.9-3.5% (Shen et al. 2019), thus these results were excluded from subsequent analysis as there were sufficient grounds to believe they were not reliable and a true value could not be obtained.

After examination of Shapiro-Wilk results and Q-Q plots, the following variables were not normally distributed: DTE on gait speed, dual-task variability, FTSTS, whole blood EPA, DHA/AA and CHAMPS score. For correlation analysis, no transformations were made as Spearman Rank testing was used on variables which did not meet the assumption of normality. For each multiple regression model, analysis of the variation inflation factors, normality of residuals P-P plots and of the standardised residuals versus predicted values of multivariate confirmed that the assumptions of residual normality, multicollinearity and homoscedasticity were met for all models (Field 2013).

CHAMPS score and dietary intake were missing for four participants. All missing data was random and was due to withdrawal from the study. All analysis was conducted on a complete case basis. Baseline serum homocysteine data was available for 48 participants, as this was the number of available samples that could be run on one ELISA plate. Inter-plate variability for bioassays is an established limitation of such methodology (Reed et al. 2002). As the coefficient of variation between ELISA kits was not able to be established, results from remaining participants at baseline, that were run on a different plate were not included in this analysis as it could not be determined if they could be reliably pooled with the prior result from the first plate.

Table 8 provides a summary of the demographic information of the participants within the study, as well as the participants mobility and cognitive outcomes.

Table 8 Descriptive statistics for the demographic information, mobility and cognitive function of the studied population. Mean values and standard deviations are given for normally distributed data and median and interquartile range for non-normally distributed.

Demographic Information n=60			
Measure	Mean/Median	SD/IQR	CI95%
Age (years)	67*	8*	66, 69
Height (m)	1.63	0.07	1.61, 1.64
Weight (kg)	69.9*	15.5*	68.6, 76.7
BMI (kg/m^2)	26.4*	6.6*	26, 28.9
NART Score	36*	6*	34, 37
MMSE Score	30	1	29, 30
Hand Grip Strength (kg)	19.5	4.6	18.3, 20.7
PASE Score	112*	52*	114, 136
Mobility n=60			
Habitual Gait Speed (m/s)	1.22	0.16	1.18, 1.26
Fast Walking Speed (m/s)	1.66	0.25	1.59, 1.72
Dual Task Gait Speed (m/s)	1.06	0.28	0.99, 1.13
DTE on Gait Speed (%)	-10.39*	32.5*	-38.93, -13.12
Dual Task Stride Length Variability (%)	4.05*	2.35*	4.06, 5.22
FTSTS (s)	12.85*	2.8*	12.12, 13.58
Cognitive Function n=60			
Verbal Memory (%)	57.3	11.5	54.3, 60.2
Spatial Working Memory (%)	75.8	6.3	74.2, 77.5
Executive Function (number of correct connections)	67.1	18.3	62.3, 71.8
Interference control (ms)	141.4*	138*	144.6, 252.6

(*)Indicates that a value is the median/interquartile range

Abbreviations: Body Mass Index (BMI), National Adult Reading Test (NART), Mini Mental State Examination (MMSE), Physical Activity Scale for the Elderly (PASE), Dual Task Effect (DTE), Five times sit to stand (FTSTS)

Dietary intake, physical activity, whole blood omega-3 status and serum homocysteine are summarised in Table 9.

Table 9 Descriptive statistics for whole blood omega-3 PUFAs, serum homocysteine, dietary intake and physical activity levels. Mean values and standard deviations are given for normally distributed data and median and interquartile range for non-normally distributed.

Whole Blood Fatty Acids n=56 and Serum Homocysteine n=48			
	Mean/Median	SD/IQR	CI95%
DHA (%)	1.66	0.51	1.52, 1.80
EPA (%)	0.30*	0.13*	0.23, 0.28
DHA/AA	0.24*	0.1*	0.23, 0.28
Serum homocysteine-bovine serum albumin (µg/ml)	3.34	0.76	3.12, 3.56
Dietary Intake and Physical Activity n=56			
Daily energy (kcal)	1644	382	1575, 1786
Carbohydrate (g)	175	43	164, 187
Protein (g/kg bodyweight)	1.03	0.32	0.94, 1.13
Fat (g)	71	22	65, 78
Diet Diary EPA (mg)	23*	40*	49, 183
Diet Diary DHA (mg)	46*	120*	109, 331
FFQ EPA (mg)	50*	9*	52, 114
FFQ DHA (mg)	85*	140*	87, 183
Vitamin B12 (µg)	4.6*	2.7*	4.7, 6.0
Folic Acid (µg)	268	78	247, 289
Vitamin B6 (mg)	1.78	0.48	1.66, 1.92
CHAMPS Score for All Activities (kcal)	3094*	3118	3097, 4324
CHAMPS Score for Moderate and Vigorous Activities (kcal)	1537*	1872*	1440, 2422
Minutes of Moderate to Vigorous activities	180*	180*	162, 265

(*)Indicates that a value is the median/interquartile range

Abbreviations: Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), Food Frequency Questionnaire (FFQ), Community Healthy Activities Model Program for Seniors (CHAMPS)

Cross sectional analysis of whole blood fatty acids, physical activity, mobility and cognitive function

Confounding factors

Age was significantly correlated with HGS, fast walking speed and dual-task gait speed. BMI was significantly correlated with HGS, fast walking speed, habitual gait variability. Dietary protein intake was significantly correlated with HGS, fast walking speed and FTSTS. For all correlations, the significant confounders listed above were controlled for using partial correlations.

After controlling for false discovery rates, analysis of the mobility outcomes found that circulating DHA was positively associated with dual-task gait speed (*Figure 30*) and the DTE on gait speed (*Figure 31*). There were no other associations between mobility outcomes and circulating fatty acids (Table 10). No significant relationships were detected between serum homocysteine and gait outcomes under single or dual-task conditions (Table 11).

Table 10 Correlation matrix plotting whole blood fatty acids against mobility outcomes, n=56.

	Habitual Gait Speed (m/s)	Fast Walking Speed (m/s)	Dual Task Gait Speed (m/s)	Dual task effect on Gait Speed (%)	Dual Task Variability (%)	Five Times Sit to Stand (s)
DHA (%)	R=0.001 (*p*=0.968)	R=0.097 (*p*=0.487)	R=0.318* (*p*=0.018)	R=0.312* (*p*=0.019)	R=-0.257 (*p*=0.056)	R=-0.095 (*p*=0.495)
EPA (%)	R=-0.048 (*p*=0.729)	R=0.137 (*p*=0.324)	R=0.200 (*p*=0.142)	R=0.180 (*p*=0.176)	R=-0.169 (*p*=0.204)	R=-0.056 (*p*=0.685)
DHA:AA (%)	R=0.041 (*p*=0.768)	R=0.291[a] (*p*=0.033)	R=0.194 (*p*=0.156)	R=0.115 (*p*=0.397)	R=-0.071 (*p*=0.603	R=0.023 (*p*=0.871)

* indicates a significant association ($p<0.05$)

[a] Indicates a result that was statistically significant before controlling for the false discovery rates.

Abbreviations: Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA).

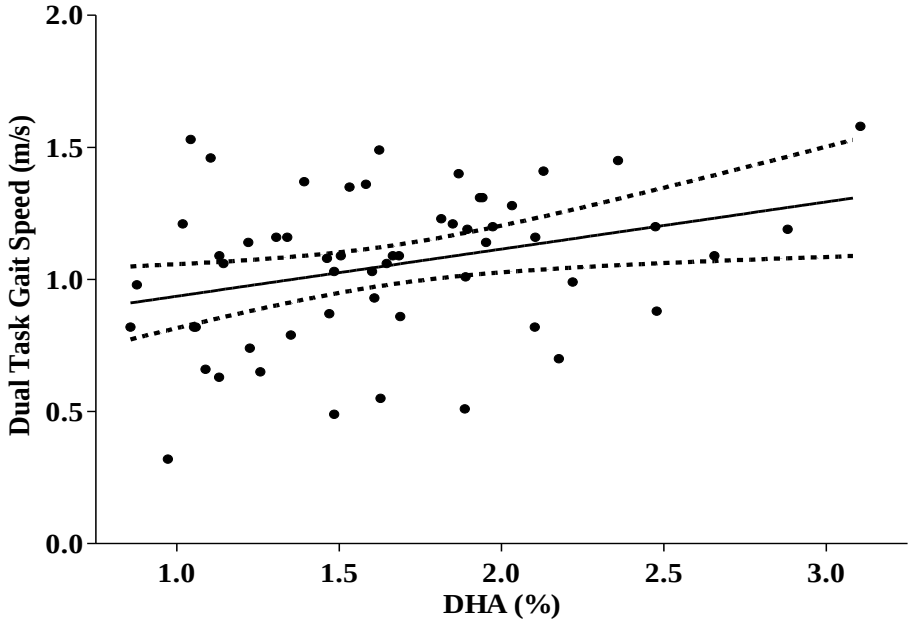


Figure 30 *Correlation between whole blood docosahexaenoic acid (DHA) and dual task gait speed controlling for age R=0.318 p=0.018 CI95% 0.059, 0.535 (n=56).*

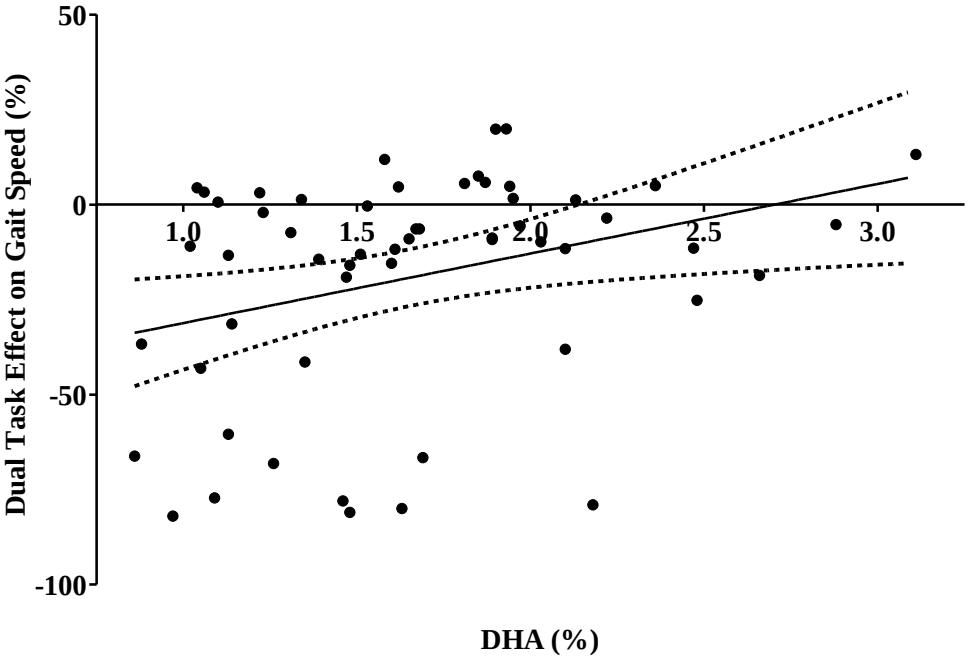


Figure 31 *Correlation between whole blood docosahexaenoic acid (DHA) and the dual task effect on gait speed R=0.312 p=0.019 CI95% 0.04, 0.537 (n=56).*

Table 11 Correlation matrix for serum homocysteine-BSA and mobility outcomes, n=48.

Outcome Measure	Homocysteine-BSA Correlation
Habitual Gait Speed (m/s)	R=0.190 (*p*=0.197)
Fast Walking Speed (m/s)	R=0.256 (*p*=0.079)
Dual Task Gait Speed (m/s)	R=-0.005 (*p*=0.975)
DTE on Gait Speed (%)	R=-0.209 (*p*=0.155)
Dual Task Variability (%)	R=0.040 (*p*=0.788)
Five Times Sit to Stand (s)	R=-0.149 (*p*=0.317)

Abbreviations: Bovine serum albumin (BSA)

DHA, Physical Activity and Cognitive Function as Predictors of Mobility

A summary of the multiple regression models using dual-task gait speed and DTE on gait speed as dependent variables can be found in Table 12. DHA, CHAMPS score, verbal memory, spatial working memory, executive function, interference control, age, BMI and dietary protein intake entered as predictors for each model.

The model for dual-task gait speed was significant F (3, 50) = 10.51 p<0.001 adjusted R^2 =0.363. spatial working memory (coefficient 0.021 [CI95% 0.010, 0.032], *p*<0.001), DHA (coefficient 0.137 [CI95% -0.003, 0.277], *p*=0.054) and interference control (coefficient 0.001 [CI95% -0.001, 0.001], *p*=0.112) remained in the model.

The model for DTE on gait speed was significant F (6, 50) = 4.87 p=0.001 adjusted R^2 =0.317. spatial working memory (coefficient 1.92 [CI95% 0.43, 3.41], *p*=0.013), interference control (coefficient -.056 [CI95% -0.11, -0.01], *p*=0.026), CHAMPS score (coefficient -11.34 [CI95% -22.27, -0.41], *p*=0.042), dietary protein intake (coefficient -26.36 [CI95% -54.63, 1.92], *p*=0.067), executive function (coefficient -0.42 [CI95% -0.94, 0.10], *p*=0.111) and DHA (coefficient 14.29 [CI95% -4.57, 33.15], *p*=0.134) remained in the model.

Table 12 Summary of the multiple regression models using mobility outcomes as dependent variables with DHA, CHAMPS score, verbal memory, spatial working memory, executive function, interference control, age, BMI and dietary protein intake entered as predictors, n=56.

Model Statistics	Variable	Regression Coefficient	β	CI95%	P Value
Dual Task Gait Speed					
F (3, 50) = 10.51 p<0.001 adjusted R^2 =0.363	Spatial Working Memory	0.021	0.45	0.010, 0.032	<0.001*
	DHA	0.137	0.23	-0.003, 0.277	0.054
	Interference Control	0.001	-0.20	-0.001, 0.001	0.112
DTE on Gait Speed					
F (6, 50) = 4.87 p=0.001 adjusted R^2 =0.317	Spatial Working Memory	1.92	0.33	0.43, 3.41	0.013*
	Interference Control	-.056	-0.31	-0.11, -0.01	0.026*
	CHAMPS Score	-11.34	-0.27	-22.27, -0.41	0.042*
	Dietary Protein Intake	-26.36	-0.23	-54.63, 1.92	0.067
	Executive Function	-0.42	-0.21	-0.94, 0.10	0.111
	DHA	14.29	0.19	-4.57, 33.15	0.134

* Indicates a statistically significant result

4.4 Discussion

Relationship between Whole Blood Fatty Acids and Gait under Single and Dual Task Conditions

To date, there is fairly limited data on the effects of omega-3 PUFAs on mobility outcomes in older adults. Previous population studies have identified a positive relationship between omega-3 PUFAs and HGS (Abbatecola et al. 2009; Frison et al. 2017), including work from our lab which showed a moderately strong (R=0.47) relationship between whole blood DHA (Strike et al. 2016) and HGS, whereas the present analysis found no relationship between whole-blood fatty acids and the primary outcome of HGS. The relationship between omega-3 PUFAS and gait has not been consistent across all the literature with Fougere et al. (2017) finding no relationship between erythrocyte omega-3 PUFAs and rate of decline in gait speed over three years and Rousseau et al. (2009) finding no relationship between self-reported intakes of omega-3 PUFAs and HGS.

This cross-sectional analysis showed for the first time a relationship between whole blood DHA and dual-task gait performance in older adults. Dual-task gait protocols have emerged as a reliable way of assessing the interplay between the cognitive and physical domains in older adults. DTE on gait speed has been associated with risk of falls in older adults (Muir-Hunter and Wittwer 2016) as well as cognitive decline (Sheridan et al. 2003; MacAulay et al. 2017). Although the results from this analysis provide some promising evidence for a positive association between whole blood DHA with dual-task gait, the strengths of these relationships are somewhat unclear. The R values for the analysis show a weak relationship between these outcomes, and the CIs have a large range making it difficult to draw definitive conclusions as to exactly how strong this relationship is. Furthermore the relationship between DHA and dual-task outcomes did not remain significant when further analysed by multiple regression including cognitive function, self-reported physical activity, dietary protein intake and demographic information Nonetheless this analysis raises the interesting possibility that increasing DHA intake could have an impact on dual-task gait in older adults.

The present study found no relationship between DHA and single task gait speed yet under the more cognitively demanding dual-task scenario a relationship was observed. Cognitive function plays an important role in maintaining gait speed and stability in older adults, and appears to be more important in maintaining a stable gait in those with more limited mobility (Beauchet et al. 2009). Omega-3 PUFAs have previously been shown to prevent cognitive decline in healthy older adults, however, this may be limited to a few domains specifically verbal memory, immediate recall and attention (Yurko-Mauro et al. 2010; Sinn et al. 2012; Lee et al. 2013; Zhang et al. 2016b; Hooper et al. 2017). Gait control has been primarily linked to executive

function and processing speed (Coppin et al. 2006; Holtzer et al. 2006; Springer et al. 2006; Bruce-Keller et al. 2012; Kearney et al. 2013; Martin et al. 2013a). Therefore, despite the evidence for DHA improving cognition in older adults and the relationship between cognition and gait with the present study, there is an incongruity between the specific cognitive domains involved, so it is unclear whether the link between DHA and dual-task gait is mediated through a cognitive dependent mechanism. It is possible the relationship between these outcomes could be via a subtle cognitive mechanism, or interplay between several cognitive domains which would be difficult to detect with cognitive testing alone. As such, more sensitive measures, such as brain imaging, may be required to investigate the link between specific brain regions, gait performance and omega-3 PUFA status. Future studies should seek to replicate the current findings in a larger sample size and across a greater variability of omega-3 PUFA levels. Studies should also seek to uncover any potential mechanisms by which DHA may influence gait in older adults, as it is currently unclear as to whether the relationship is through an effect on cognition and whether this is domain specific. Furthermore, this relationship was shown on backwards counting task it would, therefore, be interesting to investigate whether this is consistent across different types of secondary tasks that involve other cognitive domains and brain regions.

It is interesting to note that the relationships demonstrated within this analysis were between DHA and mobility outcomes with no relationships being shown for EPA or DHA/AA. Research has suggested that omega-3 PUFAS may be able to enhance MPS within older adults (Smith et al. 2011a), with *in vitro* data suggesting that it may in fact be EPA that drives this effect not DHA (Kamolrat and Gray 2013). Whilst it could be suggested that this increase in MPS could translate to mobility outcomes no relationships was detected within this sample. One possible explanations for this is low levels of EPA detected in the whole blood across the sample, indeed the experimental work that's has been conducted demonstrating an increase in MPS and muscle strength was achieved using dosages of EPA >1.5g per day (Smith et al. 2011a; Smith et al. 2015; Da Boit et al. 2016b). Having low levels of EPA across the sample and a limited range of exposure may have impacted the ability to detect relationships between the measured outcomes and whole blood omega-3 PUFAs. Some previous observational research has detected a relationship between the ratio of omega-3 PUFA to omega-6 PUFA and mobility (Abbatecola et al. 2009)**.** Whilst the omega-3 PUFA to omega-6 PUFA ratio has been investigated for its relevance to conditions where chronic inflammation may be an important underlying physiological mechanism (Simopoulos 2002). Within the human data is difficult to unravel whether any beneficial association or effect is occurring as a result in the shift in the ratio between these fatty acid types or indeed as a result of just greater levels of omega-3 PUFAs. Furthermore, we must also consider that some of the protective mechanisms by which omega-3

PUFAs may exert their positive effects on MPS through changes in intramuscular cell signalling rather than impacting levels of inflammation (Smith et al. 2011b, 2011a), thus the relevance of this marker in regards to mobility outcomes should be questioned.

All the previous studies investigating the relationship between omega-3 PUFAs and gait controlled for a number of important confounders including age, BMI, educational level, MMSE score, physical activity and comorbidities; however, protein intake was only controlled for in the studies by Frison et al. (2017) and Rousseau et al. (2009); it was not factored into the analysis by Fougere et al. (2017), Abbatecola et al. (2009) and Strike et al. (2016). In the present analysis, dietary protein intake was found to be a significant confounder for habitual and fast walking gait, as well as FTSTS. Given that protein has been shown to be an important macronutrient for the physical health of older adults (Bauer et al. 2015), and that inadequate dietary protein consumption is a concern for this population (National Diet and Nutrition Survey 2018), future studies should make this an essential criterion to factor into their analyses.

Relationships between Homocysteine and Gait under Single and Dual Task Conditions

No relationships between serum homocysteine and mobility and cognition were detected. Previously homocysteine has been inversely associated with physical function in older adults (Kado et al. 2002) however, this relationship is likely not linear in nature and may only exist when higher levels of homocysteine of >10.1 μmol/ are present (Nurk et al. 2005; Smith et al. 2010a; Wong et al. 2013). The technique used to assess homocysteine in this analysis, meant values were expressed as homocysteine-BSA, as samples were diluted in phosphate buffered saline with 0.1% BSA as per the ELISA protocol. BSA contains some homocysteine thus results are expressed as homocysteine-BSA. Homocysteine-BSA cannot be converted reliably to homocysteine in micromoles per litre, which is the standardised value for human studies. This meant it was impossible to determine how many, if any of the participants, were at a level of homocysteine which may be expected to be associated with health consequences, or indeed how broad the range of exposure was. Furthermore, the analysis of the diet diaries revealed that every participant was meeting the reference nutrient intake for B12 and B6 with 90% meeting their folic acid RNI, indicating that B vitamin intake within this group may have been adequate enough to prevent elevated homocysteine.

Strengths and Limitations

This study has several strengths within the design. Analysis of relationships between mobility and fatty acids took into consideration not only the total amount of EPA and DHA but the ratio between DHA to AA. Given the interplay between omega-3 and omega-6 PUFAs and how this may influence inflammation (Simopoulos 2002), which may in turn play a role in the

pathogenesis of many age related health conditions (Beyer et al. 2012; Steptoe et al. 2012), this was an important analysis to perform in this specific population. Another strength of the study design was measuring and subsequently controlling for dietary factors including total energy and macronutrient intake, with protein intake in particular emerging as a critical outcome to factor into the correlational analysis. Although this is an overall strength of the study it must be acknowledged that the mean total energy intake of the participants was 1644 kcals, which when we consider that the mean BMI of the cohort was 27.5 kg/m^2 could indicate underreporting of food intake. Indeed underreporting of energy intake has been demonstrated to be greater in those with a higher BMI, thus there is a need to develop more accurate methods for assessing dietary intake so that dietary factors can be accurately controlled for within similar analyses (Ravelli and Schoeller 2020). 15

Although the direct measurement of whole blood fatty acids is an overall strength of the study, the low levels of omega-3 PUFAs found across the sample makes it impossible to directly compare the results of many of the observational analyses across the body of literature. Many previous studies have included populations with a much greater range of omega-3 PUFA levels and with a larger proportion of the sample at higher levels. Furthermore, much of the literature has measured plasma or erythrocyte fatty acids (Abbatecola et al. 2009; Fougere et al. 2017) as opposed to whole blood making direct comparisons more difficult to make. Although this data does provide some novel insight, particularly into the relationship between omega-3 PUFA status and dual-task gait outcomes, this cannot be extrapolated beyond the low levels of omega-3 PUFAs found in this sample but does provide justification for future research to investigate this further across a larger range.

Another notable limitation was our inability to compare the homocysteine levels in the cohort with normative values due to the outcome for the ELISA assay being homocysteine-BSA. Initial contact with the supplier indicated that conversion to micromoles per litre could be performed with this assay, supported by early work using an identical methodology publishing converted values (Liu et al. 2016). However, after performing the analysis within our lab we noted that any conversion yielded values considerably higher than would be expected in a healthy adult population. Further contact with the supplier indicated that the outcome of the kit could only be expressed in homocysteine-BSA µg/ml. This meant that although the values from this analysis could be used for correlation analysis as the relative values would still be valid, the result could not be contextualised in comparisons to the values from other human studies that have suggested a threshold effect for homocysteine on healthy ageing outcomes (Wong et al. 2013; Nie et al. 2014).

4.5 Conclusion

Overall this cross-sectional analysis examining relationships between whole blood fatty acid levels and serum homocysteine with mobility in healthy older women showed a weak but statistically significant relationship between DHA in whole blood and dual-task gait outcomes. This result provides novel evidence for a potential interplay between whole blood DHA and dual-task walking in older adults, which could have implications for developing dietary interventions for trajectory towards the HAP. Dual-task gait outcomes have been associated with risk of falls in older adults (Muir-Hunter and Wittwer 2016); therefore evidence for a relationship between fatty acid intake and these outcomes provides a promising insight for the potential of dietary manipulation to influence the health of older adults. However, due to the observational nature of the analysis, it cannot be determined if this relationship is causal, furthermore, it is unclear whether this relationship exists due to downstream effects of DHA on cognition and through another mechanism. Future studies should look to first replicate this novel finding and establish through what mechanism DHA could influence dual-task gait. It is possible that dual-task gait performance could be influenced by multiple cognitive domains, with even subtle differences influencing performance, with evidence suggesting links between performance in dual-task scenarios with executive function, visual memory and interference control (Doi et al. 2014). Although these results provide some promising insight into the relationships between fatty acid intake and mobility outcomes in older adults, randomised controlled trials are required to determine whether increasing omega-3 PUFA intake can have a positive effect on mobility.

Chapter 5 - The Effects of a High DHA Multi-nutrient Supplement and Aerobic Exercise on Mobility and Cognitive Function in Older Women

5.1 Introduction

Ageing is associated with a progressive decline in both cognitive and physical function, which can lead to a number of age-related health conditions including frailty and dementia (Cesari et al. 2014). Walking is a complex task with a significant cognitive aspect, and changes in several gait parameters including speed often coexist with or precede the onset of cognitive decline in older adults (Savica et al. 2016). Gait speed is a clinically relevant marker in older adults, due to strong associations with physical functioning and disability (Montero-Odasso et al. 2005). Previous work in our laboratory suggests a high DHA multi-nutrient supplement providing the omega-3 PUFAs, DHA and EPA, and supporting nutrients PS, d-α tocopherol, folic acid, vitamin B12, and *Ginkgo biloba*, improves cognition and mobility in older females (Strike et al. 2016).

Providing omega-3 PUFAs in combination with other compounds indicated to support brain function may provide greater efficacy than if supplemented in isolation, although results are so far mixed. For example, the same multi-nutrient supplement as used in our previous and present studies did not improve cerebral hemodynamic or cognitive function in healthy older adults (Jackson et al. 2016a); however, the authors acknowledge that the cognitive tests were selected on the basis that they are able to activate the prefrontal cortex, and may not be sensitive to the components in the supplement. Similarly, an omega-3 PUFA multi-nutrient supplement showed no significant effects on a battery of cognitive tests of participants with prodromal Alzheimer's disease, although improvements were seen in secondary outcomes of cognitive function and hippocampal atrophy (Soininen et al. 2017).

Aerobic exercise has consistently been shown to improve both mobility and cognition in older adults (Smith et al. 2010d). Omega-3 PUFAs and exercise share a range of overlapping biological effects, including enhancing neurogenesis, neural plasticity, and reducing inflammation (Dyall 2015b; Ryan and Nolan 2016). Indeed, preliminary evidence suggests combining omega-3 PUFAs with exercise may provide additional benefit when compared to either approach alone. For example, omega-3 PUFA supplementation combined with twice weekly stationary cycle training and cognitive stimulation led to an enhanced reduction of brain atrophy in grey matter regions compared to supplementation (Kobe et al. 2016). Similarly, combining omega-3 PUFA supplementation with resistance training in older females provided an additional benefit to muscle strength compared with the exercise alone (Da Boit et al. 2017). In addition, an omega-3 PUFA multi-nutrient supplement combined with exercise was recently

shown to improve verbal recall and executive function in older men, more than supplementation alone (Bell et al. 2019). The mechanisms underpinning these interactions are currently unclear, as the studies were not been designed to elucidate these effects. However, decreasing homocysteine levels may be a potential factor, as omega-3 PUFAs regulate the expression of genes encoding enzymes involved in homocysteine metabolism (Huang et al. 2013) and exercise decreases homocysteine levels (Vincent et al. 2003).

Aims

- Investigate the effects of a high DHA multi-nutrient supplement and exercise alone and in combination on a series of outcomes related to the HAP including, gait parameters, verbal memory, spatial memory, executive function, interference control and health related quality of life versus placebo in pre-frail and non-frail older women.
- Determine whether the combination of a high DHA multi-nutrient supplement and exercise can have a broader effect on outcomes related to the HAP in pre-frail and non-frail older women by significantly impacting a greater number of outcomes than each separate intervention. 16

Hypotheses

H^1 The high DHA multi-nutrient supplement will improve mobility, cognitive and health related quality of life outcomes versus placebo in pre-frail and non-frail older women.

H^2 Aerobic exercise will improve mobility, cognitive and health related quality of life outcomes versus placebo in pre-frail and non-frail older women.

H^3 The positive effects of high DHA multi-nutrient supplement and aerobic exercise on cognitive function will be domain specific.

H^4 The combination of the high DHA multi-nutrient supplement and aerobic exercise will lead to significant benefits across a greater number of outcomes compared to each intervention alone in pre-frail and non-frail older women.

5.2 Methods

All methods on participants and data collection have been described previously in chapter 3.

Sample Size 17

Sample size was determined based on the primary outcome of habitual walking speed. Using an effect size based on previously published values, minimally significant changes in gait speed were 0.03 m/s and 0.05 m/s with substantial changes at 0.08 m/s (Kwon et al. 2009). The sample size calculation was based on a difference of 0.08 m/sec with the level of variability set at 0.1 based on our previous work (Strike et al. 2016) with a minimum sample size of 13 participants per group required to detect an effect size d of 0.8. In our original protocol, a per group sample size of 25 participants was originally proposed based on setting the variability at 0.14 based on unpublished pilot work; however this was instead updated to fall in line with published pilot work from our lab (Strike et al. 2016; Fairbairn et al. 2019). This published data had a larger sample size and the variability value was taken for changes in gait speed making it more applicable to the present study.

Statistics

Statistical analyses were performed using IBM SPSS statistics version 21 (Chicago, USA). Data were tested for normal distribution using Shapiro-Wilk test and Q-Q-plots and Levene's test of equality of error variances to check for assumptions of homogeneity. Baseline and 24-week results from the diet and physical activity assessments were also compared within groups, using paired T-tests, to determine whether participants had made any significant changes to their diet and physical activity habits during the study intervention. Diet and physical activity data, along with data collected on medication use were examined so that interpretation of results could be made within the context of potential differences of other lifestyle-related factors. A general linear model was used to compare the active intervention groups versus the placebo over time (from pre- to post-measurement) on changes on the dependent variables on an intention to treat basis. Effect size calculation (η^2 (Eta squared)) was performed. Demographic and health information, such as age, and BMI were included as covariates in the analysis if they were significantly correlated with the dependent variable. The Benjamini-Hochberg procedure was used to decrease the false discovery rate and the probability of type I errors, with a Q value of <0.25 accepted as significant, and all P values are expressed as raw values (Benjamini and Hochberg 1995). Pre-trial registration plans dictated that a 2 x 2 ANOVA would be conducted to examine treatment effects, however due to the potential interaction between the supplementation and exercise independence of these variables could not be guaranteed, thus examining treatment effects based on the assigned groups was determined to be a more robust

method. An intention to treat analysis was carried out and included all participants who decided to discontinue treatment but completed the intervention period and assessment at 24 weeks. A per-protocol analysis was also conducted to examine the effects of the interventions on participants who completed their allocated treatment.

5.3 Results

5.3.1 Intention to Treat Analysis

Descriptive Statistics, Outliers and Transformations

After examination of Shapiro-Wilk results and Q-Q plots, the mobility outcomes DTE on gait speed (p = 0.007, skew: 1.03 kurtosis: -0.61) and dual task stride length variability (p = 0.019, skew: -1.35 kurtosis: 1.46) in the placebo group and dual-task gait speed (p = 0.038, skew: 1.12 kurtosis: 4.38) in the MS+EX group were not normally distrusted. All cognitive outcomes were normally distributed in all four intervention groups. For the SF-36 outcomes health perceptions (p = 0.003, skew: 1.86 kurtosis: 3.30), physical function (p = 0.004, skew: -0.37 kurtosis: 4.31) and pain (p = 0.004, skew: -1.24 kurtosis: 3.9) in the placebo group were not normally distributed. The general linear model is quite robust to violations of normality and tends to only be disrupted by large negative values of kurtosis (Field 2013), therefore transforming this data was not necessary.

Role limitations due to physical health problems, role limitations due to emotional health problems and social functioning from the SF-36 questionnaire were not normally distributed. Over 60% of participants scored the maximum of 100 at baseline for each of these outcomes, indicating ceiling effects and a potential lack of sensitivity to show change over time, supported by the finding that 67% of the follow up changes were 0. The data sets for these variables do not meet the assumptions for the general linear model. Due to the large number of 0 scores, it is not possible to transform this data reliably, thus the planned analysis for both intention to treat and per-protocol could not be reliably conducted on these outcomes.

Three outliers were identified following a review of stem and leaf diagrams, one in the interference control data and two in the DTE on gait speed data. These outliers were corrected by adjusting them to be one unit above or below the next closest value (Kwak and Kim 2017).

Participants

Participant flow through the study is shown in *Figure 32*. Nine participants withdrew from the study; none of the withdrawals were due to the study intervention. Reasons for withdrawal included: relocation (n=2), ankle injury (n=2), family bereavement (n=2), unrelated illness (n=2) and being prescribed a medication that conflicted with the multi-nutrient supplement (n=1). Participants who withdrew during the study were invited to attend follow up assessment; however, the invitations were declined. Table 13 provides a summary of the sample

characteristics broken down by intervention group allocation. There were no significant differences between intervention arms for any of the demographic outcomes.

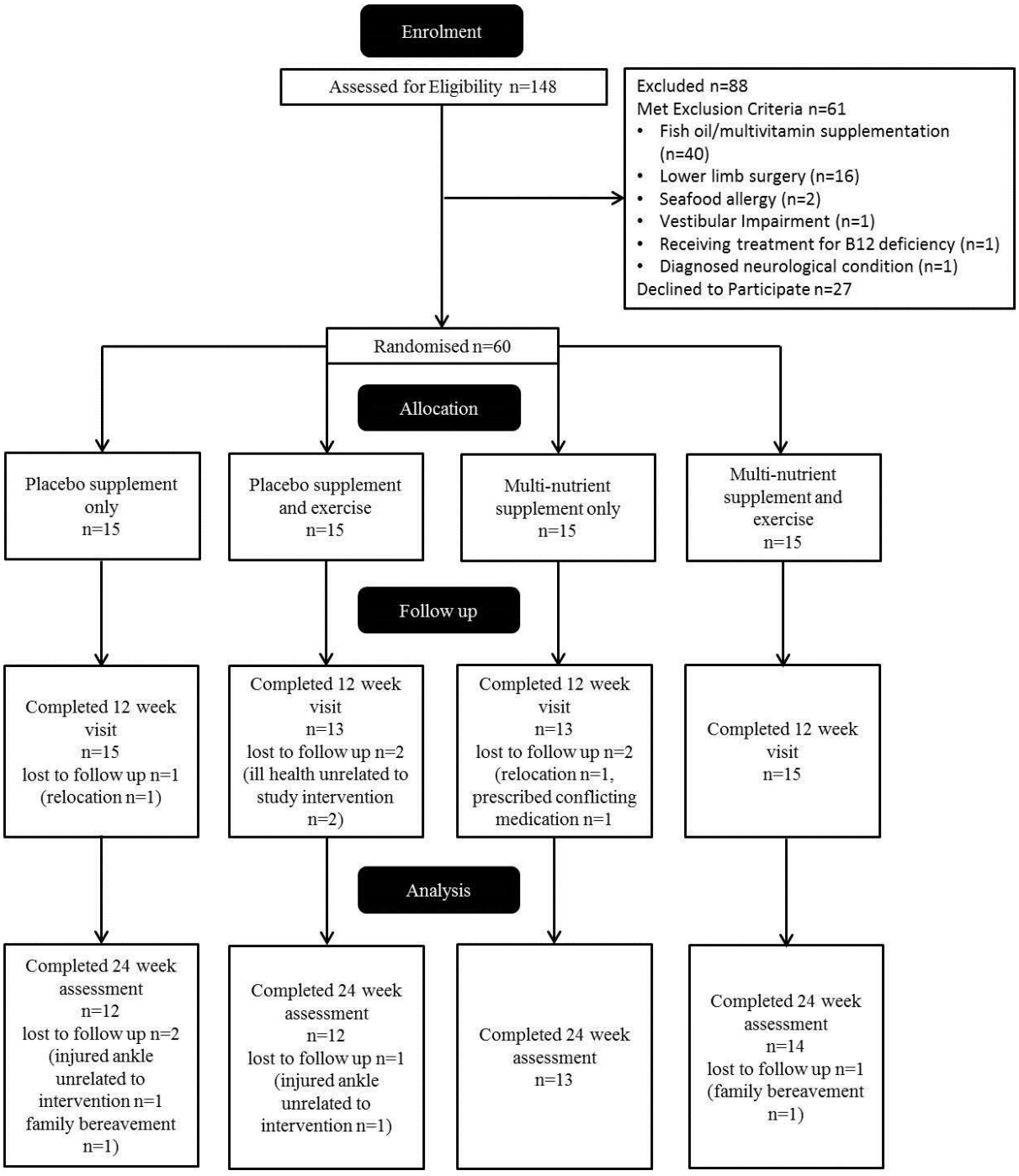


Figure 32 *Participant flow through study*

Table 13 Descriptive statistics for demographic information of study participants divided into their respective intention to treat intervention groups. Mean values and standard deviations are reported for normally distributed outcomes and median and interquartile range for non-normally distributed outcomes.

Parameter	P (N = 12)	P + EX (N = 12)	MS (N = 13)	MS + EX (N = 14)
Age (years)	67 (4)	67 (4)	69 (4)	68 (5)
Height (m)	1.63 (0.1)	1.64 (0.1)	1.62 (0.1)	1.61 (0.1)
Weight (kg)	69.5 (14.9)*	77.1 (33)*	75.6 (17.4)*	68.6 (11.1)*
BMI (kg/m^2)	26.8 (5.2)*	27.8 (12.0)*	27.4 (5.3)*	28.6 (5.9)*
Comorbidities	Hypertension (2) Hypercholesterolemia (1) Osteoarthritis (2)	Hypertension (3) Osteoarthritis (1)	Hypertension (2) Hypercholesterolemia (1) Osteoporosis (1)	Hypertension (3) Hypercholesterolemia (2) Diabetes (1) Osteoarthritis (2)
NART Score	36 (8)	36 (5)	37 (5)	36 (7)
MMSE score	30 (1)*	30 (0)*	30 (1)*	30 (2)*
Hand Grip Strength (kg)	20.5 (3.4)	21.0 (5.9)	19.3 (5.4)	19.0 (4.0)
PASE Score	114 (97)*	113 (30)*	111 (73)*	100 (55)*

*Indicates value is the median (IQR), otherwise means (SD) presented.

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX), Mini Mental Sate Exam (MMSE), National Adult Reading Test (NART), Physical Activity Scale in the Elderly (PASE)

Intervention Adherence and Blinding

There were no reported side effects in any group over the study. The multi-nutrient supplementation led to significant increases in DHA and EPA compared to baseline levels in the MS (28% and 190%, respectively) and MS+EX (43% and 140%, respectively) groups, and significant decreases in AA levels of -28% and -26%, respectively, all $p < 0.05$ (Table 14).

Compliance to the supplementation measured via changes in DHA and was 90.9% for MS+EX, and 80% for MS, 100% for P+EX and 100% for P. Values were calculated based on the percentage of participants in each group that met the criteria set out for compliance. Compliance percentages measured by capsule counting was identical to the blood measures. Total compliance to the exercise intervention measured via a register by the class instructor, expressed as a percentage of the number of sessions attending by each group, was 54.5% for MS+EX and 55.5% for P+EX. 64% of the participants in the MS+EX and 58% in the P+EX groups met the 70% threshold for compliance. Assessment of the blinding, carried out via exit questionnaire found that 31.4% of the participants correctly identified which supplement they were taking during the study. Of the participants who correctly identified which supplement they were taking seven were taking the placebo and nine were taking the active supplement.**18**

Other Lifestyle Factors

A one-way ANOVA revealed no significant differences between the intervention groups at baseline for dietary calories, carbohydrate, protein, total fat, total omega-3 PUFA, EPA, DHA or physical activity levels as measured by the three day diet diary, seafood FFQ and CHAMPS questionnaire. Furthermore paired T-Tests revealed there were no significant differences between baseline and follow up within the groups for the aforementioned dietary factors, indicating that the participants did not make any significant changes to their diet over the course of the study. There was a significant difference in physical activity for both the P+EX group (p=0.03 CI95% 102, 2521) and MS+EX group (p=0.01 CI95% 235, 1201), confirming that participants were not replacing habitual activities with the exercise class. Two participants reported changing their medications during the course of the study. Both participants were assigned to the MS+EX group and discontinued the use of analgesics and non-steroidal anti-inflammatories, which were taken for pain due to arthritis in the hip and knee.

Follow up serum homocysteine samples were run on a separate ELISA plate to the baseline samples. Follow up results were found to be four fold higher across all samples. As previously mentioned inter-plate variability for bioassays is an established limitation of such methodology (Reed et al. 2002). For this reason, the follow-up results of the homocysteine samples have been excluded from both intention to treat and per-protocol analysis.

Primary outcome measure

There was a non-significant decrease of 0.8% in the mean habitual walking speed by the P group over the study; whereas, the other groups increased their mean walking speed by 0.05 m/s (0.07 m/s) (4.0%) for the MS group, 0.03 m/s (0.09 m/s) (2.5%) for the P+EX group, and 0.01 m/s (0.12 m/s) (0.8%) for the MS+EX group. However, these changes were not statistically significant for supplementation ($p = 0.25$), exercise ($p = 0.50$) or for the combined intervention ($p = 0.79$).

Mobility Outcomes

Table 15 shows the mobility outcomes at baseline and following the 24-week intervention along with the mean difference between the two measurement points. There were no significant differences between the intervention arms for mobility outcomes at baseline. There were no significant treatment effects for any of the intervention groups on any mobility outcome under the intention to treat analysis.

Table 14 Full fatty acid breakdown of participants at baseline and follow up divided by intervention group

	P n=10[a]			P + EX n=12			MS n=11[a]			MS + EX n=14		
Measure	Baseline	24 weeks	Mean Diff	Baseline	24 weeks	Mean Diff	Baseline	24 weeks	Mean Diff	Baseline	24 weeks	Mean Diff
16:0	33.72±10.6	34.04±8.8	0.32	32.11±7.3	33.40±5.1	1.29	30.77±1.9	35.02±12.9	4.25	31.37±6.4	34.36±8.8	2.99
16:1	2.05±0.8	3.03±1.9	0.98	2.28±1.3	2.67±0.8	0.39	2.68±0.8	3.05±1.3	0.37	2.70±0.6	3.00±1.9	0.30
18:0	14.61±4.1	15.03±5.4	0.42	13.94±2.3	14.79±2.3	0.85	16.36±3.9	14.33±3.4	-2.03	14.76±2.0	14.93±5.4	0.17
18:1	21.89±7.2	23.62±10.3	1.73	22.31±4.6	22.93±5.5	0.62	20.94±4.5	20.79±8.0	-0.15	22.20±3.1	20.47±10.3	-1.73
18:2 n-6	16.74±7.4	14.85±3.7	-1.89	16.43±4.6	16.47±2.1	0.4	18.46±4.0	17.29±4.1	-1.17	18.82±4.4	15.91±3.7	-2.91
18:3 n-6	0.33±0.1	0.33±0.2	0	0.30±0.1	0.30±0.2	0	0.33±0.1	0.33±0.2	0	0.43±0.3	0.33±0.2	-0.1
18:3 n-3	0.65±0.2	0.47±0.2	-0.18	0.61±0.2	0.28±0.2	-0.33	0.82±0.6	0.42±0.3	-0.4	0.64±0.3	0.52±0.2	-0.12
20:4 n-6	6.58±1.2	6.33±1.5	-0.25	6.7±2.0	6.9±1.1	0.2	7.11±3.4	5.10±1.2*	-2.01	6.91±2.0	5.1±1.4*	-1.81
20:5 n-3	0.37±0.2	0.38±0.2	0.01	0.43±0.2	0.42±0.3	-0.01	0.32±0.1	0.93±0.2*	0.61	0.35±0.2	0.84±0.4*	0.49
22:1	0.35±0.1	0.31±0.2	-0.4	0.22±0.1	0.30±0.2	0.08	0.37±0.2	0.42±0.2	0.5	0.32±0.2	0.40±0.2	0.08
22:6 n-3	1.71±0.4	1.61±0.4	-0.1	1.67±0.6	1.54±0.4	-0.13	1.84±0.6	2.32±0.5*	0.48	1.50±0.4	2.14±0.6*	0.64

* Indicates a significant change versus baseline, assessed using paired T-test or Wilcoxon signed-rank test where appropriate

[a] Two participants whole blood fatty acid values were excluded from analysis for P and MS groups due to artefacts on analysis

16:0 Palmitic acid, 16:1 Palmitoleic acid, 18:0 Stearic acid, 18:1 Oleic acid, 18:2 Linoleic acid, 18:3 n-6 γ- Linolenic acid, 18:3 n-3 α-Linolenic acid, 20:4 n-6 Arachidonic acid, 20:5 n-3 Eicosapentaenoic acid, 22:1 Erucic acid, 22:6 n-3 Docosahexaenoic acid

Abbreviations: Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX)

Table 15 Descriptive statistics for mobility outcomes of study participants divided into their respective intention to treat intervention groups. Mean values and standard deviations are reported at baseline and at 24 weeks.

Parameter	P (N = 12)		P + EX (N = 12)			MS (N = 13)			MS + EX (N =14)		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]
Fast Walking Speed (m/s)	1.8 (0.3)	1.7 (0.2)	1.6 (0.3)	1.6 (0.2)	0.041 (0.16)	1.6 (0.2)	1.6 (0.2)	0.055 (0.11)	1.7 (0.2)	1.7 (0.2)	0.026 (0.27)
Dual-task Gait Speed (m/s)	0.9 (0.3)	1.0 (0.2)	1.1 (0.2)	1.1 (0.2)	0.037 (0.19)	1.0 (0.3)	1.0 (0.3)	0.071 (0.07)	1.2 (0.3)	1.2 (0.3)	0.028 (0.25)
Dual-task Effect on Gait Speed (%)	-41.4 (43.5)	-28.5 (13.0)	-9.3 (15.4)	8.8 (21.0)	0.098 (0.04)[b]	-39.7 (75.6)	-36.8 (26.1)	0.082 (0.06)	-12.6 (24.8)	-8.8 (39.1)	0.078 (0.07)
Dual-task Stride Length Variability (%)	5.0 (2.6)	3.9 (2.5)	4.1 (1.5)	3.6 (1.4)	0.006 (0.58)	4.9 (2.7)	5.2 (2.3)	0.048 (0.13)	4.2 (2.1)	3.7 (1.9)	0.010 (0.49)
Five Times Sit to Stand (s)	11.8 (2.1)	11.7 (2.3)	13.9 (3.9)	13.0 (3.0)	0.001 (0.95)	11.9 (2.1)	11.9 (2.2)	0.001 (0.99)	13.9 (3.9)	12.3 (2.6)	0.057 (0.10)

[a] partial η^2 and p values are expressed for the comparison of each group versus the placebo assessed using a general linear model

[b] Result did not remain significant following Benjamini-Hochberg procedure

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

Cognitive Outcomes

Table 16 shows the cognitive scores at baseline and following the 24-week intervention along with the mean difference between the two measurement points. There was no significant difference between cognitive scores at baseline between the intervention arms. There was a significant correlation between changes in verbal memory and baseline NART score (r=0.370 p=0.01 CI95% [0.09, 0.62]), therefore NART score was included as a covariate in the subsequent analysis of treatment effects on verbal memory. There were no other significant correlations between other potential confounders including age, BMI or baseline homocysteine and DHA thus these variables were not included as covariates.

There was a significant effect on verbal memory compared with the P group, by the MS [$F(1,46) = 7.59$, $p = 0.008$, partial η^2 0.144)], P+EX [$F(1,46) = 7.70$, $p = 0.008$, partial η^2 0.144] and MS+EX interventions [$F(1,46) = 15.82$, $p < 0.001$, partial η^2 0.256], all with large effect sizes, Figure 33. Significant effects versus placebo were also observed for executive function for the MS [$F(1,47) = 8.02$, $p = 0.007$, partial η^2 0.146)], P+EX [$F(1,47) = 8.37$, $p = 0.006$, partial η^2 0.151)], and MS+EX groups [$F(1,47) = 8.60$, $p = 0.005$, partial η^2 0.155)] all with large effect sizes, Figure 34. Interpretation of standardised effect size using partial η^2 was based upon definitions set out by Cohen, 2013 with small equating to partial η^2 value of 0.1, medium 0.6 and large 0.14.

Table 16 Descriptive statistics for cognitive outcomes of study participants divided into their respective intention to treat intervention groups. Mean values and standard deviations are reported at baseline and at 24 weeks.

Parameter	P (N = 12)		P + EX (N = 12)			MS (N = 13)			MS + EX (N =14)		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]
Verbal Memory (%)	58 (11)	54 (16)	61 (14)	67 (13)	0.144 (0.008)*	54 (13.0)	60 (13)	0.144 (0.008)*	58 (13)	67 (15)	0.256 (<0.001)*
Spatial Memory (%)	75 (7)	75 (6)	77 (5)	75 (6)	0.012 (0.46)	77 (7)	73 (7)	0.044 (0.15)	75 (7)	72 (10)	0.027 (0.26)
Executive Function (Correct Connections)	80 (18)	75 (19)	68 (21)	76 (23)	0.151 (0.006)*	60 (15)	67 (21)	0.146 (0.007)*	68 (18)	75 (15)	0.155 (0.005)*
Interference Control (ms)	159 (90)	152 (77)	149 (96)	118 (104)	0.010 (0.50)	210 (106)	189 (104)	0.004 (0.67)	205 (198)	169 (148)	0.015 (0.39)

* Indicates a significant effect of the intervention on changes on the specific outcome

[a] partial η^2 and p values are expressed for the comparison of each group versus the placebo assessed using a general linear model

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

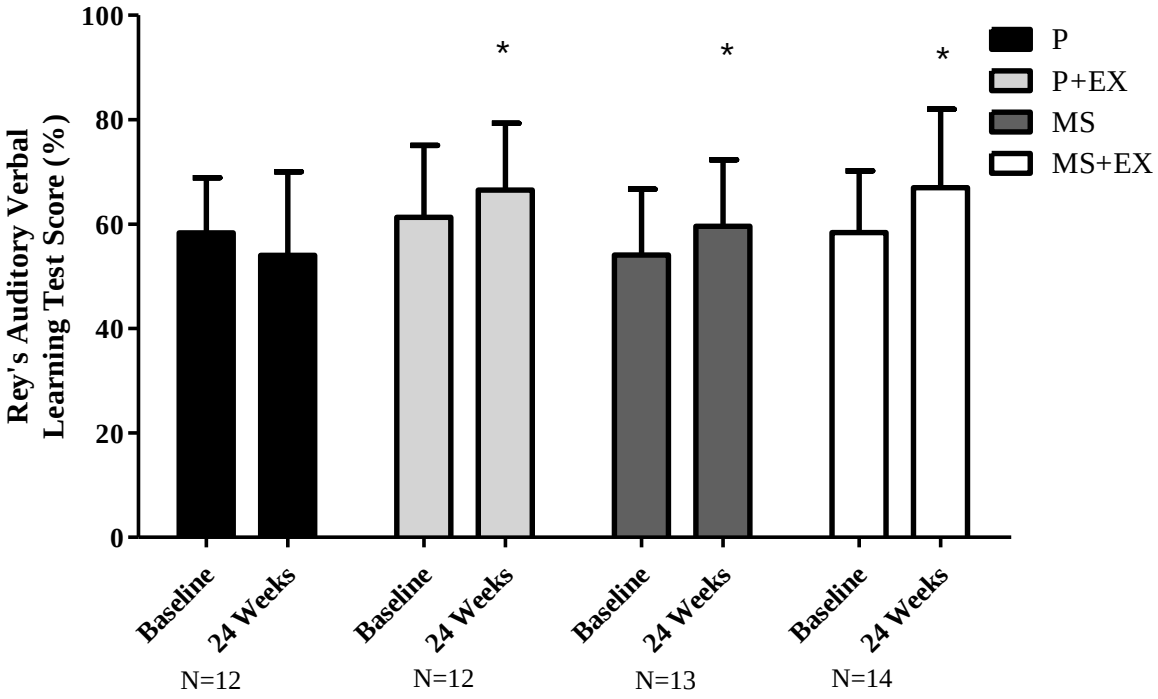


Figure 33 *Effects of multi-nutrient supplement and exercise by general linear model on Rey's auditory verbal learning test. Significant effects for multi-nutrient supplement (p = 0.008) placebo supplement and exercise (p = 0.008) and combination of multi-nutrient and exercise (p < 0.001). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in verbal memory versus placebo.*

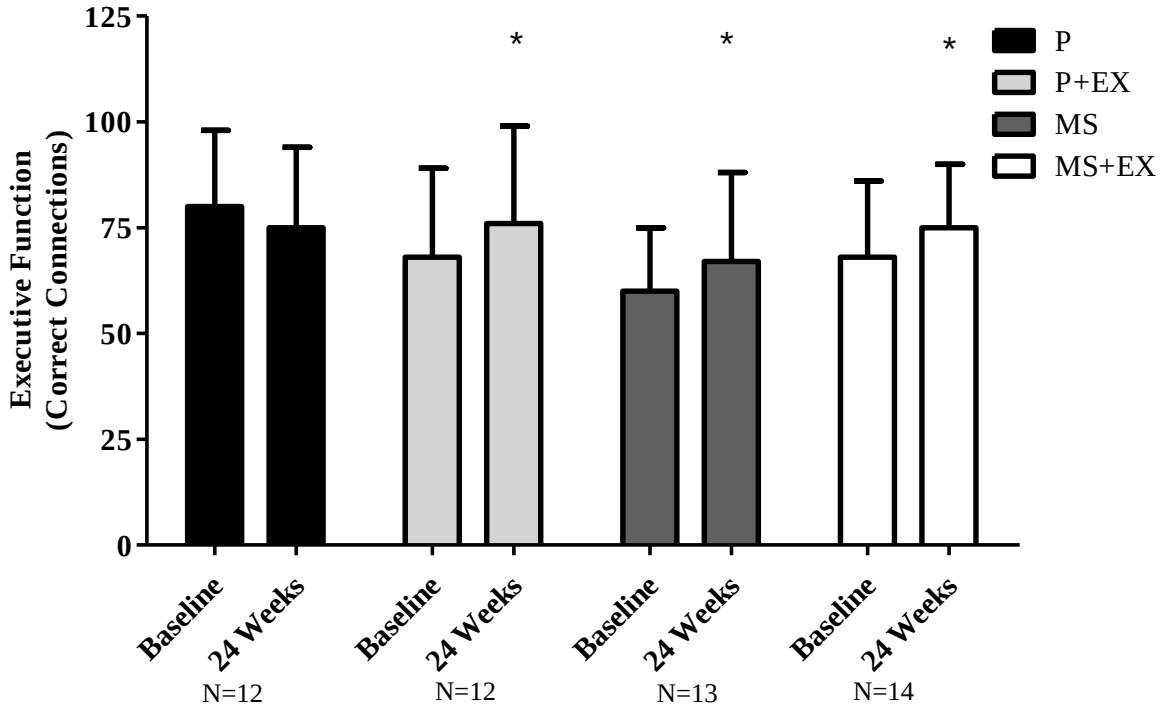


Figure 34 *Effects of multi-nutrient supplement and exercise by general linear model on executive function. Significant effects for multi-nutrient supplement (p = 0.007) placebo supplement and exercise (p = 0.006) and combination of multi-nutrient and exercise (p = 0.005). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in executive function versus placebo.*

SF-36 Questionnaire Outcomes

Table 17 shows the analysed SF-36 questionnaire outcomes at baseline and following the 24-week intervention along with the mean difference between the two measurement points. There were no significant differences between the intervention arms for these outcomes at baseline. The MS+EX group reported significant improvements in emotional wellbeing, compared to the P group [F(1,47) = 8.07, p = 0.03, partial η^2 0.146], with a large effect size, Figure 35. No other treatment effects were identified.

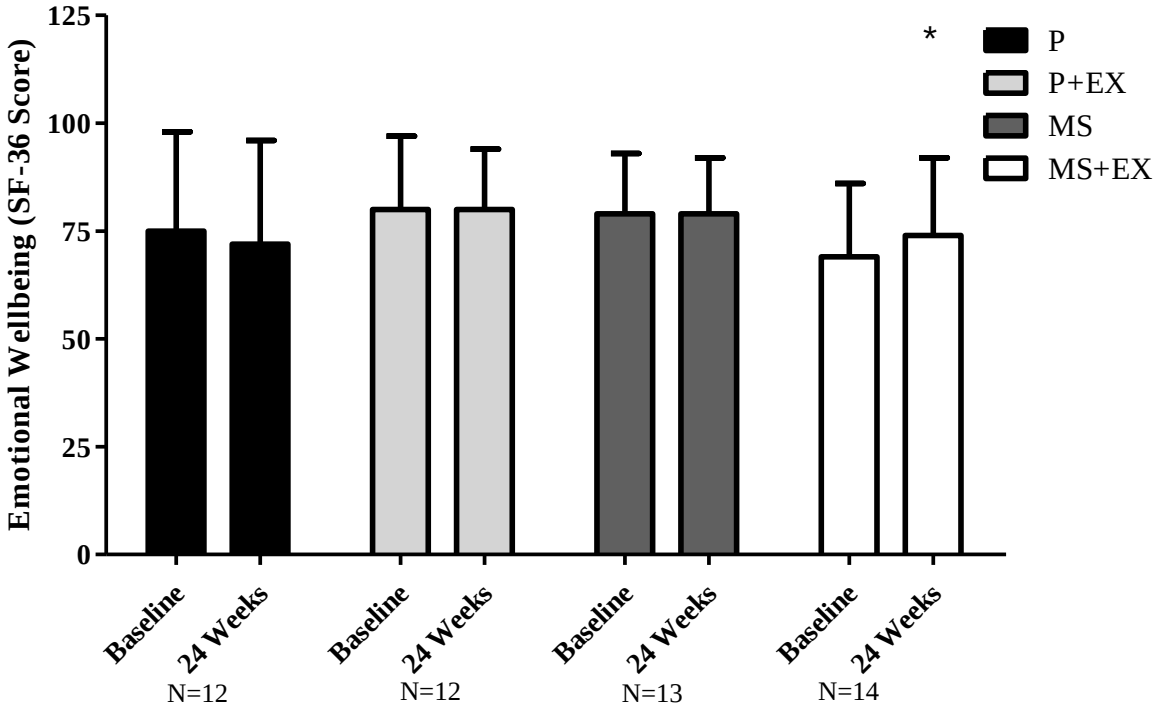


Figure 35 *Effects of multi-nutrient supplement and exercise by general linear model on self-reported emotional wellbeing. Significant effects were observed for the MS+EX group (p = 0.03). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in executive function versus placebo*

Table 17 Descriptive statistics for Short Form-36 questionnaire outcomes of study participants divided into their respective intention to treat intervention groups. Mean values and standard deviations are reported at baseline and at 24 weeks.

Parameter	**P (N = 12)**		**P + EX (N = 12)**			**MS (N = 13)**			**MS + EX (N =14)**		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (*p* value)[a]	Baseline	24 weeks	partial η^2 (*p* value)[a]	Baseline	24 weeks	partial η^2 (*p* value)[a]
Physical Function	79 (24)	78 (26)	75 (21)	79 (19)	0.029 (0.25)	82 (15)	83 (16)	0.002 (0.78)	75 (26)	77 (22)	0.006 (0.58)
Bodily Pain	75 (24)	73 (25)	75 (22)	80 (13)	0.041 (0.16)	74 (19)	77 (17)	0.019 (0.35)	66 (21)	66 (16)	0.001 (0.80)
Emotional Wellbeing	75 (23)	72 (24)	82 (17)	80 (14)	0.003 (0.71)	79 (14)	79 (13)	0.026 (0.26)	69 (18)	74 (17)	0.146 (0.007)*
Energy / Fatigue	57 (25)	55 (25)	65 (26)	67 (24)	0.016 (0.39)	62 (22)	63 (19)	0.006 (0.59)	58 (25)	58 (24)	0.003 (0.70)
General Health	66 (23)	70 (23)	75 (18)	76 (18)	0.009 (0.51)	66 (16)	66 (19)	0.018 (0.36)	62 (24)	62 (24)	0.028 (0.25)

* Indicates a significant effect of the intervention on changes on the specific outcome

[a] partial η^2 and *p* values are expressed for the comparison of each group versus the placebo assessed using a general linear model

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

5.3.2 Per-Protocol Analysis

Descriptive, Outliers and Transformations

After examination of Shapiro-Wilk results and Q-Q plots fast walking speed in the MS+EX group (p=0.003 skew: 2.27 kurtosis: 5.41) and DTE on gait speed (p<0.001 skew: 2.79 kurtosis: 8.83) in the P group were not normally distributed. From the SF-36 questionnaire physical function (p=0.005 skew:-0.41 kurtosis: 4.91) energy (p=0.002 skew:-0.32 kurtosis:2.68), bodily pain (p=0.003 skew:-1.04 kurtosis: 1.53), emotional wellbeing (p=0.028 skew: -1.67 kurtosis: 2.55) and general health perceptions (p=0.001 skew: 1.47 kurtosis: 0.96) in the P group and physical function in the P+EX group (p=0.005 skew:1.24 kurtosis: 0.38) were not normally distributed. Similarly to the intention to treat analysis, no transformations were used on these variables due to the robustness of the general linear model.

Participants

For per-protocol analysis participants were allocated based on their adherence to the interventions, anyone who started an intervention but discontinued were excluded from the analysis. Group sizes were as follows MS+EX n=9, P+EX n=7, MS n=12 and P n=14. In addition, some data were excluded from this analysis, one participant from the P group reported visiting a pain clinic and that this had influenced her walking, thus her mobility and SF-36 data were excluded. Two participants from the MS+EX group discontinued the use of analgesics and non-steroidal anti-inflammatory drugs for arthritis, therefore, their mobility and SF-36 questionnaire data were also excluded. Finally a participant from the P group had researched the RAVLT words and revised, thus her follow up score would not have been a fair reflection of her verbal memory, so this result was excluded. Table 18 provides a summary of the sample characteristics broken down by intervention group allocation. There were no significant differences between intervention arms for any demographic outcome.

Other Lifestyle Factors

A one-way ANOVA revealed no significant differences between the intervention groups at baseline for dietary calories, carbohydrate, protein, total fat, total omega-3 PUFA, EPA, DHA or physical activity levels as measured by the three day diet diary, seafood FFQ and CHAMPS questionnaire. Furthermore paired T-Tests revealed there were no significant differences between baseline and follow within the groups for the aforementioned dietary factors. There were significant difference in baseline versus 24 weeks values for self-reported physical activity

for both the P+EX group (p<0.001) and MS+EX group (p=0.001), confirming that participants were not replacing habitual activities with the exercise class.

Table 18 Descriptive statistics for demographic information of study participants divided into their respective per-protocol intervention groups. Mean values and standard deviations are reported for normally distributed outcomes and median and interquartile range for non-normally distributed outcomes.

Parameter	P (N = 14)	P + EX (N = 7)	MS (N = 12)	MS + EX (N = 9)
Age (years)	66 (4)	68 (3)	69 (5)	67 (5)
Height (m)	1.64 (0.07)	1.62 (0.05)	1.63 (0.06)	1.62 (0.08)
Weight (kg)	75.6 (20.2)	72.1 (21.1)	74.1 (13.5)	69.6 (10.9)*
BMI (kg/m^2)	25.9 (9.6)*	26.7 (4.1)	27.7 (5.4)	27.9 (6.4)
Comorbidities	Hypertension (3) Hypercholesterolemia (1) Osteoarthritis (2)	Hypertension (2) Osteoarthritis (1)	Hypertension (2) Hypercholesterolemia (1) Osteoporosis (1)	Hypertension (2) Hypercholesterolemia (1) Diabetes (1) Osteoarthritis (2)
NART Score	35 (5)	40 (5)	36 (5)	38 (7)
MMSE score	30 (0)*	30 (1)*	29.5 (1)*	30 (0.5)*
Hand Grip Strength (kg)	20.5 (3.1)	21.3 (7.8)	19.5 (5.4)	19.9 (4.4)
PASE Score	136 (52)	105 (11)	125 (41)	112 (35)

*Indicates value is the median (IQR), otherwise means (SD) presented.

Abbreviations:: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX), Mini Mental Sate Exam (MMSE), National Adult Reading Test (NART), Physical Activity Scale in the Elderly (PASE)

Primary outcome measure

There was a significant effect on the primary outcome of HGS compared to the P group, by the MS+EX [F(1,38) = 6.55, p = 0.015, partial η^2 0.158)] and P+EX interventions [F(1,38) = 7.53, p = 0.01, partial η^2 0.177], all with large effect sizes, Figure 36. A decline in HGS of 0.04 m/s was observed for the placebo group, whereas all of the active intervention groups experienced an increase with improvements of 0.03 m/s, 0.07 m/s and 0.06 m/s for MS, P+EX and MS+EX groups respectively. The improvements made by the MS+EX [F(1,38) = 6.55, p = 0.015, partial η^2 0.158)]and P+EX were statistically significant versus the placebo [F(1,38) = 7.53, p = 0.01, partial η^2 0.177], both with large effect sizes, Figure 37.

Mobility Outcomes

Table 19 shows the secondary mobility outcomes at baseline and following the 24-week intervention for each of the per-protocol groups. There were no significant differences between the intervention arms for these outcomes at baseline.

Significant effects were observed versus the placebo on fast walking speed for the MS+EX [F(1,38) = 8.55, *p* = 0.006, partial η^2 0.196)] and P+EX interventions [F(1,38) = 10.85, *p* = 0.002, partial η^2 0.237)], all with large effect sizes, Figure 38. Finally there was a significant effect of the MS+EX intervention versus the P group on FTSTS performance [F(1,37) =5.23, *p* = 0.029, partial η^2 0.133)], with a large effect size, *Figure 39*. Figure 40, Figure 41 and Figure 42 show how the results for the mobility outcomes, where treatment effects were observed, differ between the intention to treat and per-protocol analyses.

Table 19 Descriptive statistics for mobility outcomes of study participants divided into their respective per-protocol intervention groups. Mean values and standard deviations are reported at baseline and at 24 weeks.

Parameter	**P (N = 13)[b]**		**P + EX (N = 7)**			**MS (N = 12)**			**MS + EX (N = 7)[c]**		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]
Fast Walking Speed (m/s)	1.77 (0.29)	1.62 (0.15)	1.54 (0.26)	1.61 (0.25)	0.237 (0.002)*	1.65 (0.21)	1.61 (0.20)	0.101 (0.055)	1.67 (0.15)	1.71 (0.15)	0.196 (0.006)*
Dual-task Gait Speed (m/s)	0.98 (0.31)	0.97(0.24)	1.01 (0.18)	1.16 (0.19)	0.057 (0.15)	1.03 (0.32)	1.01 (0.32)	0.002 (0.80)	1.13 (0.29)	1.22 (0.24)	0.015 (0.47)
Dual-task Effect on Gait Speed (%)	-44.3 (61.4)	-30.8 (35.2)	-25.0 (15.9)	-16.3 (12.0)	0.003 (0.76)	-41.4 (78)	-40.6 (75.2)	0.025 (0.35)	-17.3 (28.8)	-12.0 (22.3)	0.008 (0.61)
Dual-task Stride Length Variability (%)	4.8 (2.1)	4.6 (1.26)	4.3 (1.2)	3.4 (0.78)	0.016 (0.46)	4.9 (2.8)	5.2 (2.0)	0.013 (0.50)	4.3 (2.4)	3.9 (2.2)	0.001 (0.84)
Five Times Sit to Stand (s)	12.0 (2.0)	12.5 (2.7)	12.7 (1.9)	12.3 (3.4)	0.001 (0.92)	10.2 (3.6)	10.6 (4.1)	0.004 (0.70)	14.7 (4.2)	12.6 (3.4)	0.133 (0.029)*

[*] Indicates a significant effect of the intervention on changes on the specific outcome

[a] partial η^2 and p values are expressed for the comparison of each group versus the placebo assessed using a general linear model

[b] One participant excluded from placebo group due to attending a pain clinic

[c] Two participants excluded due to discontinuing their use of analgesic and anti-inflammatory medications

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

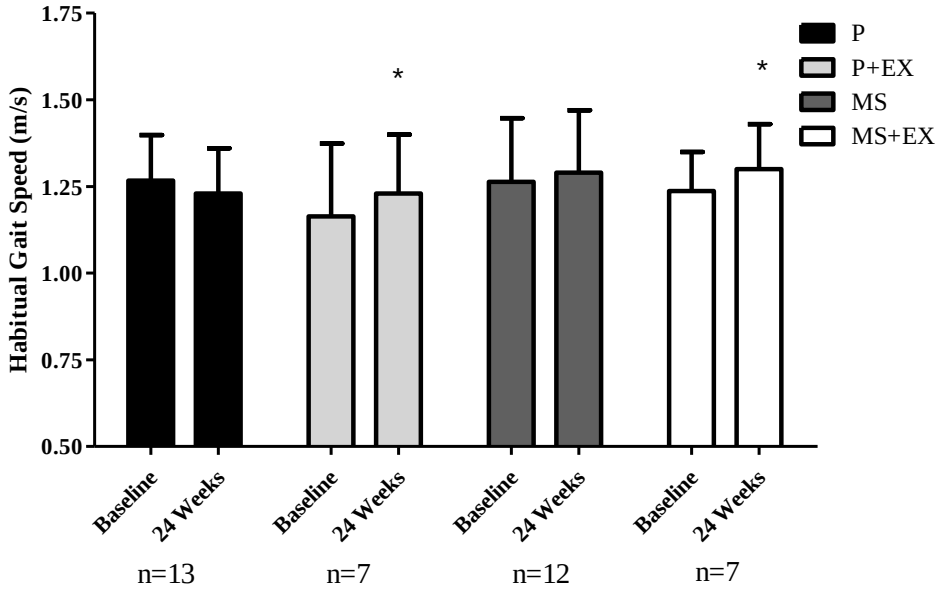


Figure 36 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on habitual gait speed. Significant effects for placebo supplement and exercise (p = 0.01) and combination of multi-nutrient and exercise (p = 0.015). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in habitual gait speed versus placebo.*

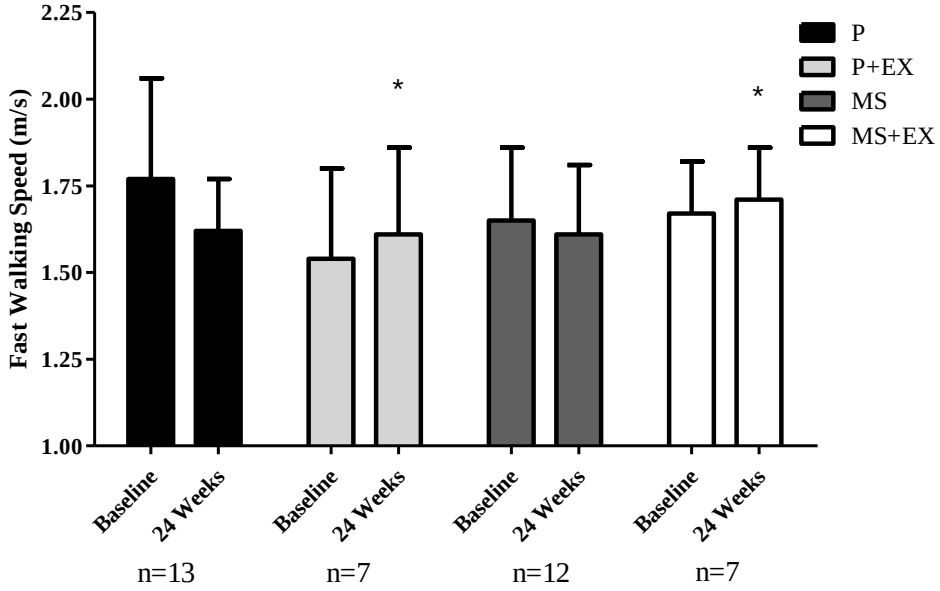


Figure 37 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on fast walking speed. Significant effects for placebo supplement and exercise (p = 0.006) and combination of multi-nutrient and exercise (p = 0.002). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in habitual gait speed versus placebo.*

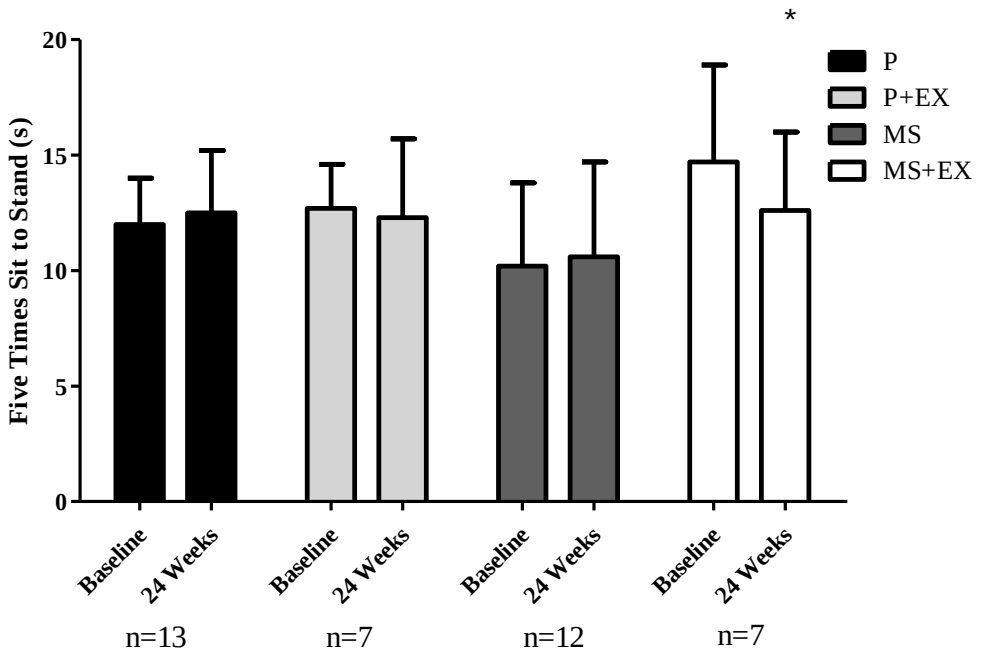


Figure 38 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on five times sit to stand. A significant effect for the combination of the multi-nutrient supplement and exercise (p = 0.029) was observed. Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in habitual gait speed versus placebo*

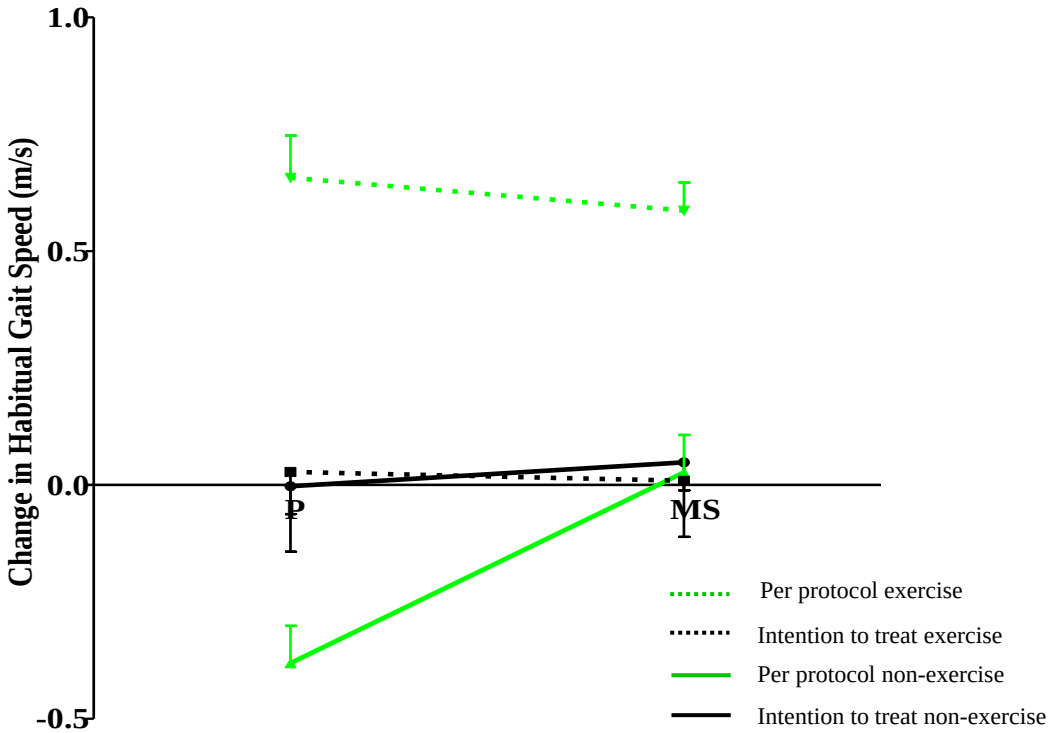


Figure 39 *Intention to treat and per-protocol analyses for changes in habitual gait speed. No significant effects of any of the interventions were shown in the intention to treat analysis, however, in the per-protocol analysis both the MS+EX (p = 0.015) and P+EX (p = 0.01) groups showed significant improvements versus the placebo. Placebo (P) Multi-nutrient supplement (MS).*

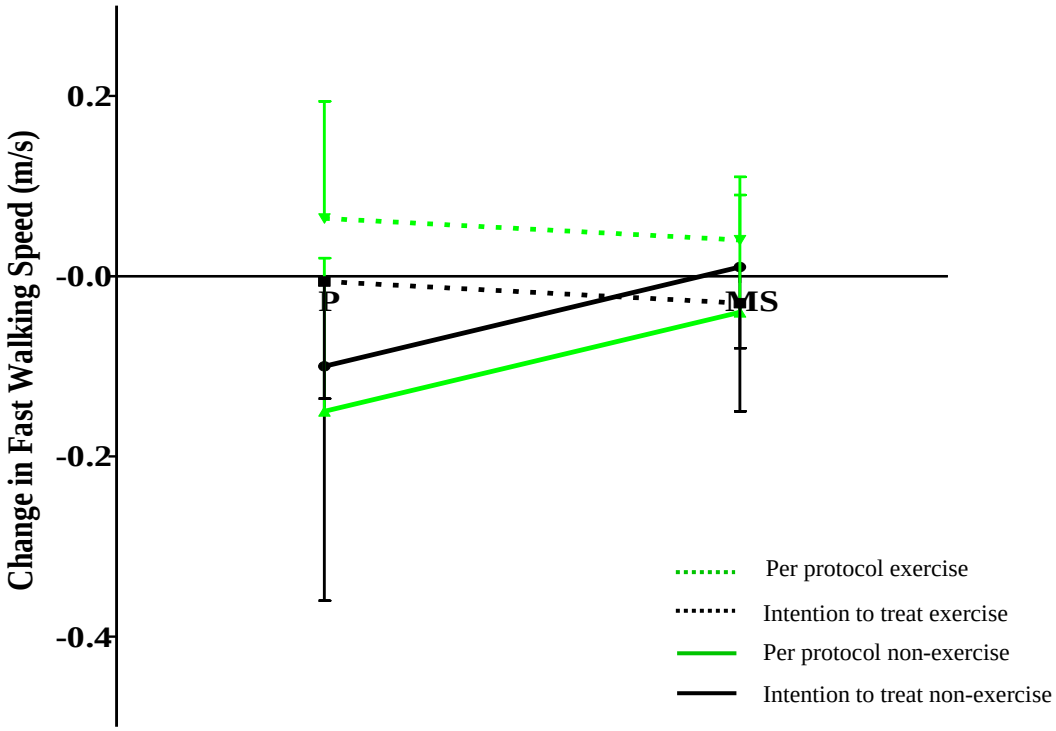


Figure 40 *Intention to treat and per-protocol analyses for changes in fast walking speed. No significant effects of any of the interventions were shown in the intention to treat analysis, however, in the per-protocol analysis both the MS+EX (p = 0.006) and P+EX (p = 0.002) groups showed significant improvements versus the placebo. Placebo (P) Multi-nutrient supplement (MS).*

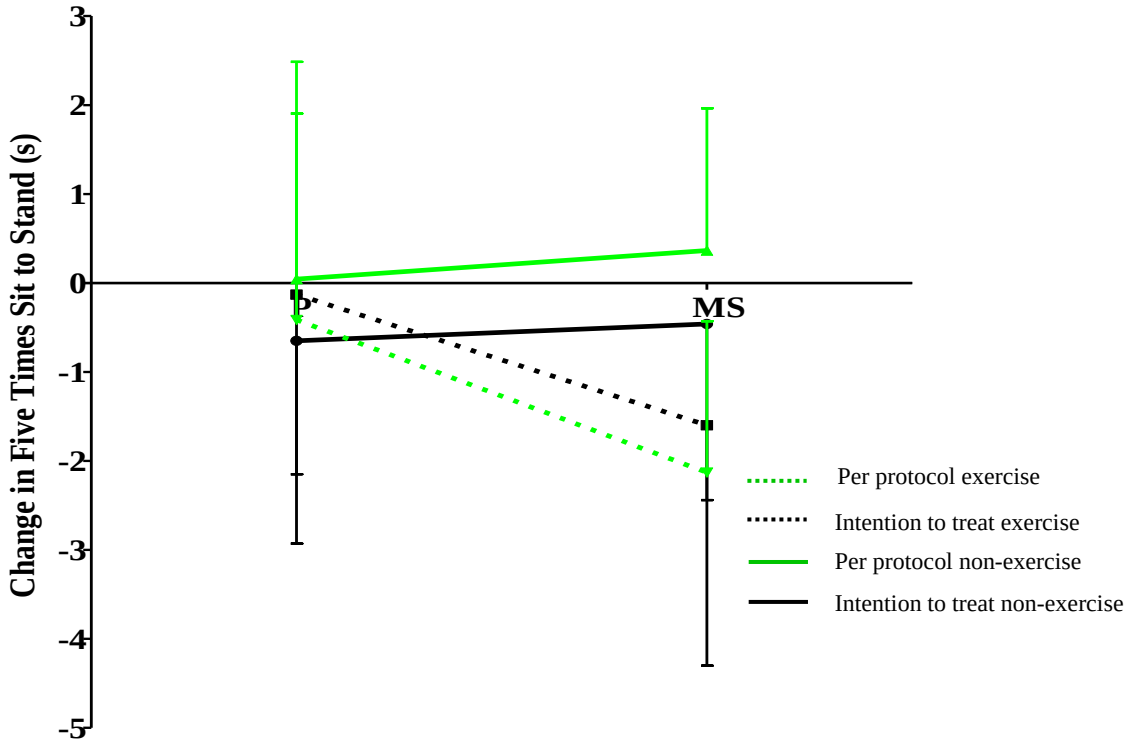


Figure 41 *Intention to treat and per-protocol analyses for changes in five times sit to stand. No significant effects of any of the interventions were shown in the intention to treat analysis, however, in the per-protocol analysis the MS+EX ($p = 0.026$) group showed significant improvements versus the placebo. Placebo (P) Multi-nutrient supplement (MS).*

Cognitive Outcomes

Table 20 shows the cognitive scores at baseline and following the 24-week intervention for each per-protocol group. There were no significant differences between cognitive scores at baseline between the intervention arms.

Significant effects were observed versus the placebo on verbal memory for the MS+EX [$F(1,40) = 34.26$, $p < 0.001$, partial η^2 0.495)] MS [$F(1,40) = 21.51$, $p < 0.001$, partial η^2 0.381)] and P+EX interventions [$F(1,40) = 20.05$, $p < 0.001$, partial η^2 0.364)], all with large effect sizes, Figure 42. Significant treatment effects were also observed versus the placebo on executive function for the MS+EX [$F(1,41) = 8.39$, $p = 0.006$, partial η^2 0.181)] MS [$F(1,41) = 5.48$, $p = 0.024$, partial η^2 0.126)] and P+EX interventions [$F(1,41) = 6.63$, $p = 0.014$, partial η^2 0.181)], all with large effect sizes, Figure 43. No other treatment effects were observed on cognitive outcomes. Figure 44 and *Figure 45* show how the results for the cognitive outcomes, where treatment effects were detected, differed between the intention to treat and per-protocol analyses.

Table 20 Descriptive statistics for cognitive outcomes of study participants divided into their respective per-protocol intervention groups. Mean values and standard deviations are reported at baseline and at 24.

Parameter	**P (N = 13)[b]**		**P + EX (N = 7)**			**MS (N = 12)**			**MS + EX (N =9)**		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]
Verbal Memory (%)	58 (11)	53 (12)	58 (11)	67 (11)	0.364 (<0.001)*	55 (13)	63 (14)	0.381 (<0.001)*	58 (12)	70 (10)	0.495 (<0.001)*
Spatial Memory (%)	75 (6)	74 (6)	75 (4)	73 (6)	0.003 (0.73)	76 (6)	72 (7)	0.028 (0.30)	76 (8)	71 (11)	0.058 (0.14)
Executive Function (Correct Connections)	78 (18)	76 (18)	66 (23)	77 (27)	0.149 (0.014)*	61 (13)	69 (20)	0.126 (0.024)*	64 (18)	75 (14)	0.181 (0.006)*
Interference Control (ms)	147 (86)	155 (68)	178 (114)	110 (73)	0.080 (0.07)	210 (112)	180 (104)	0.029 (0.29)	254 (234)	184 (176)	0.096 (0.05)

* Indicates a significant effect of the intervention on changes on the specific outcome

[a] partial η^2 and p values are expressed for the comparison of each group versus the placebo assessed using a general linear model

[b] One participant excluded as they had practiced verbal memory test at home

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

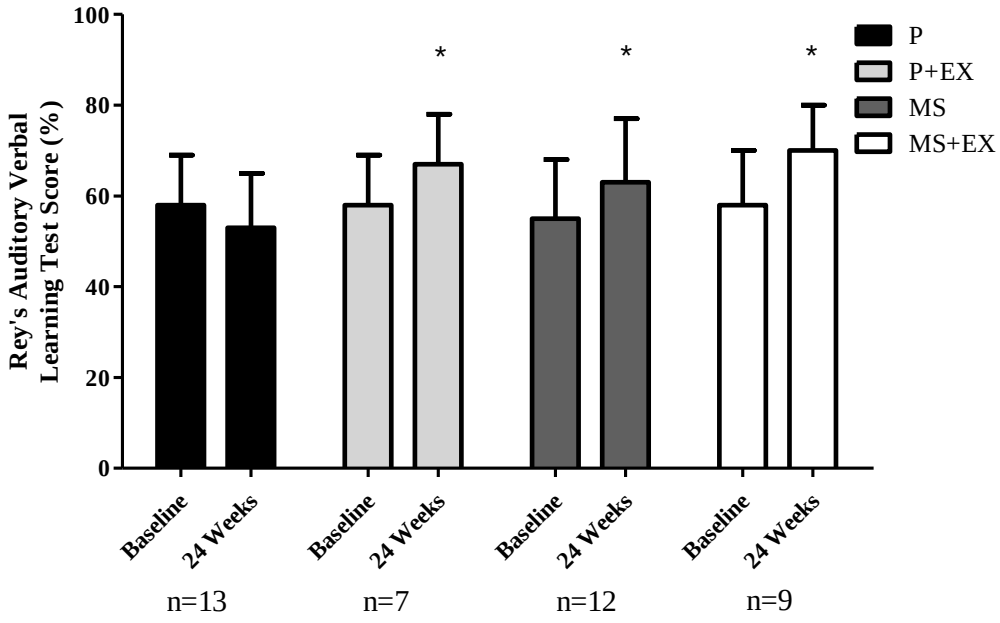


Figure 42 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on Rey's auditory verbal learning test. Significant effects for multi-nutrient supplement ($p < 0.001$) placebo supplement and exercise ($p < 0.001$) and combination of multi-nutrient and exercise ($p < 0.001$). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in verbal memory versus placebo.*

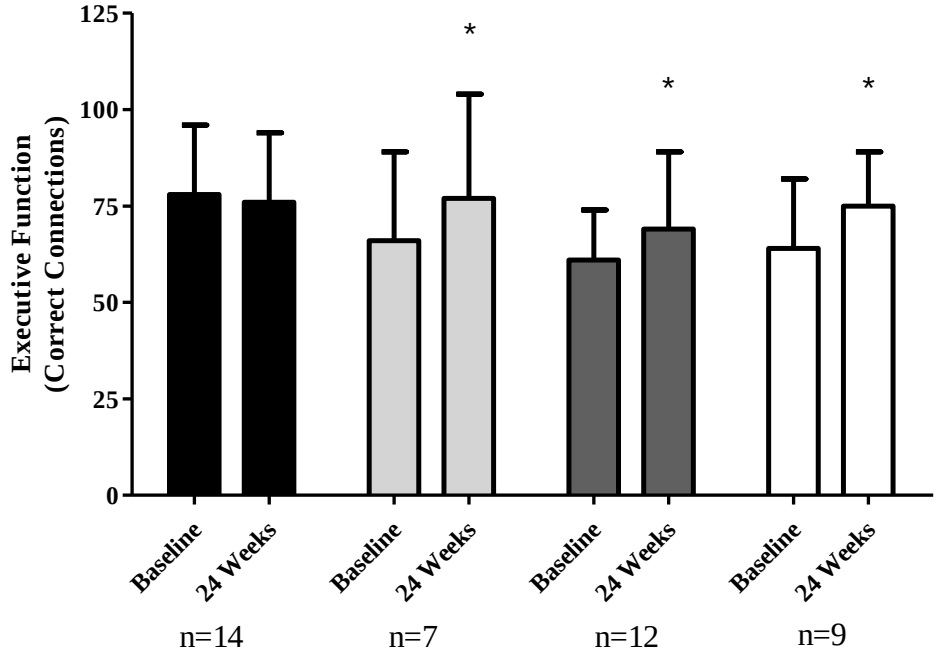


Figure 43 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on executive function. Significant effects for multi-nutrient supplement (p = 0.024) placebo supplement and exercise (p = 0.014) and combination of multi-nutrient and exercise (p = 0.006). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in executive function versus placebo.*

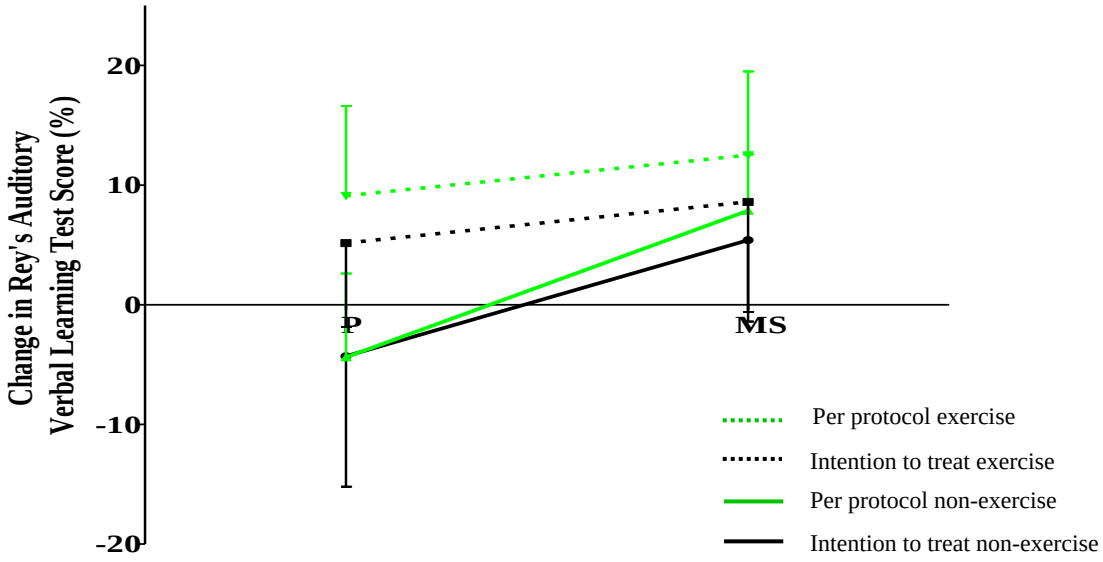


Figure 44 *Intention to treat and per-protocol analyses for changes in verbal memory. The multi-nutrient supplement alone, placebo supplement and exercise and the combination of multi-nutrient supplement and exercise were shown to have significant treatment effects in the intention to treat and per-protocol analyses. Placebo (P) Multi-nutrient supplement (MS).*

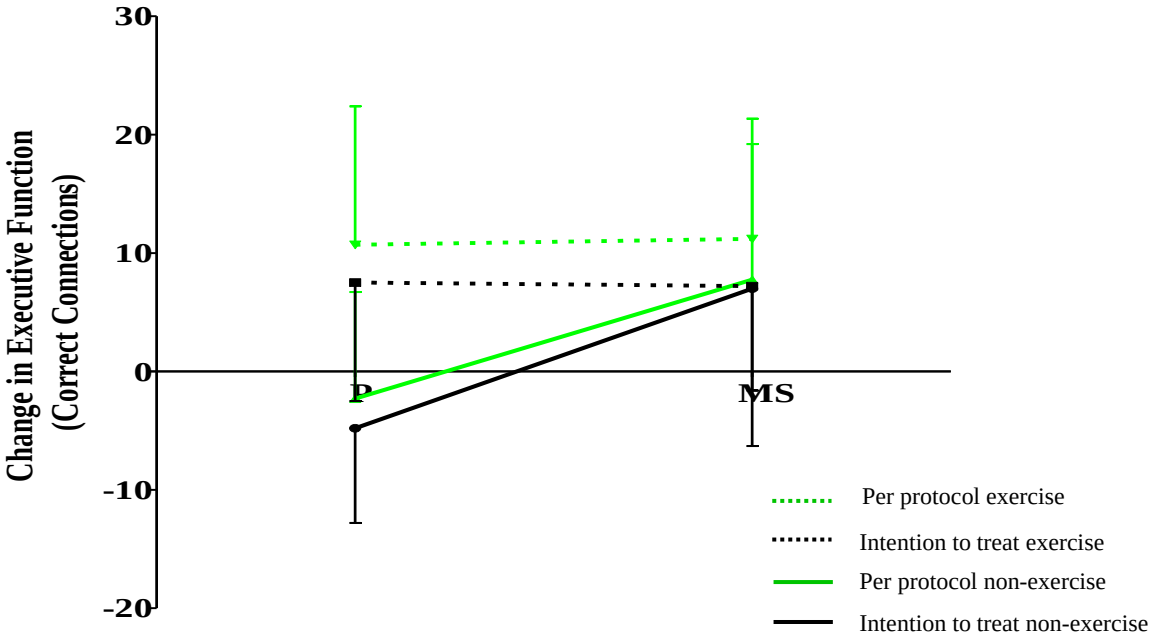


Figure 45 *Intention to treat and per-protocol analyses for changes in executive function. The multi-nutrient supplement alone, placebo supplement and exercise and the combination of multi-nutrient supplement and exercise were shown to have significant treatment effects in the intention to treat and per-protocol analyses. Placebo (P) Multi-nutrient supplement (MS).*

SF-36 Questionnaire Outcomes

Table 21 shows the analysed SF-36 questionnaire outcomes at baseline and following the 24-week intervention for each per protocol group. There were no significant differences between the intervention arms for these outcomes at baseline.

Significant treatment effects versus placebo were observed for the MS+EX group on emotional wellbeing with both exercise [$F(1,41) = 12.47$, $p = 0.001$, partial η^2 0.252)] , with a large effect size, Figure 46. Figure 47 shows a comparison of the intention to treat and per-protocol analyses for emotional wellbeing.

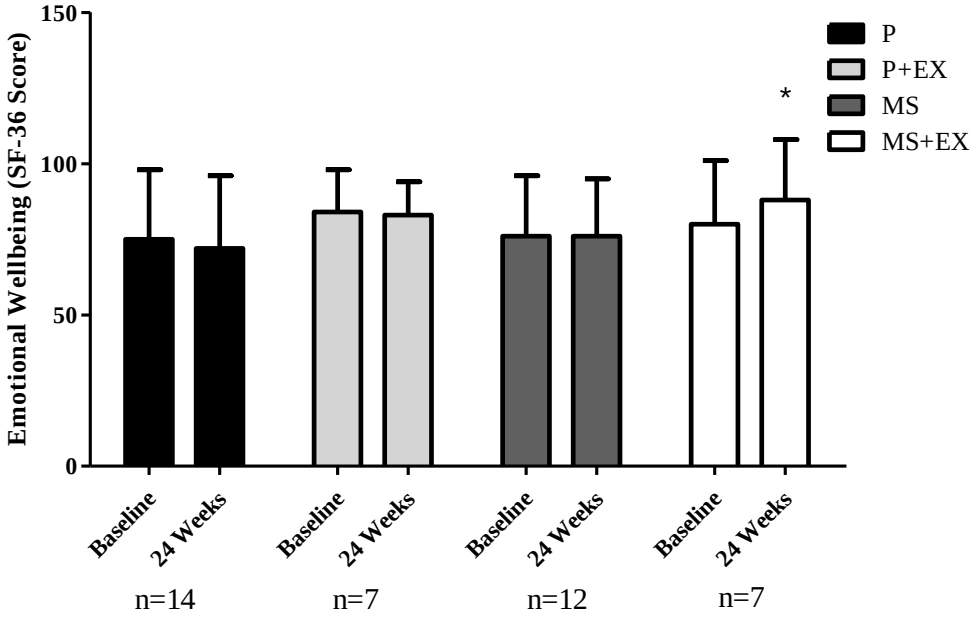


Figure 46 *Per-protocol intervention effects of multi-nutrient supplement and exercise by general linear model on self-reported emotional wellbeing. Significant effects were observed for the MS+EX group (p = 0.001). Placebo (P), Placebo supplement and exercise (P+EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS+EX).*

** Indicates a significant effect of the intervention on changes in executive function versus placebo*

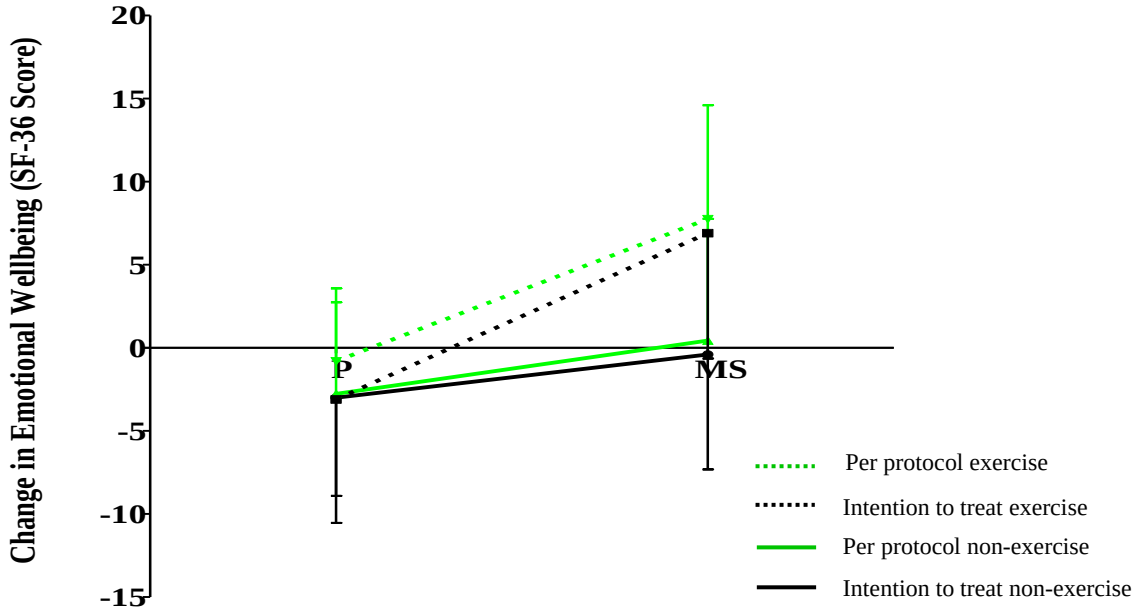


Figure 47 *Intention to treat and per-protocol analyses for changes in emotional wellbeing. There were significant effects of the multi-nutrient supplement in both the intention to treat ($p=0.016$ partial $\eta^2=0.150$) and per-protocol ($p=0.023$ partial $\eta^2=0.176$) analyses. Placebo (P) Multi-nutrient supplement (MS).*

Table 21 Descriptive statistics for Short Form-36 questionnaire outcomes of study participants divided into their respective per-protocol intervention groups. Mean values and standard deviations are reported at baseline and at 24 weeks.

Parameter	**P (N = 14)**		**P + EX (N = 7)**			**MS (N = 12)**			**MS + EX (N =7)**		
	Baseline	24 weeks	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]	Baseline	24 weeks	partial η^2 (p value)[a]
Physical Function	79 (22)	79 (23)	79 (22)	84 (18)	0.044 (0.20)	82 (15)	83 (16)	0.003 (0.75)	78 (27)	79 (17)	0.005 (0.67)
Bodily Pain	76 (23)	73 (23)	71 (27)	74 (26)	0.019 (0.41)	73 (18)	78 (16)	0.024 (0.35)	63 (24)	61 (19)	0.001 (0.928)
Emotional Wellbeing	77 (22)	74 (23)	82 (15)	82 (11)	0.023 (0.36)	74 (20)	75 (19)	0.040 (0.22)	71 (16)	77 (14)	0.242 (0.001)*
Energy / Fatigue	60 (23)	59 (22)	60 (34)	64 (31)	0.021 (0.37)	58 (24)	59 (23)	0.007 (0.60)	63 (24)	63 (22)	0.002 (0.81)
General Health	69 (21)	72 (20)	73 (22)	73 (23)	0.028 (0.31)	64 (18)	62 (20)	0.028 (0.31)	70 (16)	69 (14)	0.053 (0.16)

* Indicates a significant effect of the intervention on changes on the specific outcome

[a] partial η^2 and p values are expressed for the comparison of each group versus the placebo assessed using a general linear model

Abbreviations: Placebo (P), Placebo supplement and exercise (P + EX), multi-nutrient supplement only (MS), multi-nutrient supplement and exercise (MS + EX)

5.4 Discussion

This study explored for the first time the effects of a high DHA multi-nutrient supplement and aerobic exercise on mobility and cognition in older women. Although the supplementation led to significant increases in DHA levels, there were no significant improvements in the primary outcome measure of habitual walking speed. However, significant improvements in verbal memory and executive function were observed for both the multi-nutrient supplement and exercise interventions alone and in combination, with improvements in emotional wellbeing occurring in the MS+EX group only. Furthermore, per-protocol analysis revealed benefits of aerobic exercise on habitual and fast walking gait speed and the combined multi-nutrient supplement and aerobic exercise lead to improved time on the five times sit to stand test. Memory and executive function have been identified as two important cognitive domains for older adults, as declining performance in both have been linked to a transition from a healthy to a pathological ageing trajectory (Rajan et al. 2015; Mortamais et al. 2017). Preservation of these domains therefore supports the role of the multi-nutrient formula as well as regular exercise to preserve cognition in the older adult and possibly reduce the burden of age related disease. Combining the multi-nutrient supplement with aerobic exercise resulted in positive effects on emotional wellbeing and ability to rise from a chair, which were not observed for each intervention separately, offering the intriguing prospect that the combination of a high DHA multi-nutrient supplement and exercise may have a broader impact across multiple healthy ageing outcomes. Given that age related decline occurs across mobility and cognition, and the evidence that these two aspects of health are closely linked, the ability to act across multiple health domains is promising, as it could promote an overall healthy trajectory allowing older adults to maintain their independence late into old age.

Mobility

The effects of omega-3 PUFA supplementation on gait speed in older females has previously shown promise in RCTs when administered on its own (Hutchins-Wiese et al. 2013) and as part of the same multi-nutrient formula as used in the present study (Strike et al. 2016). It would be expected that nutritional intervention could lead to preservation of function rather than producing a clinically meaningful increase (≥0.06 m/s) in gait speed, thereby leading to a healthy ageing trajectory. Indeed, the improvements within this previous work were driven by both improvements in HGS in the treatment group and decreases in the placebo group. In the present study there were increases in HGS following the multi-nutrient supplementation of a similar magnitude to our previous study; however, the decline in the placebo group was less than predicted, with a mean decline of 0.8%, as opposed to 2% in our previous study (Alcock et al. 2013), which may reflect the previously outlined high functioning nature of the participants. Interestingly, the effect size for the combined supplementation and exercise group was modest

in comparison to each intervention on their own. This may be partly explained by the aforementioned participants who discontinued daily use of analgesic and anti-inflammatory medications for joint pain who both observed small declines in gait speed of 0.01 m/s.

The concept of a preservation of mobility over time in response to improved nutrition is further supported by observational research showing that despite an overall decrease in gait speed over 8 years, adherence to a healthy Mediterranean dietary pattern was associated with 0.04 m/s greater gait speed in 2225 healthy older adults versus lower adherence (Shahar et al. 2012). Rate of decline in gait speed in healthy older adults has been shown to vary between 0.02-0.03 m/s per year (White et al. 2013a). Based on this evidence it would appear that the participants in our previous study, as well as in the work by Hutchins-Wiese et al. (2013), experienced an above average decline in HGS over a 24 week period. This decline in gait speed may have contributed to the ability to detect a significant difference between the active and placebo groups. Combining the results from this study and our previous work it would appear that a decline in gait speed of 0.03m/s may be required to be able to detect a change in gait speed following supplementation. Although this rate of decline has been observed following a 24 week intervention, extending the intervention period to a minimum of 48 weeks may be required to better fall in line with the expected rate of gait speed attrition (White et al. 2013b).

Results from our previous analysis showed a positive relationship between DHA and dual-task gait speed and DTE on gait speed, indicating that DHA may affect these outcomes. Results from this intervention trial showed that supplementation with a high DHA multi-nutrient supplement had no effect on dual-task gait outcomes, in both intention to treat and per-protocol analysis. Furthermore, rather than showing a decline in dual-task gait, the placebo group had an overall mean improvement of 0.1m/s for dual-task gait speed, 13% on DTE on gait speed and 1.1% on dual-task variability. All dual-task outcomes had large within group standard deviations. One explanation for this is that a limitation of measuring dual-task gait performance is variation in task prioritisation. There may have been differences between the participants in whether they prioritised walking or the secondary cognitive task. The selection of a particular strategy for dual-task performance is consistent with the model of task prioritisation (Yogev-Seligmann et al. 2012), which suggests that when there is competition for attentional resources, an individual must decide how to prioritise the two tasks and that this self-selected strategy of task prioritisation is determined by factors that minimise danger and maximize pleasure. Thus, factors such as an individual's physical capacity to respond to a postural threat, often referred to as postural reserve**,** and their ability to recognise potential hazards in the environment and the situation primarily impact how attention is allocated. Although no specific instruction was given on task prioritisation, it is possible that some participants may have altered their task prioritisation between baseline and follow up making these outcomes less sensitive to change

over time. This switch in task prioritisation is more likely than a training effect as although dual-task training has shown some benefits on mobility outcomes, this was following an extensive dual-task training protocol (Brustio et al. 2018). Additionally, effect sizes were small and not comparable to the large changes observed in the present study. A focus was placed on the gait outcomes from the dual-task measurements as gait variability and DTE on gait speed are more clinically relevant outcomes due to their associations with falls in older adults (Beauchet et al. 2008a; Herman et al. 2010). However, future work should collect data on cognitive outcomes under dual-task conditions to assess whether task prioritisation changes between baseline and follow up, so this could be controlled for in the analysis. For backwards counting tasks, this could include recording the number of correct answers per minute whilst seated and during the dual-task gait assessment. This would give an indication of task performance and prioritisation and by recording the correct numbers per minute rather than just total number of errors**.** This method would control for variances in the amount of numbers that are recalled as well as accounting for any variances in task prioritisation.

Results from the intention to treat analysis found no significant effects of twice weekly aerobic exercise sessions for 12 weeks on the primary outcome of HGS or any other mobility outcome. Conversely, the per-protocol analysis revealed significant treatment effects for the exercise intervention on habitual and fast walking gait speed. Both the MS+EX and P+EX groups showed substantial clinically meaningful change for habitual gait and fast walking speed. The results from this analysis support previous studies which have found that aerobic exercise can improve mobility in the older adult (Mangione et al. 1999; Barnett et al. 2003; Denison et al. 2013). Previous work has shown positive responses to cycle ergometer training on six metre timed up and go in healthy older adults (Denison et al. 2013), and on chair rise time and 6-minute walk test in older adults with osteoarthritis (Mangione et al. 1999). It is currently not fully understood how exercise interventions improve walking ability. It appears that exercise interventions have the ability to improve neuromuscular function by ~25% which results in an ~11% increase in walking speed, though there is little to suggest that older adults walk faster because they use the newly acquired physical abilities in walking (Beijersbergen et al. 2013). It is likely that the mechanism behind the improvement in gait speed following exercise is multifactorial and interactive between biomechanical, physiological, neurological and psychological factors.

It is important to note that 69.6% of the participants reported meeting the WHO guidelines for moderate to vigorous levels of physical activity at baseline. This data therefore indicates that additional physical benefits can be obtained with cycle ergometer training in older adults who already report meeting activity guidelines. It is important to make the distinction that this effect was only observed in the per-protocol analysis. The exercise intervention for this study did not

meet the adherence goal of 70% with the overall adherence being 55%, a value that is consistent with some other exercise intervention trials in a similar demographic (Flegal et al. 2007; Sjosten et al. 2007; Nagamatsu et al. 2013b).

The per-protocol analysis found a significant improvement in FTSTS performance versus placebo for the combined multi-nutrient supplement and exercise intervention only. The difference between the MS+EX and P groups was 2.57 s. Minimal detectable change over repeated measures on the FTSTS in older women at the 95% CI has previously been defined as 2.5 s (Goldberg et al. 2012), thus providing some support for the validity of our findings on this outcome. Previously, supplementation with 2 g/day of fish oil combined with 12 weeks of resistance training was shown to increase maximum voluntary contraction and rate of torque development in leg muscles of older women versus resistance training alone (Rodacki et al. 2012). Similarly, supplementation with 2.1 g EPA and 0.6 g DHA and resistance training for 18 weeks provided an additional benefit to maximal isometric torque in knee extensor muscles versus resistance training alone (Da Boit et al. 2016b). Within these previous studies the improvements in muscle strength and power development did not translate into significant changes in functional outcomes including gait assessment and ability to rise from a chair. It is, however, important to note that these prior studies did not include a placebo only control group with the comparator arm receiving either omega-3 PUFA supplementation or exercise alone. Our analysis provides some promising data that the combination of a high DHA multi-nutrient supplement and aerobic exercise can elicit a positive change in FTSTS performance in older women. This is partly supported by data that omega-3 PUFA supplementation may re-sensitise ageing muscle to anabolic stimuli, thus enhancing MPS (Smith et al. 2011a). Although this is a promising result, it must be noted that the MS+EX group at baseline had a FTSTS of 14.7 s, which although not statistically significant from the other intervention groups, indicates a lower degree of functioning in this task in line with adults aged 80-89 years (Bohannon 2006). As such, it is recommended that this result should be considered preliminary at this stage, as it may not translate to older adults who exhibit a higher degree of functioning in this task at baseline. We also note that the sample size within the per-protocol analysis is small and thus although the result provides some rationale for further investigation, more research is required to confirm this finding within a larger sample size.

Cognitive Function

The exercise and multi-nutrient interventions alone and in combination led to improvements in verbal memory in the intention to treat analysis, with the total recall across the trials representing eight to twelve more words remembered than the placebo group, representing recall that is more consistent with a much younger age range (Gibson et al. 2014). These

findings corroborate previous observations showing positive effects on verbal memory following treatment with the same multi-nutrient supplement (Strike et al. 2016). The effect of the supplementation adds to the body of evidence that verbal or episodic memory, in particular, appears to be positively modified by omega-3 PUFA and B vitamin supplementation in older adults (Yurko-Mauro et al. 2010; de Jager et al. 2012; Lee et al. 2013; Hooper et al. 2017). Similarly, the improvements following exercise are consistent with previous research (Smith et al. 2010d). Data from 806 older adults indicates that the grey matter density of the temporal lobe, amygdala angular gyrus and hippocampus are strongly linked to performance in the immediate recall portion of the RAVLT (Moradi et al. 2017). DHA is particularly enriched in grey matter regions making up approximately one third of the total fatty acids, where it is highly enriched in PS (Brenna and Diau 2007a; Kim et al. 2014). Furthermore, supplementation with B12 and folic acid has been shown to reduce atrophy of grey matter regions in older adults by 4.6% over two years, however, this effect is modified by homocysteine levels with higher homocysteine having a greater response to supplementation (Douaud et al. 2013). This provides a potential physiological underpinning for the effect of supplementation with the specific multi-nutrient blend on this domain in this study and across several other trials.

DHA is important for neuronal functioning, maintaining membrane integrity, and reducing inflammation and oxidative stress (Dyall 2015a). The incorporation of DHA increases neuronal membrane fluidity and improves neurotransmission, which could be important for memory-related learning by increasing the neuroplasticity of nerve membranes, synaptogenesis and improve synaptic transmission (Fontani et al. 2005). EPA and DHA have been shown to potently modulate neuroinflammation by decreasing the production of eicosanoids from AA, while DHA and its derivative metabolites inhibit the secretion of pro-inflammatory cytokines (Serhan et al. 2015) while at the same time being a substrate for the production of anti-inflammatory docosanoids (Serhan et al. 2008). The changes in DHA from baseline in the present study are modest in comparison to other similar studies on omega-3 PUFA supplementation, with the MS group increasing by 0.5% and the MS+EX by 0.65% of total fatty acids in the intention to treat analysis. For example 24 weeks of supplementation with 900 mg DHA in a sample of 485 older adults lead to a 3.2% increase in percentage weight of DHA in plasma phospholipids, a change that was associated with less errors in verbal memory (Yurko-Mauro et al. 2010). Similarly, one year of supplementation with a daily dose of 1300 mg DHA and 450 mg EPA in 35 older adults with MCI resulted in improvements in delayed recall on the RAVLT, which was accompanied by a 1.73% increase in percentage weight of DHA (Sinn et al. 2012). This disparity in the changes in DHA in relation to cognitive outcomes highlights an important gap within the current literature that future work should seek to address. Although dose-response analyses of supplementation protocols is useful for study design (Yurko-Mauro et

al. 2015) the data from the present analysis supports previous work that the effects of DHA are driven by its enrichment within cells. Moving forward it is important to understand both how the degree of change in DHA and the absolute amounts at baseline and following supplementation influence results. This will provide an insight into a possible dose-response or dose threshold for DHA. This could be achieved by adopting a more uniform approach to reporting in fatty acid trials, which includes reporting baseline and end point analysis for all measured fatty acids, as well as consistency with which fatty acids are measured (Brenna et al. 2018). The measured lipid pool may also explain some of the differences between the present analysis and some of the examples provided previously as erythrocytes and plasma phospholipids do have greater amounts of DHA versus whole blood. Although uniformity in the lipid pool that is being measured would aid in the consistent reporting of fatty acid trials, this is unlikely to be achieved as pools are often selected to address specific research questions or for pragmatic reasons.

The multi-nutrient supplement and exercise interventions alone and in combination resulted in significantly improved executive function versus the placebo. The observation of exercise improving this cognitive domain is consistent with previous experimental work (Smith et al. 2010d). There is currently no clear consensus in the literature as to whether the active ingredients in the multi-nutrient supplement improve executive function in older adults. For example, 900 mg DHA for 24 weeks had no effect on executive function (Yurko-Mauro et al. 2010), whereas 1320 mg EPA and 880 mg DHA for 26 weeks in 65 healthy older adults produced significant improvements (Witte et al. 2014). Similarly, although B vitamins (Clarke et al. 2014) and *Ginkgo Biloba* (Snitz et al. 2009) do not appear to improve executive function, preliminary evidence suggests PS has some beneficial effects (Richter et al. 2013). A recent study suggested an omega-3 PUFA multi-nutrient supplement combined with exercise improves executive function in older men (Bell et al. 2019); however, as there was no exercise only control group the contribution of omega-3 PUFA multi-nutrient supplement to these effects cannot be identified. Indeed previous research combining omega-3 PUFAs and B vitamins in multi-nutrients formulas has yielded no benefit to executive function as covered in chapter 2. One factor that may contribute towards the inconsistency in the findings for nutrient interventions on the executive function is the operationalisation of the cognitive domain. Witte et al. (2014) operationalised executive function by creating a composite score on the following tasks: phonemic fluency, semantic fluency, trail making test and Stroop test. Although these are all valid tests of executive function it must be considered that these test also draw upon verbal ability, interference control and processing speed (Davidson et al. 2003b; Salthouse 2011b; Shao et al. 2014). Similarly within the previous trials of this specific multi-nutrient supplement the Stockings of Cambridge was used to assess executive function (Strike et al. 2016), and a

serial subtraction task to assess the function of the frontal cortex (Jackson et al. 2016b), a region associated with executive function (Goh et al. 2013), however these tasks have also designed to examine spatial working memory (Wild and Musser 2014) and working memory respectively (Bristow et al. 2016). Indeed within the present analysis it has been demonstrated that performance on the trail making task is also influenced by processing speed (Salthouse 2011b) a domain that had previously been improved by the same multi-nutrient formula (Strike et al. 2016). Due to the incongruence of these results when taken into the context of the wider body of literature we must consider the possibility that this effect on a test designed to assess executive function may also have been influenced by other cognitive domains with processing speed perhaps being the most likely area of interest. Because several cognitive processes are included in the domain of executive functioning, utilising multiple validated tests and calculating a composite score may be a more robust method for assessing this cognitive domain, thus future work should consider the adoption of this method (Iverson et al. 2020).

Previous work has found that an aerobic exercise intervention increased anterior hippocampal volume in 120 older adults (Erickson et al. 2011). Additionally, aerobic activity over a nine year follow up was positively associated with grey matter volume of frontal, occipital, entorhinal, and hippocampal regions in 299 older adults (mean age 78 years). This evidence would suggest that the effects of physical activity on maintaining the structural integrity of the brain during the ageing process could be an underlying mechanism for the effect of exercise on cognition. In particular, the preservation of frontal lobe could explain the protective effects observed for executive function, as this region has been specifically linked with this cognitive domain (Goh et al. 2013). Furthermore, 12 weeks of cycle ergometer training in a group of 56 healthy older adults was shown to increase brain connectivity in the default mode network, a large scale brain network that has shown links to both motor control and executive function (Damoiseaux et al. 2008). Importantly, these observed changes in functional connectively were positively correlated with performance on the Halstead-Reitan tapping test, a functional psychomotor task (McGregor et al. 2018). Although this motor task is not as physically demanding as walking, this research is supportive of our findings that 12 weeks of aerobic exercise improves executive function, which could alongside the well documented effects of exercise on muscle function and cardiovascular fitness (Onambélé-Pearson et al. 2010; Canuto Wanderley et al. 2015), contribute towards improvements gait speed and stability. Together these findings support the hypothesis that exercise can modulate several cognitive domains, which has also been demonstrated in a meta-analysis examining 29 studies conducted between 1966 and 2009 (including more than 2,000 cognitively healthy older adults and 234 effect sizes). This analysis found that individuals who were randomly assigned to aerobic exercise training showed modest improvements in both executive function and memory (Smith et al. 2010c).

Despite DHA being highly enriched in the hippocampus, and spatial working memory being a hippocampal dependent cognitive domain (Broadbent et al. 2004), evidence for beneficial effects of DHA supplementation on this domain is currently limited with multiple RCTs finding no effects of supplementation on this domain (Dangour et al. 2010; Andreeva et al. 2011a; Lee et al. 2013; Strike et al. 2016). In the present study, there was very little change across any of the intervention groups on this cognitive domain with the placebo group, in particular, seeing no change at all over 24 weeks. Although spatial abilities are known to decline with age (Harada et al. 2013), this decline is more pronounced in pathological ageing (Ritchie et al. 2017). Even though the robustness of this outcome in a healthy population may have limited the ability to detect change over time in this domain, the results of the present analysis are consistent with the literature that has found little evidence to suggest preservation of spatial working memory by omega-3 PUFAs or exercise in healthy older adults.

Health Related Quality of Life

Results from the analysis of SF-36 questionnaire outcomes revealed a significant positive effect of the combined of multi-nutrient supplementation and aerobic exercise on emotional wellbeing, with a large effect size. The results for this analysis were similar across both the intention to treat and per-protocol analyses. This provides some novel data that a high DHA multi-nutrient supplement may modify the response to exercise and vice versa on emotional wellbeing in older women. These results are relevant, as emotion regulation is a crucial resource in ageing. It allows individuals to successfully deal with the challenges and limitations imposed by the physiological ageing process. At the emotional level, older adults achieve well-being by selecting and optimizing particular emotion regulation processes to compensate for changes in internal and external resources. In particular, positive reappraisal strategies allow ageing persons to adaptively face constraints and limitations, through the acknowledgment of related negative emotions and the engagement in psychological and behavioural pathways leading to the identification of adequate and realistic problem solving strategies (Urry and Gross 2010).

It is important to note that the participants in the present study did not have a diagnosed depressive condition, as per the exclusion criteria, however, the majority of the current evidence has focused on participants who have received a formal diagnosis with depression. Several epidemiological studies on oily fish consumption and depression have reported a significant inverse correlation between intake of oily fish and prevalence (Tanskanen et al. 2001; Bountziouka et al. 2009; Suominen-Taipale et al. 2010) and incidence (Sanchez-Villegas et al. 2007; Astorg et al. 2008) of depression. This trend has also been reported in Mediterranean countries, where several studies reported a decreased prevalence (Skarupski et al. 2013) and incidence (Sanchez-Villegas et al. 2009; Rienks et al. 2013) of depression and/or depressive

symptoms in participants who were more adherent to the whole Mediterranean dietary pattern, which includes higher consumption of fish as well as B vitamins and vitamin E. This finding is support by data from an RCT that showed a 12 week Mediterranean diet intervention had a significant and clinically meaningful effect on the Montgomery–Åsberg Depression Rating Scale with 32% of the participants in the active intervention achieved a score that would be considered as complete remission (Jacka et al. 2017). Although observational evidence increasingly supports a positive association between omega-3 PUFA and emotional wellbeing, experimental research is less consistent. Previous meta-analyses report a positive effect of omega-3 PUFA intake on ameliorating symptoms of depression (Freeman et al. 2006; Lin and Su 2007; Rutkofsky et al. 2017). On the other hand, incongruent results have been reported in other systematic revisions of the literature (Appleton et al. 2015). The reasons for such variability in these findings depend on the significant heterogeneity among studies examined, weakening the results of the analyses. It has been highlighted that publication bias, unstandardised depression assessment, the variability of omega-3 PUFA dosages employed, and duration of the trial may have affected results across the trials (Appleton et al. 2015). The main limitation of the pooled analysis relied on the selection of studies to be included. Results from participants with major depressive disorder and depression were pooled with those with depression occurring from other clinical conditions (bipolar disorder, pregnancy, primary diseases other than depression). Taking into account that these conditions may differ in their underlying causes (Fraguas et al. 2017), it is possible they may also react differently to intervention with diet or exercise.

Some meta-analyses focused on the type of fatty acid used, resulting in a positive effect on symptom severity in depressed patients of EPA rather than DHA content of the supplement regime (Ross et al. 2007; Martins 2009). This is supported by evidence from a meta-analysis of clinical trials where it was concluded that supplements containing EPA ≥60% of total EPA + DHA, in a dose range of 200 to 2200 mg/d of EPA in excess of DHA, were effective against primary depression with a large effect size (Sublette et al. 2011a). This incongruity between findings highlights the importance of considering the omega-3 PUFAs as biologically distinct molecules. Although the supplement in the present study did contain EPA this was at a relatively low level compared to DHA, nonetheless, blood markers of EPA status did increase significantly in the active groups. The effect could also be attributed to some of the other nutrients in the supplement. Although the current body of evidence surrounding B vitamin supplementation on emotional wellbeing is unequivocal, a cross-sectional analysis of 3752 older men showed that those with plasma homocysteine of 2.03 mg/L had increased risk for depression (OR 1.70) (Almeida et al. 2008). Furthermore, a 60 day intervention in participants with depression and elevated plasma homocysteine (>10 μmol/L) with a supplement containing

1 mg B1, 1.6 mg B2, 30 mg B3, 3.3 mg B5, 3 mg B6, 263 µg B12, and 1000 µg folate improved scores on the Beck depression index as well as the emotional welling on the SF-36 questionnaire (Lewis et al. 2013).

Similarly, over the last decade, there have been several extensive reviews of the literature on the effects of exercise on mental wellbeing, which together offer support for the role that exercise can play in the promotion of positive mental health even within non-clinical populations (Rebar et al. 2015). Indeed, a series of exercises including aerobic, strength, balance, coordination, and flexibility exercises performed once per week for four months was shown to increase self-reported emotional wellbeing in a sample of fourteen older adults (Delle Fave et al. 2018).

There is some overlap in the proposed mechanisms mediating the influence of omega-3 PUFAs and exercise on emotional wellbeing. Elevated levels of tumour necrosis factor-α, interleukin-6 and interleukin-1 have been shown to be associated with depressed mood (Reichenberg et al. 2001). Moreover, higher levels of positive well-being have been associated with lower concentrations of inflammatory markers (Steptoe et al. 2012). Furthermore, there is evidence to suggest that this relationship between inflammation and depressed mood may only occur in females (Moieni et al. 2015), thus this result may not be applicable to males. Moderate intensity exercise has been shown to decrease interleukin-6 and tumour necrosis factor-α whilst improving depression scores (Paolucci et al. 2018) and similarly the immunomodulatory properties of omega-3 PUFAs have been prosed as a primary mechanism of action driving changes in mental health (Sublette et al. 2011a).

Although this result does provide a level of promise for the role of supplementation with the multi-nutrient supplement in combination with exercise, this result should still be considered preliminary at this stage. Emotional wellbeing is one sub-section of the SF-36 questionnaire and derives its score from five questions. As such it is advisable that this result is further explored using questionnaires that measure emotional wellbeing in greater depth. Furthermore emotional wellbeing has been previously positively correlated with physical functioning indicating that higher levels of physical function may lead to better reported psychological health (Garatachea et al. 2009). Given that the per-protocol analysis showed positive effects of the exercise on mobility outcomes this may have partially contributed towards these greater emotional wellbeing scores.

The null effect of the exercise intervention on SF-36 outcomes is surprising given that exercise interventions have consistently shown benefits to quality of life measures. This result could be in part due to the self-reported nature of the SF-36 questionnaire. As previously mentioned, 39 of the 55 participants reported meeting WHO exercise recommendations at baseline. This made the positive findings on objective measures of mobility and cognitive function of particular

interests, and they indicate that further benefits could be achieved from exceeding the recommendations. In this case, if the perception of the participants was that they were already physically active, this may have reduced the likelihood of the exercise intervention of having an effect on self-reported health outcomes. This nocebo effect of exercise has been demonstrated on self-reported pain and may have played a role in the null effects of exercise in this study (Colloca et al. 2018).

Strengths and Limitations 19

This study has several strengths within the design. The intervention trial was a randomised, placebo controlled semi-blinded design, with a high retention rate, thereby increasing the reliability of the data. The trial design was reviewed and made publicly available on the clinical trials registry and has been subject to peer review during the protocol publication process. The study used a range of sensitive outcomes spanning across cognition, mobility, health related quality of life and biochemical domains, which better encapsulates the trajectory towards the HAP. Individual cognitive domains where tested, which are more sensitive than commonly used measures of global cognition such as the MMSE (Mitchell 2009; Tucker-Drob 2011). Furthermore, the selected cognitive domains appear to be important for the maintenance of gait control (Bruce-Keller et al. 2012) and have previously shown some promise for modulation by physical activity or omega-3 PUFAs (Smith et al. 2010b; Witte et al. 2014).

Several important confounders were controlled for in this analysis this including age, BMI, verbal intelligence and use of medications. It is important in this type of study to monitor participant's dietary intake and physical activity; these were assessed by diet diary and FFQ, and CHAMPS questionnaire, respectively. This meant it could be confirmed that the participant's diet and physical activity outside of the interventions remained consistent. The only notable, although not statistically significant, change in diet was a decrease in fat intake across all four groups; however, we have no reason to believe this decrease across the groups would have influenced the results of the analysis. The direct and objective measure of whole-blood fatty acids is an index of metabolism and incorporation, providing a quantitative measurement compared to relying on self-reported dietary intake data as some previous studies have previously relied upon (Rousseau et al. 2009; Takayama et al. 2013), which can be influenced by recall bias. This direct measurement of fatty acid incorporation meant that adherence to the supplementation and placebo allocation could be accurately assessed. Indeed the baseline data from this studied showed no association between self-reported nutrient intakes and objectively measured biomarkers.

Whilst this study has several strengths there are also a number of important limitations that must be noted. The low participant numbers meant that it was not possible to establish whether the

combined multi-nutrient and exercise intervention offered additional benefits versus each intervention alone. Although the study was initially designed to test this hypothesis, during the peer-review process of the study results it was highlighted that the initial sample size calculation was not sufficient to assess additive or synergistic effects of the interventions. Changes in the primary outcome of HGS were similar for the multi-nutrient supplement and aerobic exercise alone and in combination. Based on this observation it is likely that if there is an additional benefit to combing the interventions the effect size would be expected to be small, therefore, in order to be able to detect any additional benefit of combining the multi-nutrient supplement and exercise power calculations should be based upon the minimal clinically meaningful change in HGS of 0.03 m/s (Kwon et al. 2009). On a similar note it is important to acknowledge that whilst the per-protocol analysis provided some interesting insight the sample size with both exercise groups were extremely small. Whilst this data could be possibly be used to generate new hypotheses based on the direction and magnitude of the effect sizes, definitive conclusions cannot be drawn from this analysis due to the lack of statistical power.

Whilst the cognitive testing procedures used within the present study are validated measures of their respective cognitive domains each cognitive domain was assessed using a single test. We now have data suggesting that using composite scores from a battery of neuropsychological testing may be a more sensitive tool for detect changes in cognition (Vellas et al. 2008). Indeed the present study was only adequately powered to detect changes in the primary outcome of HGS. Although utilising a composite score from multiple cognitive tests may be more sensitive we must also consider the feasibility of using such methods, due to the additional time required to perform such testing. In the case where multiple other outcomes are being assessed participant burden must be considered. As such in future work that seeks to measure both mobility and cognition as a way of better encapsulating the HAP, we recommend adopting a primary outcome from each category and subsequently performing a power calculation on each outcome to ensure adequate statistical power for testing of each health domain.

The high functional capacity of the participants within the study and the shorter intervention length may have contributed towards some of the null findings on the primary outcome of HGS. A longer follow up period may be required to observe any notable decline in HGS to accurately test whether lifestyle interventions can preserve function in this population group.

Adherence targets for the exercise intervention did appear to limit its effectiveness, evidenced by the differences in results across the intention to treat and per-protocol analysis. Based on the feedback received from the exit questionnaire this lack of attendance was largely driven by the timing of the classes. Although every effort was made to schedule classes at convenient times, availability of the facilities, instructor and varying participants' schedules did provide a challenge in terms of maximising adherence. Moving forward, focus certainly needs to be

placed on addressing barriers to exercise in older adults. Furthermore, the enrolled participants reported above average levels of physical activity versus population norms. 69.6% of the participants, who completed the CHAMPS questionnaire, reported meeting the WHO guidelines for moderate to vigorous aerobic physical activity. Data from the Health Survey for England (Scholes 2017) states that the average adherence to these guidelines in older women is 54%. 45% of the participants reported meeting the WHO recommendations for engaging in specific muscle strengthening exercises twice per week with national averages being 15% adherence for older women. Finally, 41% reported meeting the guidelines for both aerobic and muscle strengthening activities with national averages being 14%. Although a positive response to aerobic exercise was detected across physical and cognitive domains, further benefits or larger effects sizes may have been observed in a sample of less active or sedentary participants, indeed there is data which suggests that the largest benefits to physical activity comes from moving between the sedentary to lightly active brackets (Sofi et al. 2011; Dondzila et al. 2015). Participants in the present research were not excluded based on their baseline activity levels thus there must be caution when interpreting the result from this work. Whilst the multi-nutrient supplement and exercise interventions each produced effects on cognition of a similar magnitude to cognition in the intention to treat analysis, the whole blood fatty acid analysis suggested there was low levels of omega-3 PUFAs across the sample. These lower levels of omega-3 PUFAs at baseline may have enhanced the likelihood of response to the nutrient intervention whereas the higher levels of activity could lower the odds for a positive response to the aerobic exercise condition, thus we suggest that any comparison of these results should be avoided. Future work should screen participants based on their baseline activity levels and seek to recruit individuals who currently do not meet WHO guidelines for physical activity so that more robust comparisons can be made between nutrient and exercise interventions. 20

A further limitation was that apolipoprotein epsilon genotypes were not assessed, as this may have affected participant's response to the high DHA multi-nutrient supplementation (Hennebelle et al. 2013), and has been shown to be associated with gait speed decline in ageing (Verghese et al. 2013). Unfortunately, due to methodological issues we were unable to monitor changes in serum homocysteine levels over the study and therefore cannot identify the potential role of folic acid and vitamin B12 in the treatment effects. However, a major unifying factor in the multi-nutrient intervention is DHA, and the supplementation increased circulating DHA and EPA levels. Future work should use alternative methods such as high performance liquid chromatography to quantify homocysteine (Sawula et al. 2008).

5.5 Conclusion

Overall, these results suggest that the high DHA multi-nutrient supplement or aerobic exercise produces improvements in verbal memory and executive function in older women. These

improvements are clinically relevant and were identified in able female adults. Due to the range of compounds in the multi-nutrient supplementation, it is not possible to ascribe the effects to any single factor, and the present results further support the use of a combination of dietary factors in ageing (Dunn-Lewis et al. 2011; Strike et al. 2016). Treatment effects were not identified in the primary outcome of HGS, nonetheless the improvements in verbal memory and executive function do provide some promising insight into the benefits of dietary supplementation and exercise for the promotion of healthy ageing. Interestingly positive effects of the combined multi-nutrient supplement and aerobic exercise intervention were observed for emotional wellbeing and FTSTS, with neither intervention alone influencing these outcomes. Given that healthy ageing is related to maintaining multiple health domains, this provides some novel data that the combination of the multi-nutrient supplement and aerobic exercise may be able to have a broader impact on health outcomes in older adults, and that exercise may modify the response to supplementation and vice versa within these domains. Further work should seek to explore the effects of the multi-nutrient supplement on participants who may be most likely to respond, i.e. those with low DHA and high homocysteine levels, and also explore supplementation for a longer period, or in a more frail population.

Chapter 6 - Conclusion, Implications and Future Perspectives

The data presented within this thesis demonstrates novel and promising insights into the role of nutrition and exercise in the promotion of healthy ageing. The relationship between DHA and dual task gait has previously never been shown and provides more evidence for a link between omega-3 PUFAs and gait in older adults. This link is deserving of further exploration as we seek to understand the role nutrition can play in preserving both cognition and mobility in the older adult.

Results from the intervention trial further support the role of supplementation with DHA and supporting nutrients on verbal memory and executive function, as well as the benefits of exercise across both cognition and mobility in healthy older women.

Due to the small sample size analysis to detect superiority of the active interventions over one another was not possible. Potential additive on synergistic effects of the combination of omega-3 PUFAs, B vitamins and exercise on healthy ageing outcomes warrants further exploration in future work. It may be hypothesised that the effects of the multi-nutrient supplement and exercise operate via overlapping biological mechanisms. This includes decreasing inflammation, for example, interleukin-6 levels are linked to declines in verbal memory (Beydoun et al. 2019), and both omega-3 PUFAs and aerobic exercise have both been shown to decrease IL-6 levels (Zheng et al. 2019). Alternatively, exercise and factors in the multi-nutrient supplement, such as DHA and *Ginkgo biloba* increase BDNF levels, which may contribute to enhancing synaptic plasticity and cognitive function (Janssen and Kiliaan 2014; Belviranli and Okudan 2015; Chieffi et al. 2017). However, further work is needed to explore these potential mechanisms, if they mediate any of the observed effects and if indeed the combination of the two interventions can provide an additional benefit to the older adult.

Whilst the evidence from this thesis has provided some promising insight into the effects of the multi-nutrient supplement and exercise in healthy older adults, future work should consider applying these interventions to a population group that is less robust. Previous work has identified older adults with MCI as a subset of the population that may respond to omega-3 PUFA supplementation with regards to their cognitive function (Freund-Levi et al. 2006). Similarly there is evidence, albeit preliminary, suggesting omega-3 supplementation may be useful for preserving muscle function during periods of disuse (McGlory et al. 2019). This could mean older adults who are frail or have sarcopenia/sarcopenia obesity may be more ideal candidates for omega-3 PUFA supplementation. Furthermore, we know that from observational research older adults with MCI and physical frailty typically have higher concentrations of homocysteine (Wong et al. 2013; Beydoun et al. 2014) and are more sedentary than healthy controls (Fried et al. 2001; Sofi et al. 2011). Taken together this would suggest that those

identified as having cognitive frailty, the coexistence of cognitive impairment as well as physical frailty, would be a population group that could response well to intervention with the multi-nutrient formula and exercise separately or in combination.

An important consideration for future trials on the effects of fatty acids in healthy ageing is to better understand how tissue enrichment with omega-3 PUFAs influences outcomes. Future work should adopt a consistent method for measuring and reporting fatty acid status at baseline and following a fatty acid intervention to establish whether a dose-repose curve or dose threshold exists. This has relevance for both physical and cognitive health as many of the underlying mechanism of omega-3s have been demonstrated to be as a result of their enrichment within cells (Fontani et al. 2005; Kamolrat and Gray 2013). A focus on increasing tissue enrichment with omega-3 PUFAs in supplemental trials could likely be a more efficacious approach. Supplemental trials typically provide a single uniform dosage of omega-3 PUFA supplementation to participants who have low baseline levels. An alternative approach where a high dose of omega-3 PUFAs is provided over a short period of time to increase tissue enrichment rapidly followed by a moderate dose to maintain levels could potentially be advantageous, in a similar vein to how studies on creatine monohydrate have been performed with "loading" and "maintenance" doses (Kreider et al. 2017). Dosages of up to 5000 mg total EPA+DHA have been shown to be safe and well tolerated in older adults, and membrane composition is elevated in a dose-dependent manner through dietary intake of omega-3 PUFA (Rees et al. 2006), so using higher dosages for short periods is certainly possible (Stavrinou et al. 2020). Using this method, participants would spend more time with greater levels of omega-3 PUFA. If there is indeed a dose threshold effect for omega-3 PUFAs this approach may be particularly useful for trials with a shorter duration.

More studies are required to better understand how and why individuals may or may not respond to specific nutrition interventions. The concept of personalised dietary intervention has been gaining considerable traction within the literature. It has become apparent that there is a considerable inter-individual variation in the response to nutrition interventions (Tierney et al. 2011). This individualised response to intervention could be explained by a number of factors including age, sex, baseline dietary intake, genetics, epigenetics and the microbiome. All of these factors can influence absorption, distribution, metabolism, and excretion of compounds and metabolites, and thus affect bioavailability and biological responsiveness (Manach et al. 2017). Although it is possible to control for some of these factors in RCT design, it would not be feasible to consider every individualised factor that may influence responsiveness to a dietary intervention. Homocysteine has rarely been considered in these studies on omega-3 PUFA supplementation in older adults yet homocysteine levels have been consistently associated with risk of cognitive decline and frailty (Nurk et al. 2005; Wong et al. 2013; Xu et al. 2015). A lack

of consistency shown across some trials of omega-3 PUFA supplementation could be due to a lack of consideration for homocysteine levels. Higher homocysteine could reduce the effectiveness of omega-3 PUFA supplementation, by disrupting DHA enriched phospholipid metabolism (Selley 2007), therefore controlling for this would be important for any intervention trial. The present study gave a multi-nutrient supplement which would theoretically raise omega-3 PUFA levels whilst lowering homocysteine creating a biological state where supplementation may be more efficacious. As nutrition science moves forward it is important to not only find out what dietary interventions are effective but in what subsets of the population, they are effective. This ability to identify responders versus non-responders will help practitioners to an individualised approach to dietary programming/interventions. In future studies could use strict exclusion criteria to control for some of these variables to focus on a subset of individuals who are most likely to respond to a similar intervention. This approach has already been taken with some of trials into B vitamin supplementation and omega-3 PUFAs alone (Durga et al. 2007; Hooper et al. 2017). Alternatively given the already heterogeneous nature of older adults and the difficulty with recruitment, pre-determined sub analysis of intervention responsiveness based on stratification by DHA, homocysteine and physical activity may be a more pragmatic approach whilst still providing unique and valuable insight. Together this would help to better understand the role nutrition can play in promoting healthy ageing allowing practitioners to give more personalised advice.

Ageing and age related health conditions are very complex and likely require multifaceted interventions for both preventative and palliative care. The data from this thesis provides more evidence for the role that nutrition and exercise can play in preventing age-related decline, identifying novel relationships between DHA and dual task gait as well as treatment effects a high DHA multi-nutrient supplement and aerobic exercise on verbal memory and executive function. As we move toward a more personalised patient-centred care approach to healthy ageing, the data provided from this thesis can provide a foundation upon that future research within this area can be based upon.

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Appendices

Appendix 1 Conference abstract: The combined effects of Omega-3 Polyunsaturated Fatty Acids and B vitamins on Cognition in the older adult: A Systematic Review and Meta-analysis

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There is now evidence to suggest that there may be an interaction between B vitamins and omega-3 polyunsaturated fatty acids (PUFAs), with suggestions that optimising intake of both nutrients being key to eliciting benefits to cognition within older adults.

The aim of this systematic review was to investigate whether supplementation with a combination of omega-3 PUFAs and B vitamins can prevent cognitive decline in older adults. Randomised control trials conducted in older adults that measured cognitive function were retrieved. The included trials provided a combination of omega-3 PUFAs and B vitamins alone, or in combination with other nutrients and trials which tested for interactions between omega-3 PUFAs and B vitamins by providing omega-3 PUFA alone and also measuring B vitamin status or provided B vitamin supplementation alone and measured omega-3 PUFA status. The databases searched were The Cochrane Library, EMBASE, CINAHL, Scopus, and MEDLINE. A total of 14 papers were included in the analysis (n=4913; age: 60-70 y; follow up 24 weeks to 4 years). The meta-analysis results found a significant benefit of nutrient formulas, which included both omega-3 PUFAs and B vitamins, versus placebo on global cognition assessed using composite scores from a neuropsychological test battery (G=0.23, P=0.002), global cognition using single measures of cognition (G=0.28, P=0.004) and episodic memory (G=0.32, P=0.001). The results indicate that providing a combination of omega-3 PUFA and B vitamins benefits cognition in older adults versus a placebo, the potential for an interaction between these key nutrients should be considered in future experimental work.

Appendix 2 Conference abstract: Preliminary analysis suggests a high DHA multi-nutrient supplementation and aerobic exercise produce similar improvements in verbal memory in older females

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We have previously shown that a high DHA multi-nutrient supplement benefits verbal memory and habitual gait speed in older women. Similarly, exercise improves cognition and physical ability in this group. Combining omega-3 fatty acid supplementation with exercise may provide additional benefits to cognition and mobility. Therefore, this study explored whether the addition of an exercise intervention enhanced the effects of the supplementation on cognition

and mobility. Results presented are non-final analysis of the Omega-3 Fatty Acids and Exercise on Mobility and Cognition in Older Women (MOBILE) Study (NCT03228550).

The multi-nutrient supplement (1000 mg DHA, 160 mg EPA, 20 µg vitamin B12, 1 mg folic acid, 124 mg phosphatidylserine, 240 mg *ginkgo biloba* and 20 mg vitamin E) was given for 24 weeks, whereas the exercise intervention (two supervised stationary exercise bike sessions per week) was for the final 12 weeks. Participants were healthy women ≥ 60 years. Groups comprised: multi-nutrient supplement and exercise (n=11), placebo and exercise (n=9), multi-nutrient supplement (n=10), and placebo (n=10). Verbal and spatial memory, executive function, processing speed and single and dual-task gait conditions were assessed. Blood fatty acids were measured by pin-prick analysis.

There were significant improvements for both supplementation (p=.033) and exercise (p=.049) on verbal memory versus placebo. Improvements were similar between supplementation and exercise groups. No additional benefits were observed with the combination of interventions.No further treatment effects were identified in this non-final analysis. These findings provide further support that a high DHA multi-nutrient positively impacts verbal memory in older females, and that these effects are similar to those observed following exercise. This trial is ongoing and these results should be considered preliminary.

Appendix 3 Conference abstract and poster: Circulating DHA levels as a predictor of gait performance under single and dual-task conditions in older females

Paul Fairbairn[1], Fotini Tsofliou[1], Andrew Johnson[2], Simon C Dyall[1]

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There is a complex interplay between cognition and gait in older people, with this relationship becoming more apparent when required to conduct a simultaneous secondary task (dual-task paradigm). An inability to maintain a conversation while walking is a strong predictor of falls in older adults and thus, dual-task gait protocols are an established way to assess the relationship between cognition and gait. We and others have shown that the omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) plays an important role in cognition and is positively associated with gait outcomes in older adults.

The aim of the study was to investigate the relationship between whole-blood DHA levels and gait variables under single and dual-task in women aged 60 years and over. DHA content was quantified from pin-prick samples using gas chromatography and expressed as percentage of total fatty acids. Gait analysis was performed using APDM motion sensors.

49 women (mean age 67 year S.D. 5.3 years) completed the assessments. Circulating DHA was not associated with habitual gait speed (r=.210, p=.171, CI 95% [-.116, .453]) or stride length variability (r=-.263, p=.081, CI 95% [-.523, .042]). However it was associated with outcomes under the cognitively demanding dual-task condition. Significant associations were observed between DHA and both dual-task gait speed (r=.360, p=.015, CI 95% [.074, .590]) and dual-task effect on gait speed (r=.323, p=.031, CI 95% [.023, .569]).

These results show for the first time that DHA is positively associated with dual-task gait outcomes in older women. Decreased performance in dual-task gait outcomes in older adults has been indicated as a sensitive measure to detect older adults at high risk of a dementia. These results therefore have important clinical implications and further studies will seek to explore the nature of this relationship and assess whether increasing DHA status improves performance in these activities.

Circulating DHA levels as a predictor of gait performance under single and dual-task conditions in older females

446369

Bournemouth University

Fairbairn P, Tsofliou F, Dyall SC

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Introduction

There is a complex interplay between cognition and gait in older people, with this relationship becoming more apparent when required to conduct a simultaneous secondary task (dual-task paradigm)[1]. An inability to maintain a conversation while walking is a strong predictor of falls in older adults and thus, dual-task gait protocols are an established way to assess the relationship between cognition and gait. The omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) plays an important role in cognition [2] and has also been shown to reduce joint pain[3] and increase muscle protein synthesis (MPS) [4] and leg strength [5] in older adults which could explain the observed positive association with gait outcomes in older adults [6] (Figure 1).

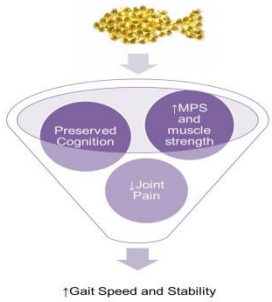

Figure 1 Effects of omega-3 PUFAs on cognition, muscle strength and joint pain could contribute towards improved gait speed and stability.

Methods

The aim of the study was to investigate the relationship between whole-blood DHA levels and gait variables under single and dual-task in women aged 60 years and over. DHA content was quantified from pin-prick samples using gas chromatography and expressed as percentage of total fatty acids. Gait analysis was performed using APDM motion sensors.

Results

49 women (mean age 67 years S.D. 5.3 years) completed the assessments (Figure 2). Circulating DHA was not associated with habitual gait speed (r=.210, p=.171, CI 95% [-.116, .453]) or stride length variability (r=-.263, p=.081, CI 95% [-.523, .042]). However it was associated with outcomes under the cognitively demanding dual-task condition. Significant associations were observed between DHA and both dual-task gait speed (r=.360, p=.015, CI 95% [.074, .590]) (Figure 3) and dual-task effect on gait speed (r=.323, p=.031, CI 95% [.023, .569]) (Figure 4).

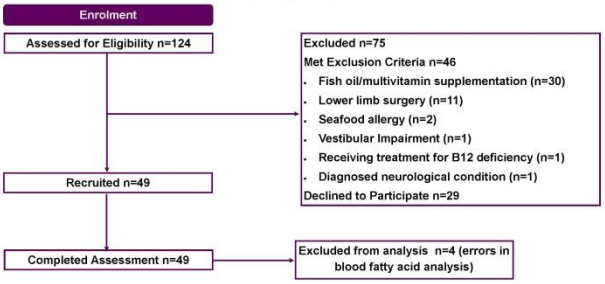

Figure 2 Participant flow diagram.

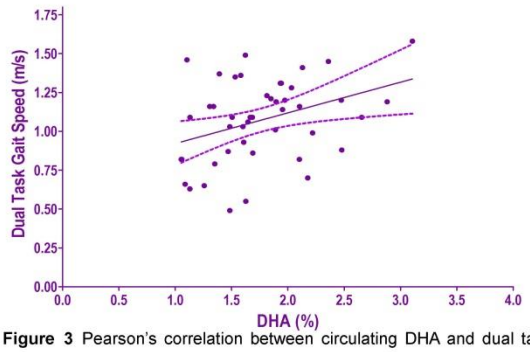

Figure 3 Pearson's correlation between circulating DHA and dual task gait speed r=.360, p=.015, CI 95% [.074, .590]).

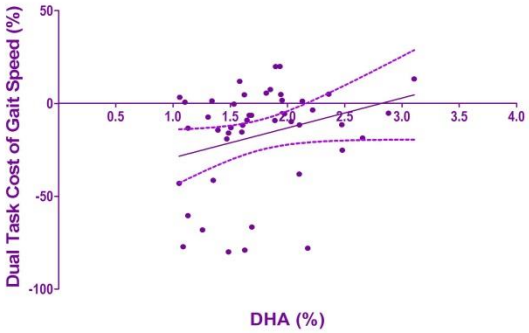

Figure 4 Pearson's correlation between circulating DHA and the dual task cost of gait speed (r=.323, p=.031, CI 95% [.023, .569]).

Conclusion

These results show for the first time that DHA is positively associated with dual-task gait outcomes in older women. Decreased performance in dual-task gait outcomes in older adults has been indicated as a sensitive measure to detect older adults at high risk of a dementia and falls. These results therefore have important clinical implications and further studies will seek to explore the nature of this relationship and assess whether increasing DHA status improves performance in these activities.

Acknowledgements

We express our sincere thanks to Efamol Ltd and The Sylvia Waddilove Trust for supporting this study.

We would also like to express or gratitude towards the participants who volunteered their time to take part in this study.

The authors declare no conflicts of interest. The supporters have no role in the study design, data collection, analysis, interpretation of the data, or the decision to publish the results.

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Appendix 4 Conference abstract and poster: The relationship between circulating omega-3 polyunsaturated fatty acids, cognitive function and dual task gait speed is different in non-frail and pre-frail older women

Fairbairn P., Tsofliou F. and Dyall S.C.

Faculty of Health and Social Sciences, Bournemouth University, Dorset, U.K.

Background: Gait performance is affected by age-related neurodegeneration and slower gait speed is a strong predictor of cognitive decline [1] particularly under cognitive load [2]**.** Furthermore, decreased gait performance under dual task (DT) challenges is associated with progression to dementia [3]. Dietary supplementation with omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly docosahexaenoic acid (DHA) has been shown to preserve cognitive function [4] and mobility [5] in older adults. This study investigated the relationships between n-3 PUFAs, cognitive function and DT gait speed in non-frail and pre-frail older women [6].

Method: Healthy older women aged 60 years and above were assessed according to the frailty criteria [6] (non-frail score = 0, pre-frail score = 1 to 2) and given a battery of cognitive tests measuring verbal memory, spatial working memory, executive function and processing speed. The DT gait protocol consisted of walking whilst simultaneously completing a backward counting task. Whole-blood fatty acid levels were measured from pin-prick samples.

Results: 15 pre-frail (mean age 69 y, S.D. 7 y) and 32 non-frail (66 y, S.D 4 y) women completed the analysis (124 screened, 29 declined to participate and 48 excluded). There were no significant differences in blood fatty acid levels between the groups. Significant positive correlations were identified between n-3 PUFA index and verbal memory (R=.376, P=.037) and DT gait speed (R=.468, P=.008). DHA (R=.437 P=.014) and DHA/arachidonic acid (ARA) (R=.471 P=.008) were also positively associated with DT gait speed. A linear regression model of DT gait speed in non-frail participants was significant, F (5, 25) = 2.85 P=.036 Adjusted R^2 =.236, with DHA accounting for a unique variance of P =.036. No significant relationships were identified for pre-frail participants, or for any of the other cognitive outcomes in either group.

Conclusion: These results show for the first time that there are differences the relationships between n-3 PUFA levels and cognitive and mobility measures between non-frail and pre-frail older women, such that higher n-3 PUFAs are associated with greater verbal memory and DT gait speed in non-frail individuals only. We have previously shown that a high DHA multi-nutrient supplement significantly improves verbal recognition memory and gait speed in older women [5], and these results confirm the role of n-3 PUFAs in these areas, but suggest supplementation should begin at an early stage before the onset of frailty symptoms. However, due to the small sample size these results should be considered preliminary and further work will seek to investigate the nature of these relationships.

The relationship between circulating omega-3 polyunsaturated fatty acids, cognitive function and dual task gait speed is different in non-frail and pre-frail older women

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Introduction

Gait performance is affected by age-related neurodegeneration and slower gait speed is a strong predictor of cognitive decline [1] particularly under cognitive load [2]. Dietary supplementation with omega-3 polyunsaturated fatty acids (n-3 PUFAs), particularly docosahexaenoic acid (DHA) has been shown to preserve cognitive function [3] and mobility [4] in older adults. This study investigated the relationships between n-3 PUFAs, cognitive function and dual task (DT) gait speed in non-frail and pre-frail older women [5].

Methods

Healthy older women aged 60 years and above were assessed according to the frailty criteria [5] (non-frail score = 0, pre-frail score = 1 to 2) and given a battery of cognitive tests measuring verbal memory, spatial working memory, executive function and processing speed. The DT gait protocol consisted of walking whilst simultaneously completing a backward counting task. Whole-blood fatty acid levels were measured from pin-prick samples. Non-normally distributed data was transformed, by fractional rank, and then by inverse distribution to meet assumptions of parametric statistical analysis.

Results

Forty seven women mean age 67±5.4 years (non frail n=32 pre-frail n=15) were recruited and completed all measurements (Figure 1). In non-frail individuals only, significant positive correlations were identified between n-3 PUFA index and verbal memory (R=0.376, P=0.037) (Figure 2) and DT gait speed (R=0.468, P=0.008). DHA (R=0.437 P=0.014) (Figure 3) and DHA/arachidonic acid (R=0.471 P=0.008) were also positively associated with DT gait speed. A linear regression model of DT gait speed in non-frail participants was statistically significant (F (5, 25) = 2.85 P=0.036 Adjusted R^2=0.236), with DHA accounting for a unique variance of P =0.036 (Table 1). No significant relationships were identified for pre-frail participants, or for any of the other cognitive outcomes in either group.

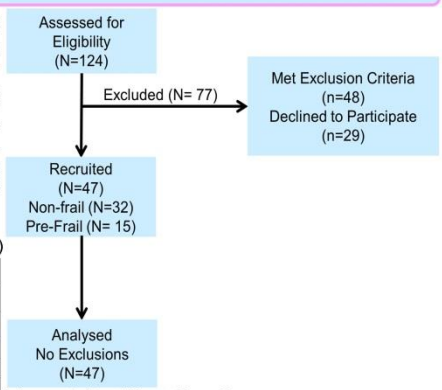

Figure 1 Participant flow diagram

Table 1 Regression analysis of dual task gait speed in non-frail participants (N=32)

Variable	Regression Coefficient	β	95% CI	P Value
Transformed DHA	0.066	0.136	0.01, 0.218	0.036
Spatial Working Memory	0.013	0.291	-0.004, 0.031	0.124
Verbal Memory	0.002	0.125	-0.006, 0.013	0.462
Executive Function	-0.004	0.297	-0.010, 0.003	0.274
Transformed Processing Speed	-0.223	-0.232	-0.575, 0.129	0.204

R=0.603 R^2=0.363 CI: Confidence Interval

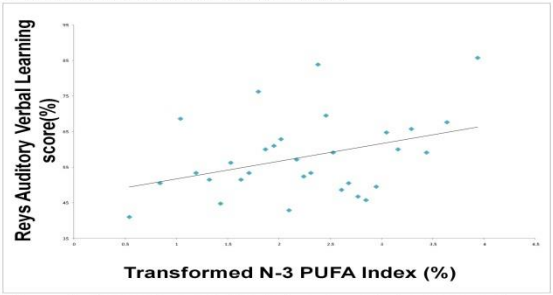

Figure 2 Pearson's correlation between verbal memory and transformed N-3 PUFA index (R=0.376 P=0.037) (N=32)

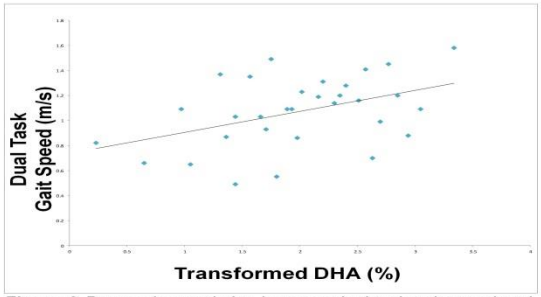

Figure 3 Pearson's correlation between dual task gait speed and transformed DHA percentage (R=0.437 P=0.014) (N=32)

Conclusion

These results show for the first time that there are differences in the relationships between n-3 PUFA levels with cognitive and mobility measures between non-frail and pre-frail older women. These results support the potentially positive role of n-3 PUFAs in these areas, but suggest supplementation should begin at an early stage before the onset of frailty symptoms. However, due to the small sample size further work is required to investigate the nature of these relationships.

Acknowledgements

We express our sincere thanks to Efamol Ltd and The Sylvia Waddilove Trust for supporting this study.

We would also like to express or gratitude towards the participants who volunteered their time to take part in this study.

The authors declare no conflicts of interest. The supporters have no role in the study design, data collection, analysis, interpretation of the data, or the decision to publish the results.

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Appendix 5 Conference abstract A Feasibility Study to Develop Dual Task Gait and Cognitive Testing Protocols in Women Aged ≥60 years

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[1]Faculty of Health and Social Sciences, Bournemouth University, Dorset, U.K.

[2]Department of Psychology, Faculty of Science and Technology, Cognition and Cognitive Neuroscience Research Centre, Bournemouth University, Dorset, U.K.

Research indicates an important interplay between cognition and gait in older people, with slower gait a predictor of cognitive decline. Walking is a complex task and cognitive aspects become increasingly important in older adults, particularly when required to conduct a simultaneous secondary task (dual-task paradigm). Inability to maintain a conversation while walking is a strong predictor of falls in older adults. Consequently dual-task gait protocols have become an established way to assess the relationship between cognition and gait.

In order to assess cognition and performance in the dual-task paradigm in our future studies it was necessary to develop appropriate experimental protocols. Five healthy women aged ≥60 years completed cognitive tests assessing verbal memory, spatial memory, executive function and interference control. They also completed normal and dual-task gait under a range of protocols. Gait testing was analysed using APDM motion sensors.

The results will be presented at the conference.

Appendix 6 Participant recruitment newspaper advertisement

Bournemouth University

Are you interested in how nutrition and exercise can influence healthy

Here at Bournemouth University we are seeking female volunteers aged 60+ to take part in an exciting new study.

The supplement we are using may improve memory and walking ability on its own. We are now combining it with exercise to see if this can increase the beneficial effects.

All who take part will receive 6 months' supply of the multinutrient supplement and free diet and health assessment

If you are interested in taking part or have any questions please don't hesitate to contact a member of the research team

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Appendix 7 Inclusion and Exclusion Criteria and MOBILE trial

Inclusion

Females aged 60 years and above

Able to walk at least 50 m unaided, (2)

Classified as non-frail or pre-frail according to the Fried (2001) criteria

Community-dwelling

Exclusion criteria

Vestibular impairments,

Diagnosed neurological disorder inclusive of Alzheimer's disease, other forms of dementia, mild cognitive impairment, depression, generalised anxiety disorder, all psychotic illnesses or other clinically diagnosed psychiatric disorders likely to affect the cognitive measures (as judged by a the research team)

Cognitive impairment (Mini Mental Status Examination score of 24 or below)

Lower limb surgery

Seafood allergy

Regular consumption of multivitamin or fish oil supplements within six months prior to baseline measurements

Previously received advice from a health care professional not to undertake strenuous exercise.

HIV positive

History of alcohol or drug dependency

Body mass index of more than 40 kg/m^2

Subjects with any other existing medical conditions likely to influence the study measures (as judged by a member of the research team)

Appendix 8 Fatty Acid Composition and Oil blend used for the Placebo Capsules

Fatty Acid Profile		% Fatty Acid Methyl Ester	Milligram triglyceride per Capsule
14:0	Myristic	0.4	3.3
16:0	Palmitic	7.3	67.8
16:1	Hexadecenoic	0.5	4.2
16:3	Heptadecatrienoic	0.1	0.5
16:4	Hexadecatetraenoic	0.1	0.8
17:0	Heptadecanoic	0.1	0.5
18:0	Stearic	6.6	60.3
18:1 (n-9)	Oleic	72.4	656.5
18:1 (n-7)	CIS-vaccenic	0.8	6.8
18:2 (n-7)	Linoleic	8.2	74.1
18:3 (n-3)	Alpha-linoleic	0.1	1.1
18:4 (n-3)	Octadecatetraenoic	0.1	1.2
20:0	Icosanoic	0.3	2.7
20:1	Icosenoic	0.3	3.0
20:4 (n-6)	Arachidonic	0.0	0.4
20:5 (n-3)	Icosapentaenoic	0.8	6.8
22:0	Docosanoic	0.9	7.6
22:1 (n-11)	Cetoleic	0.1	0.8
22:5 (n-3)	Docosapentaenoic	0.1	0.7
24:0	Tetracosanoic	0.3	2.5

22:6 (n-3)	Docosahexaenoic	0.5	4.3

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MASTER FORMULA

Efalex Active 50+ Placebo
Bulk Code: 34205
Efamol Code: 032080

Actives

Raw Material Code	Raw Material Name	Per Capsule		Label Claim			Purpose
		Quantity	Units	Equiv.	Quantity	Units	
105A012	High Oleic Sunflower Seed Oil (EU Food grade)	558,52	mg	Sunflower Seed Oil Typically Providing: Oleic Acid (OA) Linoleic Acid (LA)	558,52 430 22	mg mg mg	
11721	Fish Oil 18:12 (EU Food grade)	29,808	mg	Fish Oil Typically Providing: Eicosapentaenoic Acid (EPA) Docosahexaenoic Acid(DHA)	28,808 5,4 3,6	mg mg mg	Active
11105	d-alpha-Tocopherol (USP/US Food Chemical Codex)	7,825 (5% Overage)	mg	Vitamin E	7,45 5	IU mg α-TE	Active

Inactives

Raw Material Stock Code	Raw Material Name	Quantity	Purpose of Inactive
105Z005	Glyceryl monostearate (Ph.Eur)	52,847 mg	Carrier
000A102	*Bovine Gelatin – Halaal (USP/Ph.Eur/EDQM)	157,10 mg	Capsule shell
000A502 11745	*Glycerin (BP/USP/EP) *Glycerin (BP)	42,79 mg	Plasticizer
11848	*Purified Water (BP2011 with additional micro testing)	7,93 mg	Solvent
11419	*Sorbitol 70% (BP)	42,79 mg	Diluent
11205	*Iron Oxide Red (JECFA – 2008)	2,66 mg	Colourant
11207	*Iron Oxide Yellow (JECFA – 2008)	1,73 mg	Colourant

*Capsule shell constituents.
Capsule shell quantities represent approximate masses after drying.

Appendix 9 Borgs rating of perceived exertion scale

Rating	Descriptor
6	No exertion at all
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Appendix 10 The Rey's Auditory Verbal Learning Test

Immediate Recall	Interference
Drum	Desk
Curtain	Ranger
Bell	Bird
Coffee	Shoe
School	Stove
Parent	Mountain
Moon	Glasses
Garden	Towel
Hat	Cloud
Farmer	Boat
Nose	Lamb
Turkey	Gun
Colour	Pencil
House	Church
River	Fish

Appendix 11 Venous Blood Sampling Risk Assessment and Standard Operating Procedure

Quality control

1. The phlebotomist will work in a quiet, clean, well-lit area. A clean working surface with two chairs along with a hand wash basin with soap, running water and paper towels will be provided.
2. All phlebotomists will have received full training including an understanding of anatomy, awareness of the risk of the blood exposure, and the consequences of poor infection prevention and control.
3. Standard operating procedure will be issued to each phlebotomist and will be printed and available on the day of blood sampling.
4. All storage tubes and aliquots will be labelled accurately to ensure blood samples are stored and analysed properly and to make sure samples are identifiable and traceable to ensure that the sample is correctly matched with the result and with the patient or donor.
5. Samples will be required to be of a high quality so that the results from the subsequent testing is satisfactory.
6. A log book for the study is available to report any adverse events. This would include an accurate account of the incident, possible causes, management and follow up of adverse events.

Safety

1. All necessary protective equipment for phlebotomists will be provided this includes, hand hygiene materials (soap and water or alcohol rub), well fitted non-sterile gloves, single use disposable needles, syringes or lacing devices in sufficient numbers to ensure that each patient has a sterile needle and syringe or equivalent for each blood sampling.
2. Adequate numbers of samples tubes will be provided to prevent dangerous practices (e.g. decanting blood to recycle laboratory tubes).
3. To avoid contamination, any common-use items, such as tourniquets, should be checked to ensure they are clean before use on a participant, and single-use items will not be reused.
4. Each participant will be given a verbal explanation of the blood sampling procedure prior to having blood drawn, to promote participant cooperation and to make the whole procedure as comfortable as possible.

Infection Prevention and Control Practices

Do	Do not
DO carry out hand hygiene (use soap and water or alcohol rub), and wash carefully, including wrists and spaces between the fingers for at least 30 seconds	DO NOT forget to clean your hands
DO use one pair of non-sterile gloves per procedure or patient	DO NOT use the same pair of gloves for more than one patient DO NOT wash gloves for reuse
DO use a single-use device for blood sampling and drawing	DO NOT use a syringe, needle or lancet for more than one patient
DO disinfect the skin at the venepuncture site	DO NOT touch the puncture site after disinfecting it
DO discard the used device (a needle and syringe is a single unit) immediately into a robust sharps container	DO NOT leave an unprotected needle lying outside the sharps container
DO seal the sharps container with a tamper-proof lid	DO NOT overfill or decant a sharps container
DO place laboratory sample tubes in a sturdy rack before injecting into the rubber stopper	DO NOT inject into a laboratory tube while holding it with the other hand
DO immediately report any incident or accident linked to a needle or sharp injury, and seek assistance; start post-exposure prophylaxis as soon as possible, following protocols	DO NOT delay post-exposure prophylaxis after exposure to potentially contaminated material; beyond 72 hours, post-exposure prophylaxis is NOT effective

Procedure

Setting up of Equipment

Collect all the equipment needed for the procedure and place it within safe and easy reach on a tray or trolley, ensuring that all the items are clearly visible. The equipment required includes:

- A supply of laboratory sample tubes, which should be stored dry and upright in a rack; blood can be collected in. These tubes will have a clotting factor added to them to ensure the sample clots within 30 minutes. Ensure that the rack containing the sample tubes is close to you, the health worker, but away from the patient, to avoid it being accidentally tipped over

- Sterile glass or plastic tubes with rubber caps
- Well-fitting, non-sterile gloves;
- Blood-sampling devices (Vacutainers)
- A tourniquet;
- Alcohol hand rub;
- 70% alcohol swabs for skin disinfection;
- Gauze or cotton-wool ball to be applied over puncture site;
- Sample labels;
- Writing equipment;
- Laboratory log book
- Leak-proof transportation bags and containers;
- A puncture-resistant sharps container.

Prepare the Participant

1. Introduce yourself to the patient, and ask the patient to state their full name.
2. Ask whether the patent has allergies, phobias or has ever fainted during previous injections or blood draws.
3. If the patient is anxious or afraid, reassure the person and ask what would make them more comfortable.
4. Place a clean paper or towel under the patient's arm.
5. Discuss the test to be performed and obtain verbal consent. The patient has a right to refuse a test at any time before the blood sampling, so it is important to ensure that the patient has understood the procedure.

Select the Site

1. Extend the patient's arm and inspect the antecubital fossa or forearm.
2. Locate a vein of a good size that is visible, straight and clear, The median cubital vein lies between muscles and is usually the most easy to puncture. Under the basilic vein runs an artery and a nerve, so puncturing here runs the risk of damaging the nerve or artery and is usually more painful. DO NOT insert the needle where veins are diverting, because this increases the chance of a haematoma.
3. The vein should be visible without applying the tourniquet. Locating the vein will help in determining the correct size of needle.
4. Apply the tourniquet about 4–5 finger widths above the venepuncture site and re-examine the vein.

Perform Hand Hygiene and Put Gloves On

1. Wash hands with soap and water, and dry with single-use towels; or if hands are not visibly contaminated, clean with alcohol rub – use 3 ml of alcohol rub on the palm of the hand, and rub it into fingertips, back of hands and all over the hands until dry.
2. After performing hand hygiene, put on well-fitting, non-sterile gloves.

Disinfect the entry site

1. Clean the site with a 70% alcohol swab for 30 seconds and allow to dry completely (30 seconds)
2. Apply firm but gentle pressure. Start from the centre of the venepuncture site and work downward and outwards to cover an area of 2 cm or more.
3. Allow the area to dry. Failure to allow enough contact time increases the risk of contamination.
4. DO NOT touch the cleaned site; in particular, DO NOT place a finger over the vein to guide the shaft of the exposed needle. It the site is touched, repeat the disinfection.

Take blood

Perform venepuncture as follows.

1. Anchor the vein by holding the patient's arm and placing a thumb BELOW the venepuncture site.
2. Ask the patient to form a fist so the veins are more prominent.
3. Enter the vein swiftly at a 30 degree angle or less, and continue to introduce the needle along the vein at the easiest angle of entry.
4. Once sufficient blood has been collected, release the tourniquet BEFORE withdrawing the needle. Some guidelines suggest removing the tourniquet as soon as blood flow is established, and always before it has been in place for two minutes or more.
5. Withdraw the needle gently and apply gentle pressure to the site with a clean gauze or dry cotton-wool ball. Ask the patient to hold the gauze or cotton wool in place, with the arm extended and raised. Ask the patient NOT to bend the arm, because doing so causes a haematoma.

Fill the Laboratory Sample Tubes

1. Best practice is to place the tube into a rack before filling the tube
2. Pierce the stopper on the tube with the needle directly above the tube using slow, steady pressure. Do not press the syringe plunger because additional pressure increases the risk of haemolysis.
3. Where possible, keep the tubes in a rack and move the rack towards you. Inject downwards into the appropriate coloured stopper. DO NOT remove the stopper because it will release the vacuum.

Clean contaminated surfaces and complete patient procedure

1. Discard the used Vacutainer sampling device into a puncture-resistant sharps container.
2. Check the labelling on the sample tubes to make sure they are correct. The label should be clearly written with the information required by the laboratory, which si the participant number and the date upon which the blood sample is taken.
3. Discard used items into the appropriate category of waste.
4. Perform hand hygiene again
5. Inform the patient when the procedure is over.
6. Ask the participant how they are feeling. Check the insertion site to verify that it is not bleeding, and then thank the participant.
7. Wipe down all surfaces with Bioclense.

Clean up spills of blood or body fluids

If blood spillage has occurred (e.g. because of a laboratory sample breaking in the phlebotomy area or during transportation, or excessive bleeding during the procedure), clean it up. An example of a safe procedure is given below.

1. Put on gloves
2. Mop up liquid from large spills using paper towels, and place them into the clinical waste.
3. Remove as much blood as possible with wet cloths before disinfecting.
4. Use Bioclense disinfectant on all areas affected by the spillage.

<u>Serum Extraction Procedure</u>

1. Make sure all storage aliquots are labelled appropriately with the same details as the blood sample (participant number and date of collection.
2. Allow each blood sample to clot at room temperature this should take 60 minutes. Samples should not be left longer than 60 minutes before being centrifuged to prevent lysis of cells in the clot, releasing cellular components not usually found in serum samples.
3. Centrifuge the sample at 1000-2000 x g for 10 minutes
4. Extract the serum using a Pasteur pipette into storage aliquots
5. Dispose of the remaining sample into the clinical waste bins
6. Immediately place the samples into storage at -20 to -80 degrees Celsius

Appendix 12 Three day food diary template

3 Day Diet Diary

Name ……

Guidelines for Recording Dietary Intake

This booklet should contain all the information you need to fill out the diet diary

Do's

- Record accurately all that you eat and drink at the time that it is eaten. Only write down what you actually eat, not what you start off with on your plate.
- Write down the foods and drinks immediately after you have eaten them, NOT from memory at the end of the day.
- Take your booklet with you if you will be eating or drinking away from home.
- Do record everything you eat and drink for the whole day
- Use as many pages as you like.

Don'ts

- Don't change what you normally eat
- Don't write from memory at the end of the day – you may forget some foods you have eaten.
- Don't forget to record any between meal snacks like sweets, chocolate, crisps, biscuits, fruit……
- Don't forget to record drinks, mixers and **alcohol**

Describing what food and drink you have eaten

Please write down what you eat and drink giving as much detail as possible about each item.

Amounts

You can use general kitchen utensils to record your foods e.g.

- Teaspoon (tsp) – heaped/ level/ one/ two
- Dessert spoon
- Tablespoon
- Ladle

- Teacup
- Mug
- Half pint/ pint
- Soup Bowl
- Dessert Bowl
- Cereal Bowl
- Side Plate
- Estimate – large/med/small portion

Detail
Try to give as much detail as possible e.g.

- Bread – was it white/ brown? Thick, medium or thin sliced?
- Milk – was it full fat or semi skimmed?
- Drinks – was it diet, fizzy, natural fruit juice?
- Tea/ Coffee – did you add sugar/ milk?

Cooking methods	- Give the method of cooking. - Are your eggs boiled, poached or scrambled? - Are your potatoes boiled, mashed, baked or chipped? - Is your bacon grilled of fried?
Brand names	- Write down the brand names of foods and drinks where you can e.g. *Flora margarine, Kellogg's cereal, Heinz baked beans.*
Names	- Name the type of biscuit, cake or cereal you eat e.g. *Digestive biscuit, fruit cake, Weetabix. Name the type of cheese,* *fish, meat you eat e.g. Dairylea cheese spread, haddock, pork chop.*
Meat/ Fish	The type of animal, number of slices/ portions, the part of the animal, cooking method
Fruit & Veg	Cooked/ Raw? Peeled or eaten with skin? Fresh/frozen/ canned? How much consumed?
Pasta, Rice & Noodles	White/ Wholemeal, number of bowls/ spoonfuls when cooked
Cereals	Brand name, spoonfuls, what kind of milk.
Eggs	Size and cooking method.
Spreads/ jams	Number of portions/ teaspoons

Confectionary & Savoury Snacks	Weight from packet, size, number of biscuits/ sweets
Drinks	Include all beverages- tea, fizzy drinks, milk, diluting juice. Alcohol

Number of cups/ glasses/ mugs/ cans

Made up dishes	Write down what the dish is called and give the ingredients if
	you can, e.g. stew (beef, carrots, onions, gravy)
Sauces/dressings	Please do not forget to include sauces e.g. tomato ketchup cheese sauce, salad dressings and gravy when completing your diet diary

Recipes and Labels

Please write down any recipes you have used while filling in this record.

Please cut out the Nutritional Information from the labels of any commercial made up foods you have eaten, not forgetting the brand name.

Please attach recipes and labels to this diary and hand in with the completed record

Thank you for all your help and co-operation.

Food Diary (example)

DAY: DATE:

Write 1 item per line.
Start a separate page for each day.

Time	Amount Eaten	Details of food and Drink
6.00am	1 cup	Tea with milk-semi skimmed
9.00am	2 cups 2 medium slices 2 teasp 2 teasp	Tea with milk semi skimmed Mothers Pride white, toasted Butter marmalade
11.00am	1 mug 3	Coffee black Safeway custard cream biscuits
11.45am	1	Mars bar
12.00noon	2 small 3tbsp 3 scoops 2 tbsp thin slice	Grilled lamb chop with fat cut off Thick gravy Mashed potato Peas (frozen) Fruit cake, home made, using basic sponge recipe with added raisins
3.00pm	1 1 mug	Mcvities digestive biscuit coffee black
5.30pm	2 x matchbox size 20cm 2 medium thick slices 2 thick slices 2 tbsp 3 tbsp	Scottish cheddar cheese Cucumber not peeled Cold boiled ham with fat Mothers pride white bread no butter Home made apple crumble Birds tinned custard
7.00pm	1 mug 2	Tesco's drinking chocolate made with milk (semi skimmed) Oatcakes plain

Food Diary

DAY: ONE **DATE:**

Time	Amount Eaten	Details of food and Drink

Food Diary

DAY: TWO **DATE:**

Time	Amount Eaten	Details of food and Drink

Food Diary

DAY: THREE **DATE:**

Time	Amount Eaten	Details of food and Drink

Appendix 13 Seafood food frequency questionnaire

DATE:_____ RATER___ PT INIT

Omega 3 Fatty Acid Questionnaire

1) Gender: ❒ Male (1) ❒ Female (2)

2) About how long ago was your last meal? _____hours

3) Have you eaten any fish or shellfish--such as shrimp, crab, lobster etc.—in the past 24 hours? (include sushi made with fish or shellfish)
1) ❒ No
2) ❒ Yes

4) How many times have you eaten fish or shellfish in the past week?
1) ❒ 0 times
2) ❒ 1-3 times
3) ❒ More than 3 times

5) Over the past 6 months, about how often have you eaten fish or shellfish in any form?
1) ❒ Never
2) ❒ Less than 1 time each month
3) ❒ 1 time each month
4) ❒ 2-3 times each month
5) ❒ 1 time each week
6) ❒ 2 times each week
7) ❒ 3-4 times each week
8) ❒ 5-6 times each week
9) ❒ 1 time each day
10) ❒ 2 or more times each day

If NEVER, Skip to Question 8.

6) Each time you ate fish or shellfish, how much did you eat?
1) ❒ Less than 2 ounces or less than one fillet or less than 4 pieces of sushi
2) ❒ 2 to 7 ounces or about 1 fillet or 4 -14 pieces of sushi
3) ❒ More than 7 ounces or more than 1 fillet or more than 14 pieces of sushi

7) Please check off the **types of fish or shellfish you eat most frequently** (Check off as many as are appropriate for you) .
1) ❒ Bass
2) ❒ Bluefish
3) ❒ Catfish
4) ❒ Clams
5) ❒ Cod
6) ❒ Crab
7) ❒ Flounder
8) ❒ Haddock
9) ❒ Halibut
10) ❒ Herring
11) ❒ Kingfish
12) ❒ Lobster
13) ❒ Mackerel
14) ❒ Mahi Mahi
15) ❒ Mussels
16) ❒ Oysters
17) ❒ Salmon
18) ❒ Sardines
19) ❒ Scallops
20) ❒ Sea Trout
21) ❒ Shark
22) ❒ Shrimp
23) ❒ Skate
24) ❒ Snapper
25) ❒ Sole
26) ❒ Swordfish
27) ❒ Tilapia
28) ❒ Tilefish
29) ❒ Trout (freshwater)
30) ❒ Tuna
31) ❒ Turbot
32) ❒ Whitefish
33) ❒ Whiting

DATE:_____ RATER___ PT INIT

8) In the past 6 months, about how often did you eat walnuts?

1) ❒ Never
2) ❒ Less than 1 time each month
3) ❒ 1 time each month
4) ❒ 2-3 times each month
5) ❒ 1 time each week
6) ❒ 2 times each week
7) ❒ 3-4 times each week
8) ❒ 5-6 times each week
9) ❒ 1 time each day
10) ❒ 2 or more times each day

If NEVER, Skip to Question 10.

9) Each time you ate walnuts, how much did you eat?

1) ❒ Less than ¼ cup
2) ❒ ¼ to ½ cup
3) ❒ More than ½ cup

10) In the past 6 months, about how often did you use canola cooking oil?

1) ❒ Never	6) ❒ 2 times each week
2) ❒ Less than 1 time each month	7) ❒ 3-4 times each week
3) ❒ 1 time each month	8) ❒ 5-6 times each week
4) ❒ 2-3 times each month	9) ❒ 1 time each day
5) ❒ 1 time each week	10) ❒ 2 or more times each day

If NEVER, Skip to Question 12.

11) Each time you used canola cooking oil, how much did you use?

1) ❒ Less than 1 teaspoon
2) ❒ 1-2 teaspoons
3) ❒ 2 teaspoons
4) ❒ 3 teaspoons (1 tablespoon)
5) ❒ More than 1 tablespoon

12) In the past 6 months, about how often did you eat flaxseed?

1) ❒ Never
2) ❒ less than 1 time each month
3) ❒ 1 time each month
4) ❒ 2-3 times each month
5) ❒ 1 time each week
6) ❒ 2 times each week
7) ❒ 3-4 times each week
8) ❒ 5-6 times each week
9) ❒ 1 time each day
10) ❒ 2 or more times each day

If NEVER, Skip to Question 14.

13) Each time you ate flaxseeds, about how much did you eat?

1) ❒ Less than 1 teaspoon
2) ❒ 1-2 teaspoons
3) ❒ 2 teaspoons
4) ❒ 3 teaspoons (1 tablespoon)
5) ❒ More than 1 tablespoon

DATE:_____ RATER___ PT INIT

14) In the past 6 months, about how often did you use flaxseed oil?

1) ❒ Never
2) ❒ Less than 1 time each month
3) ❒ 1 time each month
4) ❒ 2-3 times each month
5) ❒ 1 time each week
6) ❒ 2 times each week
7) ❒ 3-4 times each week
8) ❒ 5-6 times each week
9) ❒ 1 time each day
10) ❒ 2 or more times each day

If NEVER, Skip to Question 16.

15) Each time you used flaxseed oil, how much did you have?

1) ❒ Less than 1 teaspoon
2) ❒ 1-2 teaspoons
3) ❒ 2 teaspoons
4) ❒ 3 teaspoons (1 tablespoon)
5) ❒ More than 1 tablespoon

16) In the past 6 months, about how often did you use cod liver oil?

1) ❒ Never
2) ❒ Less than 1 time each month
3) ❒ 1 time each month
4) ❒ 2-3 times each month
5) ❒
6) ❒ 2 times each week
7) ❒ 3-4 times each week
8) ❒ 5-6 times each week
9) ❒ 1 time each day
10) ❒ more times each day

If N

17) Each time you used cod liver oil, how much did you have?

1) ❒ Less than 1 teaspoon
2) ❒ 1-2 teaspoons
3) ❒ 2 teaspoons
4) ❒ 3 teaspoons (1 tablespoon)
5) ❒ More than 1 tablespoon

CHAMPS Activities Questionnaire for Older Adults

Date:_____

Name or ID:_____

This questionnaire is about activities that you may have done in the past 4 weeks. The questions on the following pages are similar to the example shown below.

INSTRUCTIONS

If you DID the activity in the past 4 weeks: Step #1 Check the YES box.

Step #2 **Think about how many TIMES a week you usually did it, and write your response in the space provided.**

Step #3 **Circle how many TOTAL HOURS in a typical week you did the activity.**

Here is an example of how Mrs. Jones would answer question #1: Mrs. Jones usually visits her friends Maria and Olga twice a week. She usually spends one hour on Monday with Maria and two hours on Wednesday with Olga. Therefore, the total hours a week that she visits with friends is 3 hours a week.

In a typical week during the past 4 weeks, did you…

1. Visit with friends or family (other than those you live with)?

☒ **YES** How many TIMES a week? 2

☐ **NO**

How many TOTAL hours a week did you usually do it?

Less than 1 hour | 1-2½ hours | 3-4½ hours | 5-6½ hours | 7-8½ hours

If you DID NOT do the activity:

- **Check the NO box and move to the next question**

In a typical week during the past 4 weeks, did you …						
1. Visit with friends or family (other than those you live with)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
2. Go to the senior center? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
3. Do volunteer work? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
4. Attend church or take part in church activities? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
5. Attend other club or group meetings? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
6. Use a computer? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …						
7. Dance (such as square, folk, line, ballroom) (do not count aerobic dance here)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
8. Do woodworking, needlework, drawing, or other arts or crafts? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
9. Play golf, carrying or pulling your equipment (count walking time only)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
10. Play golf, riding a cart (count walking time only)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
11. Attend a concert, movie, lecture, or sport event? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

12. Play cards, bingo, or board games with other people? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …						
13. Shoot pool or billiards? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
14. Play singles tennis (do not count doubles)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
15. Play doubles tennis (do not count singles)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
16. Skate (ice, roller, in-line)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

17. Play a musical instrument? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
18. Read? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
19. Do heavy work around the house (such as washing windows, cleaning gutters)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …						
20. Do light work around the house (such as sweeping or vacuuming)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
21. Do heavy gardening (such as spading, raking)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

22. Do light gardening (such as watering plants)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
23. Work on your car, truck, lawn mower, or other machinery? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

****Please note: For the following questions about running and walking, include use of a treadmill.**

24. Jog or run? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
25. Walk uphill or hike uphill (count only uphill part)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …	

26. Walk fast or briskly for exercise (do not count walking leisurely or uphill)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
27. Walk to do errands (such as to/from a store or to take children to school (count walk time only)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
28. Walk leisurely for exercise or pleasure? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
29. Ride a bicycle or stationary cycle? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
30. Do other aerobic machines such as rowing, or step machines (do not count treadmill or stationary cycle)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

31. Do water exercises (do <u>not</u> count other swimming)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL <u>hours a week</u> did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …						
32. Swim moderately or fast? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL <u>hours a week</u> did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
33. Swim gently? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL <u>hours a week</u> did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
34. Do stretching or flexibility exercises (do <u>not</u> count yoga or Tai-chi)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL <u>hours a week</u> did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
35. Do yoga or Tai-chi? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL <u>hours a week</u> did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

36. Do aerobics or aerobic dancing? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours
37. Do moderate to heavy strength training (such as hand-held weights of more than 5 lbs., weight machines, or push-ups)? **YES** How many TIMES a week? _____ ⓞ **NO**	How many TOTAL hours a week did you usually do it? ⓞ	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours

In a typical week during the past 4 weeks, did you …							
38. Do light strength training (such as hand-held weights of 5 lbs. or less or elastic bands)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
39. Do general conditioning exercises, such as light calisthenics or chair exercises (do not count strength training)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
40. Play basketball, soccer, or racquetball (do not count time on sidelines)? **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
41. Do other types of physical activity not previously mentioned (please specify)? _____ **YES** How many TIMES a week? _____ ⊙ **NO**	How many TOTAL hours a week did you usually do it? ⊙	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

Appendix 15 The Standard Form-36 questionnaire

Standard Form – 36 (SF-36)	
Participant Number:	Date:

INSTRUCTIONS: This survey asks for views about your health. This information will help keep track of how you feel and how well you are able to do your usual daily activities. Answer every question marking the answer as indicated. If you are unsure about how to answer a question, please give the best answer you can.

1. In general, would you say your health is: (Circle One)	1. Excellent 2. Very Good 3. Good 4. Fair 5. Poor

2. Compared to one year ago, how would you rate your health in general at this time? (Circle One)	1. Much better now than one year ago 2. Somewhat better now than one year ago 3. About the same as one year ago 4. Somewhat worse that one year ago 5. Much worse now than one year ago

3. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

(Circle the appropriate number for each question)

Activities	Yes, limited a lot	Yes, limited a little	No, not limited
a. Vigorous activities, such as running, lifting heavy Objects, or participation in strenuous sports	1	2	3
b. Moderate activities, such as moving a table, Vacuuming, bowling or golfing	1	2	3
c. Lifting or carrying groceries	1	2	3
d. Climbing several flights of stairs	1	2	3
e. Climbing one flight of stairs	1	2	3
f. Bending, kneeling, or stooping	1	2	3
g. Walking more than a mile	1	2	3

h. Walking a long distance	1	2	3
i. Walking a short distance	1	2	3
j. Bathing or dressing yourself	1	2	3

4. During the past 4 weeks, have you had any of the following problems with your work or other regular activities as a result of your physical health? (Circle the appropriate number for each question)

a. Cut down on the amount of time you spent on work or other activities	Yes = 1	No = 2
b. Accomplished less than you would like	Yes = 1	No = 2
c. Were limited in the kind of work or other activities	Yes = 1	No = 2
d. Had difficulty performing the work or other activities (For example – requiring an extra effort)	Yes = 1	No = 2

5. During the past four weeks, have you had any of the following problems with your work or other regular daily activities as result of any emotional problems (such as feeling depressed or anxious)? (Circle the appropriate number for each question)

a. Cut down on the amount of time you spent on work or other activities	Yes = 1	No = 2
b. Accomplished less than you would like	Yes = 1	No = 2
c. Didn't do work or other activities as carefully as usual	Yes = 1	No = 2

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours or groups? (Circle one)	1. Not at all 2. Slightly 3. Moderately 4. Quite a bit 5. Extremely

7. How much bodily pain have you had during the past 4 weeks? (Circle one)	1. None 2. Very mild 3. Mild 4. Moderate 5. Severe 6. Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)? (Circle one)	1. Not at all 2. Slightly 3. Moderately 4. Quite a bit 5. Extremely

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks: (Circle one number on each line)

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
a. Did you feel full of pep?	1	2	3	4	5	6
b. Have you been a very nervous person?	1	2	3	4	5	6
c. Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
d. Have you felt calm and peaceful?	1	2	3	4	5	6
e. Did you have a lot of energy?	1	2	3	4	5	6
f. Have you felt downhearted and blue?	1	2	3	4	5	6
g. Did you feel worn out?	1	2	3	4	5	6
h. Have you been a happy person?	1	2	3	4	5	6
i. Did you feel tired?	1	2	3	4	5	6

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives etc.)?(Circle one)	1. All of the time 2. Most of the time 3. Some of the time 4. A little of the time 5. None of the time

11. How TRUE or FALSE is each of the following statements to you?(Circle one for each line).

	Definitely True	Mostly True	Don't Know	Mostly False	Definitely False
a. I seem to get sick easier than other people	1	2	3	4	5
b. I am as healthy as anybody I know	1	2	3	4	5
c. I expect my health to get worse	1	2	3	4	5
d. My health is excellent	1	2	3	4	5

Appendix 16 Bournemouth University ethics for the randomised control trial

Bournemouth University

Research Ethics Checklist

Reference Id	10788
Status	Approved
Date Approved	23/06/2016

Researcher Details

Name	Paul Fairbairn
School	Health and Social Care
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, DEng)
Course	Postgraduate Research
Have you received external funding to support this research project?	No
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Bournemouth University

Project Details

Title	The effect of an omega 3 fatty acid multinutrient supplement and physical activity on mobility and cognition in older adults
Proposed Start Date of Data Collection	01/06/2016
Proposed End Date of Project	01/02/2018
Original Supervisor	Ethics Programme Team
Approver	Research Ethics Panel

Appendix 17 Participant Agreement Form

Participant Agreement Form

Title of project: The effect of an Omega-3 fatty acid multi-nutrient supplement and physical activity on mobility and cognition in older adults.

Name and contact information of chief investigator: Paul Fairbairn PhD student at Bournemouth University pfairbairn@bournemouth.ac.uk 01202 965008

PhD supervisory team details
Dr. Simon Dyall sdyall@bournemouth.ac.uk
Dr. Fotini Tsofliou ftsofliou@bournemouth.ac.uk
Dr. Jane Murphy jmurphy@bournemouth.ac.uk

	Please Tick or Initial
I have read and understood the participant information sheet for the above research project with the version number 001 dated 25/11/15	
I confirm that I have had the opportunity to ask questions.	
I understand that my participation is voluntary.	
I understand that I am free to withdraw up to the point where the data are processed and become anonymous, so my identity cannot be determined.	
I understand that during the task or experiment, I am free to withdraw without giving reason and without there being any negative consequences.	
I understand that should I not wish to answer any particular question(s), complete a test or give a sample, I am free to decline at any point.	
I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the outputs that result from the research.	
I understand that I will not be able to have access to any individual participant results from any of the testing protocols	
I understand that if in the unlikely event that I lose the ability to consent I will be withdrawn from the study, any collected results and blood samples given prior to this will still be analysed and included in the study.	
I agree to take part in the above research project.	

_____ _____ _____

Name of Participant Date Signature

_____ _____ _____

Name of Researcher Date Signature

This form should be signed and dated by all parties after the participant receives a copy of the participant information sheet and any other written information provided to the participants. A copy of the signed and dated participant agreement form should be kept with the project's main documents which must be kept in a secure location.

Consent Form Version 001 25/11/15

Appendix 18 Participant Information Form

Version 002
Date 25/11/15

Participant Information Sheet

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being conducted and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Contact us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Study Title
The effect of an omega-3 fatty acid multi-nutrient supplement and exercise on mobility and cognition in women aged 60+.

Purpose of the study
The main purpose of the study is to investigate the effects of a dietary supplement containing omega-3 fatty acids, vitamin B12, folic acid, phosphatidylserine, ginkgo biloba and vitamin E and exercise on mobility and brain function in women aged 60+.

Do I have to take part?
No, it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a participant agreement form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

Am I eligible to take part?
In order to take part in the study you must be female and aged 60+ years. Due to the nature of the mobility testing and you must be able to walk without a walking aid, this is to ensure safety of all participants involved in the study.

The following would exclude an individual from taking part in the study: diagnosed neurological disorder, vestibular impairments, previous lower limb surgery, prior diagnosis or receiving treatment for pernicious anaemia, allergy to seafood, regular consumption of fish oil supplements in the last 6 months or if you have been advised by a doctor not to take part in strenuous physical activity.

What will happen if I take part?
Before taking part in the study you will be asked to sign a participant agreement form. Before participant agreement forms are collected you will be given an opportunity to ask any questions you have to the chief investigator.

<u>The Interventions</u>
All participants will be randomly assigned to one of four groups:

- Multi-nutrient supplement and exercise
- Placebo supplement and physical exercise
- Multi-nutrient supplement with no exercise intervention
- Placebo supplement with no exercise intervention.

Participants will be required to take four capsules per day alongside food for a period of 24 weeks. Four capsules of the active supplement contains: 2000mg fish oil (1400mg total omega 3, 1000mg docosahexaenoic acid and 160mg eicosapentaenoic acid), 20µg B12, 1000µg folic acid, 124mg phosphatidylserine, 240mg gingko biloba standardized leaf extract, 20mg vitamin E. The placebo capsules will contain an oil blend that is typical of the UK diet. The supplement is being provided by Efamol.

The exercise intervention will involve a participation in a group exercise class which will be led by a qualified instructor.

The Outcome Measure Testing

All testing will take place at Bournemouth University. You will be asked to attend sessions at the start of the study, and then after 12 and 24 weeks, with each session lasting around 2 hours. Reimbursement for travel expenses will be offered for all testing sessions.

On the first visit the following additional information will be measured and/or recorded: Age, height, weight and national adult reading test score

To measure brain function, you will need to complete a series of tests including memory and information processing. Tests of mobility will include walking speed and a five times sit to stand test. The walking tests will consist of the following tasks, and each test will be performed 5 times. These will be assessed using motion sensors which can be seen below.

- Walking at a habitual pace
- Walking at a fast pace
- Walking at a habitual pace whilst simultaneously doing a counting task
- Walking at a habitual pace whilst simultaneously doing a more difficult counting task

The sit to stand task will involve rising from a chair and sitting back down again five times.

Diagram of Motion Sensors

You will be asked to complete a short questionnaire to assess health status, which will involve answering 36 multiple choice questions.

A blood sample will be required at the start of the study and after 24 weeks. A pinprick blood sample from the fingertip will be used to assess the levels of fats in the blood. A venous blood sample will be taken at to determine serum homocysteine. All blood sampling will be performed by a fully trained member of the research team, to limit any discomfort.

To help control for other factors which may impact results, you will be asked to fill out a short food frequency questionnaire, three day diet diary and physical activity assessment questionnaire.

Loss of Ability to Consent
In the unlikely event that a participant loses the ability to consent to any of the study protocols, they will immediately be withdrawn from the study and no further testing will be performed. However any results that has already been recorded or blood samples that have been taken prior to losing the ability to consent, will still be analysed and included in the study.

What are the possible benefits of taking part?
In past research each of the interventions used in this study has shown promise in preventing age related decline in physical and brain function. By taking part you will be helping to contribute towards the body of evidence for the potential health benefits of these interventions when applied separately or in combination.

What are the possible disadvantages and risks of taking part?
Side effects associated with consuming this type of dietary supplement are rare, however if you do experience any side effects you can report this to a member of the research team or you can withdraw from the study with no explanation. The only common side effect associated with this type of supplement as mild digestive discomfort. For the purposes of monitoring adverse events you will be asked by the principle investigator for a reason for withdrawing, so this can be reported in the research, however you are not required to give this information if you do not wish to.

There is a risk of experiencing pain, discomfort or injury during mobility tasks or by taking part in the exercise intervention. All procedures are valid measures of mobility and have regularly been used in studies with older adults, and will be carried out by trained researchers in a safe environment. The exercise is low impact and will be led by a fully qualified instructor. In the unlikely case of any adverse event a first aider will be on hand throughout the testing day and the exercise sessions.

The collection of blood samples will involve some discomfort. A trained researcher will conduct these to minimize any burden to the participant.

Will my taking part in the study be kept confidential?
All the information that we collect about you during the course of the research will be kept strictly confidential. You will not be able to be identified in any reports or publications. All data relating to this study will be kept for 5 years on a BU password protected secure network

What will happen to the results of the study?
Results from the study will be written up as part of a PhD programme at Bournemouth University. Results will also be disseminated through peer reviewed journals and conference presentations. Once analysed results from the study can be reported back, to you, if you wish to receive them. Individual results will not be reported back for any of the outcome measures. You will be contacted after conclusion of the study with the necessary contact details if you wish to receive a summary of the study results.

Who is organising the research?

This study has been organised by Paul Fairbairn as part of the completion of a PhD degree programme and will be supervised by Dr. Simon Dyall Dr. Fotini Tsofliou and Dr. Jane Murphy

Who has reviewed the study?

The study proposal has been reviewed by Dr. Simon Dyall Dr. Fotini Tsofliou and Dr. Jane Murphy. This study proposal has also been reviewed in line with Bournemouth University's Research Ethics Code of Practice.

Complaints

If you have any complaints regarding any part of the research process please do not hesitate to contact Professor Vanora Hundley the deputy dean of research and professional practice at vhundley@bournemouth.ac.uk

If you have any questions regarding participation in the study please do not hesitate to contact Paul Fairbairn at pfairbairn@bournemouth.ac.uk or on the phone number 01202965008

Furthermore if you wish to contact any of the academic supervisors for the study their contact details are as follows:

Dr. Simon Dyall sdyall@bournemouth.ac.uk

Dr. Fotini Tsofliou ftsofliou@bournemouth.ac.uk

Dr. Jane Murphy jmurphy@bournemouth.ac.uk

Appendix 19 List of duties included within the development and data collection processes of the thesis along with who carried out each task

Method Development	Paul Fairbairn, Simon Dyall, Fotini Tsofliou and Andrew Johnson
Pilot/feasibility data collection and analysis	Paul Fairbairn
Participant Recruitment	Paul Fairbairn
Clinical Visit Coordination	Paul Fairbairn
Participant screening	Paul Fairbairn
Clinical Measurement (Cognitive and Mobility Testing)	Paul Fairbairn
Distribution and analysis of CHAMPS, FFQ, 3 day diet diary and SF-36 questionnaire	Paul Fairbairn
Counting and distribution of supplementation	Paul Fairbairn
Processing of supplements and exercise letters for randomisation	Simon Dyall
Venous Blood Sampling Phlebotomy	Fotini Tsofliou
Fingerpick blood sampling	Paul Fairbairn
Blood Sampling processing and analysis	Paul Fairbairn
Design of exercise class structure	Paul Fairbairn
Leading of Exercise Classes	Emma Burton
Bi-weekly participant check-ins	Paul Fairbairn
Data processing and analysis	Paul Fairbairn