The interactive impact of omega-3 fatty acids, APOE genotype and sex hormones on cognition

Matthew Graeme Pontifex

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

The impact of sex and menopause in Alzheimer's disease remains understudied despite increasing evidence of greater female risk, particularly in middle aged *APOE4* carriers. Exploratory analysis of data generated from the Cognitive Ageing Nutrition and Neurogenesis (CANN) study in humans revealed that *APOE* carrier-status and its interaction with hormone-therapy alter both circulating n-3 PUFA status and cognitive performance. Cognitive performance was greater in individuals receiving hormone-therapy, with specific *APOE4* benefits apparent. To further study the influence of sex hormones on *APOE4* status, we utilised a female *APOE*-TR mouse model, and induced ovarian failure (VCD injections). Recognition memory and spatial memory were assessed using object recognition, Y-maze, and Barnes maze. VCD abolished recognition memory in *APOE4*-TR mice ($p<0.05$), whilst *APOE4* genotype alone led to ~45% and ~15% reductions in Barnes and Y-maze performance. Molecular analysis indicated both VCD and genotype related deficits in synaptic plasticity, which were more evident in *APOE4*-VCD treated animals, with *Bdnf* gene-expression and protein levels reduced 30% and 2-fold respectively. Brain DHA levels were 13% lower in VCD treated animals, independent of genotype. Model animals were provided with DHA-rich n-3 PUFA supplementation at two physiologically relevant doses to explore treatment efficacy. Deficits in recognition memory observed in *APOE4* VCD treated mice were restored by high fish-oil supplementation ($p < 0.05$). Conversely, despite nominally increasing Barnes maze performance, high fish-oil supplementation did not significantly improve spatial memory impairment. Protein and gene-expression analysis again supported the behavioural findings with BDNF/Akt pathway significantly increased in response to high fish-oil in *APOE4* mice ($p < 0.05$). High fish-oil also increased *Igf-1*, *Mapk1* and *Ntrk2* expression, potentially explaining BDNF/Akt modulation. Both doses were effective in restoring Brain DHA levels, however the higher dose increased DHA:AA further. Together, these results indicate cognitive and neurological impacts of menopause, which are exacerbated by *APOE4,* and ameliorated through high fish-oil supplementation.

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Statement of publication

My thesis integrates three potential papers (**Published:** chapter 1, **Prepared for publication:** chapters 3 and 4) in which I am the primary author. These are combined with experimental (chapter 2) and discussion chapters (chapter 5).

Chapter 1 **Literature review: The Effect of *APOE* genotype on Alzheimer's disease risk is influenced by sex and docosahexaenoic acid status**

A large proportion of my literature review presented below has been published in Neurobiology of aging as a narrative review article in 2018 (Pontifex, Vauzour et al. 2018) and updated and expanded with literature published in the last two years.

Alzheimer's disease a global health burden

Dementia is an umbrella term encompassing a number of complex neurological and neurodegenerative disorders that manifest progressively over time, with deleterious behaviours and genetic predisposition contributing to compromised cognitive functions. Of these diseases, Alzheimer's disease (AD) is the most common, accounting for ~70% of dementia cases. AD incidence doubled from 1990 to 2016 (Nichols, Szoeke et al. 2019), making the disease a serious global health concern. Current projections estimate a further tripling of cases (152 million) by 2050 (Patterson 2018), and with no disease modifying treatments currently available, the societal, health care and economic pressures relating to this debilitating disease will undoubtedly escalate. Many promising drug candidates, most of which have been focused towards preventing amyloid beta (Aβ) pathology (production, aggregation or clearance) have unfortunately failed to convey any clinical benefit (Panza, Lozupone et al. 2019), either as a result of poor efficacy or safety concerns. Therefore, it may be time to rethink our approach, whether that be discovering more suitable targets and drug candidates or utilising validated lifestyle strategies to prevent or delay the onset.

It is well established that lifestyle factors such as diet and exercise have a modulatory impact on AD risk (Nichols, Szoeke et al. 2019). Therefore, successful implementation of an intervention in "at-risk" individuals, based upon these low-risk lifestyle factors, may offer a plausible approach to at-least reduce AD onset. Strategies capable of delaying disease onset by as little as two years, would have profound implications on current disease burden (Brookmeyer, Gray et al. 1998), with recent predictive UK models suggesting that a two or five year delay would result in a respective 19% or 33% reduction in the predicted AD prevalence by 2050 (Lewis F 2014).

APOE4 mediated AD risk

APOE4 risk, a consequence of altered protein structure

The apolipoprotein E ε4 allele (*APOE4*) is the strongest prevalent genetic risk factor for sporadic late-onset Alzheimer's disease (LOAD). Possession of one or two *APOE4* alleles, confers respectively 3-4, and 8-12-fold increased risk, and substantially reduces age of disease onset (Davidson, Gibbons et al. 2007, Heffernan, Chidgey et al. 2016).

Structurally, the mature 299 amino acid APOE protein consists of three distinct regions: 1) The N-terminal domain (1-167), an anti-parallel four-helix bundle containing the receptor-binding region (136-150); 2) The C terminal domain (192-299), a α-helical structure encompassing the lipid-binding region (260-299), and 3) a flexible hinge region (192–222) which connects the two (Hatters, Peters-Libeu et al. 2006, Phillips 2014). Three common APOE variants exist in the population. Despite each differing by only one or two amino acid changes at positions 112 and 158 (APOE2: Cys, Arg; APOE3: Cys Cys; APOE4: Arg Arg) (Hatters, Peters-Libeu et al. 2006, Phillips 2014), each are structurally distinct, with contrasting biophysical properties, such as stability, protein folding and affinity for specific molecules (e.g. lipoprotein and receptor preference) (Hatters, Peters-Libeu et al. 2006, Phillips 2014). Arginine at position 112, is unique to APOE4 and enables re-orientation of Arg-61 away from the N-terminal 4 helix bundle assisting in the formation of a salt bridge between Arg-61 (N terminal domain) and Glu-255 (C- Terminal domain) (Dong, Wilson et al. 1994). This so called domain interaction has subsequently been suggested as the molecular basis for the deleterious effects of APOE4 (Dong and Weisgraber 1996, Hatters, Peters-Libeu et al. 2006, Zhong and Weisgraber 2009, Phillips 2014). Interestingly, studies exploring the use of small molecule correctors, which aim to transform or nullify the aberrant APOE4 domain interaction have produced promising results, providing evidence for this theory (Wang, Najm et al. 2018).

Contribution of *APOE* genotype to AD risk

The human *APOE* gene, located on chromosome 19, has three common alleles, ε2, ε3 and ε4. Relative to the most common isoform *APOE3* (allele frequency 78%), the rarer *APOE2* (allele frequency 7%) is considered protective (Liu, Kanekiyo et al. 2013), whilst the *APOE4* isoform (allele frequency 14%) predisposes to AD. Systematic reviews and

meta-analyses consistently describe strong *APOE4*-AD associations. For example the *AlzGene* database reports increased odds ratios (ORs) for both heterozygous (OR: 2.8, 95% CI: 2.3–3.5) and homozygotes (OR: 11.8, 95% CI: 7.0–19.8) *APOE4* relative to 'neutral' *APOE3* homozygotes (Bertram, McQueen et al. 2007). The *APOE4* allele is concentrated within the AD population, with prevalence reaching in excess of 50% relative to the global frequency estimated at 14% (in people <65 years of age) (Hallman, Boerwinkle et al. 1991, Eisenberg, Kuzawa et al. 2010, Ward, Crean et al. 2012). Such associations are also apparent in mild cognitive impairment (MCI). *APOE-* ε*4* carriers are 3.0-3.7 times more likely to develop MCI compared to all other groups (Viticchi, Falsetti et al. 2017) and *APOE4* MCI are more likely to convert to a more severe state of MCI (mMCI) or AD (Scarabino, Broggio et al. 2016). *APOE4* has been associated with hippocampal, amygdala and medial-temporal lobe atrophy (Manning, Barnes et al. 2014, Lupton, Strike et al. 2016), which underlies the greater development and conversion rates in this genotype subgroup.

A consensus is developing that the impact of the *APOE4* allele on AD risk diminishes upon reaching extreme ages (>90 years) (Corrada, Paganini-Hill et al. 2013, Valerio, Raventos et al. 2014). Such a trend is unexpected given that the *APOE4* variant is attributed to an increased risk and reduced age of onset. The phenomenon has been attributed to the survivor effect and the fact that these individuals have other phenotypic attributes, which offer protection with many *APOE4* carriers reaching extreme ages with normal cognition (Corder, Saunders et al. 1994, Rebeck, Perls et al. 1994). The study of such individuals is likely to provide valuable insights into strategies to mitigate the effect of genotype at younger ages.

***APOE4* persistence is a product of its environment?**

Although controversial, protective, and evolutionary advantages for *APOE4* in pre-industrial societies have been reported (Oriá, Patrick et al. 2007). In such populations where malnutrition and infection is rife, carriers of *APOE4* appear to receive a survival advantage (Oriá, Patrick et al. 2007, Fujioka, Phelix et al. 2013, Huebbe and Rimbach 2017, Trumble, Stieglitz et al. 2017), and carrier prevalence is markedly increased (Abondio, Sazzini et al. 2019). This advantage is associated with *APOE4's* ability to respond to environmental stresses such as inhibiting parasite growth. In complete contrast to developed populations, Trumble and colleagues showed this can lead to protection

from cognitive impairment (Trumble, Stieglitz et al. 2017), and is a prime example of how *APOE4* mediated pathophysiology is a product of its environment.

In addition, whilst a significant risk factor in developed societies, possession of *APOE4* does not categorically determine AD outcome (Corder, Saunders et al. 1993, Liu, Kanekiyo et al. 2013). Indeed, although *APOE4* prevalence within global AD populations varies considerably ranging from 41% - 61% (Farrer, Cupples et al. 1997, Corbo and Scacchi 1999, Crean, Ward et al. 2011), only half of *APOE4* homozygotes develop AD by age 90 years (Henderson, Easteal et al. 1995). This indicates that the penetrance of the *APOE4* allele, its influence on the rate of cognitive decline and the likelihood of transitioning to MCI and AD, is variable and therefore potentially modifiable (Singh, Singh et al. 2006, Ward, Crean et al. 2012, Fenesi, Fang et al. 2017, Moser and Pike 2017).

***APOE4* is pleiotropic in AD pathogenesis**

Although *APOE4* is a well-established determinant of AD, a fundamental understanding of its role in AD pathophysiology, and clarity as to which processes are causative rather than associative, remain unclear. Instead, research has identified a range of plausible disease mechanisms, ranging from amyloid beta (Aβ) pathology to lipid and cholesterol metabolism, which highlights the pleiotropic nature of *APOE4*.

Aβ dependant mechanisms: The influence of *APOE* on Aβ metabolism and pathology has been widely described (Table 1.1). Containing an Aβ-binding motif (244-272) (Liu, Wu et al. 2011), APOE is able to interact directly with Aβ (Bales, Verina et al. 1999), and can be found co-localised with senile plaques and neurofibrillary tangles, indicating a likely involvement in the process (Strittmatter, Saunders et al. 1993). Indeed, carriers of *APOE4* are routinely found to have higher cortical Aβ concentrations (Fouquet, Besson et al. 2014), more extensive senile plaque formation (Rodriguez, Tai et al. 2014), and earlier pathological onset (Kok, Haikonen et al. 2009), which correlates with greater synapse loss and neurodegeneration (Koffie, Hashimoto et al. 2012). This has been elegantly demonstrated, in vivo by Wu and colleagues (Wu, He et al. 2017), who observed greater cognitive decline after administering intra-cerebrovenrticular co-injection of both Aβ and ApoE4 simultaneously, compared to Aβ, ApoE4 or saline alone. Overall *APOE* mediated effects lead to changes in Aβ processing affecting: aggregation, degradation and clearance, in a genotype dependant manner (*APOE- ε4 > APOE- ε3 > APOE- ε2*) (Liu, Kanekiyo et al. 2013).

Table 1.1 Complexity of the amyloid beta (Aβ)**APOE* interaction

Affected process	**Mechanism**
Accumulation	APOE receptor binding increases amyloid precursor protein transcription and Aβ secretion, in a genotype dependant manner with ApoE4 having the greatest effect (Huang, Zhou et al. 2017)
Accumulation	APOE binds Aβ oligomers and fibrils (at high concentrations) stabilising the molecule and reducing aggregation/fibril growth. APOE4 is the least effective and therefore promotes aggregation (Garai, Verghese et al. 2014)
Accumulation	APOE4 acts as a scaffold aiding and increasing oligomerization of Aβ (Hashimoto, Serrano-Pozo et al. 2012)
Clearance	APOE4 reduces apoE/Aβ complex stability, increasing soluble oligomeric Aβ levels (Tai, Bilousova et al. 2013)
Clearance	APOE4 has reduced affinity for toxic Aβ and is therefore a poor vehicle for clearance in comparison to ApoE3 (Petrlova, Hong et al. 2011)
Clearance	APOE4 disrupts Aβ clearance across the BBB (Deane, Sagare et al. 2008, Verghese, Castellano et al. 2013)
Degradation	APOE4 reduces levels of Aβ degradation enzymes (e.g. insulin-degrading enzymes) (Jiang, Lee et al. 2008, Du, Chang et al. 2009)

Aβ, Amyloid beta, BBB, Blood brain barrier

TAU phosphorylation: Defective TAU with abnormal phosphorylation, characterised by its hyper-phosphorylated state and capacity to form neurofibrillary tangles is a hallmark of many dementias, including AD. Transgenic mouse models and in vitro investigation have revealed that this deleterious tau phosphorylation increases as a result of *APOE4* expression (Tesseur, Van Dorpe et al. 2000, Meng, Tianwen et al. 2016). Interestingly diet may exacerbate this aberrant phosphorylation induced by *APOE4*, with high cholesterol intake linked to upregulation of tau kinases, and increased tau phosphorylation in *APOE* Knockout mice (Rahman, Akterin et al. 2005). However, this tau-*APOE* interaction may be reliant upon Aβ deposition (Farfel, Yu et al. 2016). Indeed

blocking the APOE-Aβ interaction appears to ameliorate both tau and Aβ pathology (Liu, Breitbart et al. 2014).

Mitochondrial dysfunction and Endoplasmic reticulum (ER) stress: The APOE4 domain interaction is fundamental to the deleterious effects the protein imposes on mitochondria and ER. Consistent with this, removal of the interaction (e.g. via mutation) restores typical non-pathological function (Zhong, Ramaswamy et al. 2009, Chen, Ji et al. 2011). In the ER, APOE4 prompts quality control processes to activate unfolded protein stress responses, whilst simultaneously targeting APOE for degradation (Zhong, Ramaswamy et al. 2009), resulting in diminished functional APOE, increased APOE fragmentation, increased ER stress and ultimately neurotoxicity. In addition both mitochondria (Chang, ran Ma et al. 2005, Nakamura, Watanabe et al. 2009), and the ER (Liang, Xue et al. 2019), are susceptible to fragmented APOE4. Although not fully understood, APOE4-induced mitochondrial dysfunction, has been proposed to occur via disrupting expression of mitochondrial energy metabolism genes (Chen, Ji et al. 2011), with both fragmented and whole APOE4 reducing mitochondrial respiratory complex expression. This may explain why cerebral glucose hypo-metabolism is observed in *APOE4* carriers before clinical AD develops (Chen, Ji et al. 2011).

Blood brain barrier (BBB) and cerebrovascular dysfunction: *APOE4* is increasingly recognised for its implications on cerebrovascular (CV) and blood brain barrier (BBB) integrity (Tai, Thomas et al. 2016). These structures are crucial in maintaining efficient nutrient supply to the energy demanding brain, and selectively restricting access to the brain parenchyma. *APOE4* results in altered cerebral blood flow rates in humans, and mouse models (Michels, Warnock et al. 2016, Wiesmann, Zerbi et al. 2016) which correlate to brain dysfunction in AD (Bangen, Restom et al. 2012). *APOE4* targeted replacement (TR) mice display visible vascular atrophy, evident from reduced blood vessel density and thickness, as revealed by immuno-histofluorescence and perfusion transport coefficients (glucose, diazepam) (Bell, Winkler et al. 2012, Alata, Ye et al. 2015). Similarly, cerebral amyloid angiopathy is increased by *APOE4* inducing inflammatory and necrotic processes (Tai, Thomas et al. 2016). The integrity of the BBB relies heavily upon *APOE*, with knockout mice developing significant BBB membrane permeability, which is highly sensitive to injury (N. Methia 2001). Interestingly, regulation of the BBB may be *APOE* dependent (Nishitsuji, Hosono et al. 2011), with *APOE4* TR mice exhibiting BBB transport deficiencies (29% reduction in ^{3}H-glucose transport across the BBB) (Alata, Ye et al. 2015), and an increased membrane leakiness (dextran and diazepam) (Bell, Winkler et al. 2012), as well as reduced cerebral

vascularisation and thinner basement membranes (Alata, Ye et al. 2015). *In vitro*, BBB models indicate dysregulated phosphorylation of the tight junction protein occludin, with APOE4 altering kinase activation in a lipoprotein receptor-related protein (LRP) dependant manner, ultimately impacting tight junction integrity (Nishitsuji, Hosono et al. 2011). Research conducted by Halliday and colleagues utilising human frontal cortex samples, further supports *APOE4*'s involvement in BBB breakdown with biomarkers of BBB degeneration significantly increased with the *APOE4* genotype (Halliday, Rege et al. 2016).

Neuroinflammation: Neuroinflammation plays a fundamental role in neurodegeneration and AD pathology (Heneka, Carson et al. 2015). It is apparent that APOE is a key regulator of inflammatory responses, with removal of *APOE* leading to greater inflammatory responses and macrophage activation (Dorey, Chang et al. 2014). Neuroinflammation is altered in an isoform dependant manner with ApoE4 behaving similarly to *APOE knockout*. The *APOE4* genotype is associated with greater pro-inflammatory responses, both generally and after neurotoxic insult, stimulating the production of cytokines (IL-1β, IL-6, IL12p40, TNF-α) and nitric oxide species (Guo, LaDu et al. 2004, Jofre-Monseny, Loboda et al. 2007, Vitek, Brown et al. 2009). These findings are reflected in mouse models in which *APOE4* mice display greater neuroinflammation and immune cell activation in comparison to *APOE3* counterparts (Belinson and Michaelson 2009, Rodriguez, Tai et al. 2014, Rodriguez, Tai et al. 2014). The increased inflammatory response exhibited by apoE4 offers an explanation as to why only *APOE4* carriers appear to more responsive to the dementia risk reduction associated with anti-inflammatory drug use (Szekely, Breitner et al. 2008).

Cholesterol and fatty acid metabolism: As a key component of lipoproteins, APOE is associated with the metabolism and transport of lipids, cholesterol and PUFA in the periphery (Chouinard-Watkins and Plourde 2014). Produced mainly by astrocytes, APOE also has a significant presence in the brain as the major and almost exclusive apolipoprotein in the central nervous system (CNS), where it has an essential role in maintaining cholesterol supply and recycling/distributing lipids. Thus, abnormalities associated with APOE4 have profound effects in the brain. Indeed APOE4 is associated with decreased lipid and cholesterol CNS transport, which has detrimental implications on neuronal repair, remodelling and synaptic plasticity (Mahley 2016). This may in part be driven by APOE4's inability to form lipid-rich APOE particles in the brain; it instead produces lipid-poor APOE particles, which are deplete of cholesterol (Hu, Liu et al. 2015).

Interestingly, *APOE4* is similarly associated with n-3 PUFA dysregulation, which in turn may modulate cholesterol homeostasis (Pincon, Coulombe et al. 2016). The interaction involving *APOE4* and n-3 PUFA will be explored extensively in the following sections.

Synaptic Plasticity: Considered as a prerequisite to developing AD (Selkoe 2002), synaptic plasticity loss of function is reportedly influenced by *APOE* genotype. *APOE4* is associated with reduced dendritic spine density and length in both human, and mouse models (Ji, Gong et al. 2003, Dumanis, Tesoriero et al. 2009). In mice such effects have been reported to occur as early as 4 months of age (Sun, He et al. 2017). Similarly APOE4 has been shown to attenuate long term potentiation in the hippocampus reducing activation of CAMK2A and p-CREB (Qiao, Gao et al. 2014). These effects may be exacerbated by Aβ and tau (Koch, Di Lorenzo et al. 2017, Wu, He et al. 2017).

Impact of Sex on AD risk

Sex disparity in MCI and AD incidence

Sex influences dementia risk and prevalence (Podcasy and Epperson 2016). Above the age of 65 years there are approximately twice as many female AD cases (Seshadri, Wolf et al. 1997). Although the higher prevalence has been attributed to longevity, the global five year longer lifespan in females (2017) can only partially explain this phenomenon (Snyder, Asthana et al. 2016), with age-adjusted prevalence and death rates consistently higher in females. The reasons for this are still unclear, although it has been suggested that increased incidence in women may be related to the loss, after menopause, of the neuroprotective effect of oestrogens, important in maintaining synaptic plasticity, neurotransmission and BBB integrity (Maggioli, McArthur et al. 2016, Karp, Mason et al. 2017, McEwen and Milner 2017). However, current clinical trials using hormone replacement therapy (HRT) have failed to yield any promising results (Marjoribanks, Farquhar et al. 2017). Interestingly, analysis of murine hippocampal expression profiles reveals that key AD associated genes affecting energy (e.g. *Foxo1*, *Igf-1*, *Ldhb* of the 37 genes found) and amyloid deposition (e.g. *Bace1* and *App* of the 10 genes found) are considerably altered prematurely in females, predisposing them to the development of the disease (Zhao, Mao et al. 2016).

A greater penetrance of an *APOE4* genotype in females, first reported in the early 90s (Payami, Montee et al. 1994), could also explain these higher AD rates. A subsequent meta-analysis, found that carrying one *APOE4* allele had a substantial effect on AD risk in females relative to non-carriers (OR: ≈4 at 65 years), whilst their male counterparts remained at similar risk (OR: ≈1 at 65 years) (Farrer, Cupples et al. 1997) (Table 1.2 and Figure 1.1). This somewhat 'understudied' association, has been reiterated over the years (Gao, Hendrie et al. 1998, Bretsky, Buckwalter et al. 1999, Holland, Desikan et al. 2013, Xing, Tang et al. 2015), including work conducted by Altmann and colleagues who observed that the conversion of healthy controls to MCI/AD in *APOE4* carriers was stronger in women (HR: Female = 1.81 Male = 1.27), with female *APOE-* ε*3/* ε*4* more likely converting from MCI to AD (HR: Female = 2.17 and Male = 1.51 versus *APOE-* ε*3/* ε*3*) (Figure 1.1) (Altmann, Tian et al. 2014). A contemporary meta-analysis from the *Global Alzheimer's Association Interactive Network* (n=27 studies, 58,000 participants) has offered novel insight into this interaction. Despite no overall significant difference between men and women on *APOE4* -AD in 55-85 year olds, the influence of sex emerged as being age-dependent. *APOE4* females were at higher risk of MCI at ages 55-70 years and of AD at 65-75 years relative to *APOE4* males, with the sexual dimorphism disappearing after 75 years (Neu, Pa et al. 2017) (also Figure 1.1). This indicates that a higher susceptibility to the *APOE4* allele in females is most evident in the decade(s) following menopause. As with the overall reduction of penetrance of genotype on AD risk at older ages described above, a loss of effect of sex may be due to selective survival of those females less sensitive to genotype or the effect of genotype being lessened by an overall higher AD risk profile (Corrada, Paganini-Hill et al. 2013).

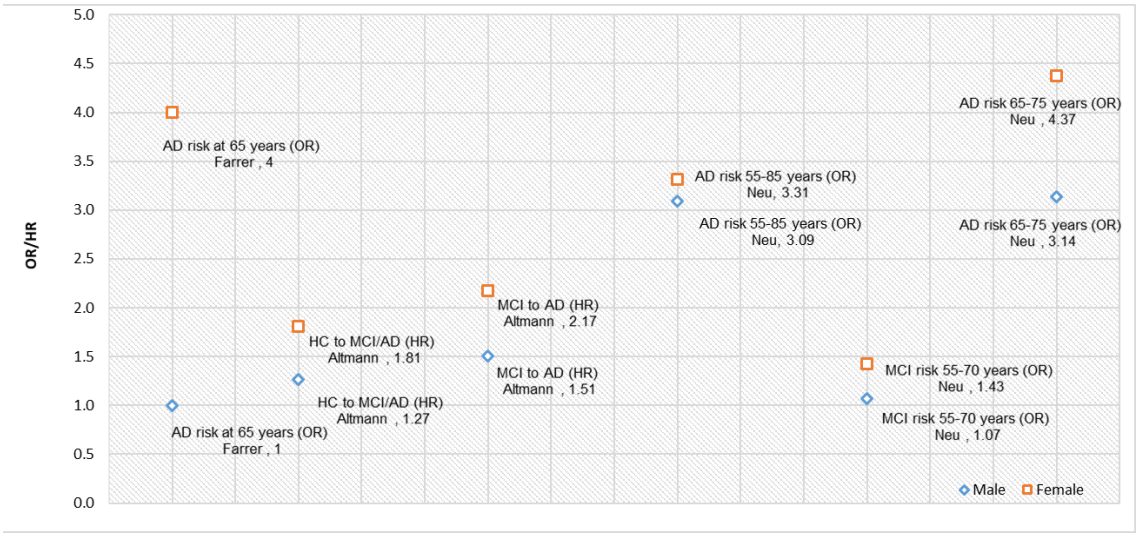

Figure 1.1 The impact of sex on Mild cognitive impairment (MCI) and Alzheimer's disease (AD) risk in *APOE-E4* carriers relative to non-carriers

From left to right in chronological order:

Farrer LA et al. 1997: Odds ratios (OR) for AD risk compared to *APOE-E3/E3*.

Altmann A. et al., 2014: Report the hazard ratios (HR) for the conversion from (a) healthy controls (HC) to MCI/AD in *APOE-E4* carriers vs. non-carrier, or (b) MCI to AD conversion in *APOE-E3/E4* vs. *APOE-E3/E3*

Neu SC et al. 2017: OD for MCI or AD compared to *APOE-E3/E3*. Sex did not influence the sensitivity of *APOE-E3/E4*-AD association in the overall group (aged 55-85 years) (P=0.53). Females versus males *APOE-E3/E4* had a higher OR of MCI (P=0.05) at ages 55-70 years (P=0.05) and of AD at 65-75 years (P=0.002).

Table 1.2 Key studies supporting the notion of an APOE-E4 *sex interaction

Study	Study type	Basic participant criteria	Follow up length	Outcome (OR/RR/HR)
Farrer (Farrer, Cupples et al. 1997)	Meta-analysis N=14,537	40 research groups contributed patient data: • *APOE* genotype • Sex • Age at disease onset • Ethnic background	NA	• In comparison with *APOE*- ε*3/* ε*3*, the sexual dimorphism in AD risk was 1.5 times greater in *APOE*- ε*3/* ε*4* individuals (P=.01) • See Figure 1.1
Altmann (Altmann, Tian et al. 2014)	Prospective cohort N=8,084 Biomarkers N=980	• Subjects were healthy controls or MCI at initial assessment • *APOE* genotype available	Minimum one follow-up at 12 months or later	• Female *APOE*- ε*4* carriers more likely to develop MCI/AD (HR=1.81 women; HR=1.27 men; P=0.011) • Compared to *APOE*- ε*3/* ε*3*, *APOE*- ε3/ ε*4* females more likely to convert from MCI to AD (HR=2.17 women; HR=1.51 men, P=0.022) see figure 1.1 • In MCI, *APOE*- ε*4* -sex interaction, significant for tau load (total tau: P=0.009; TAU:Aβ ratio P=0.020)
Sampedro (Sampedro, Vilaplana et al. 2015)	Cross-sectional study N=274	• AD Neuroimaging Initiative (ADNI) database • aged 55 to 90 years • healthy elderly control individuals • available CSF and/or MRI and/or a FDG-PET analyses	NA	• Female *APOE*- ε*4* carriers showed brain hypo-metabolism in the temporal cortex P=0.001 • Female *APOE*- ε*4* carriers showed cortical thinning in AD vulnerable areas, P<0.001

Table 1.2 Continued

Breitner (Breitner, Wyse et al. 1999)	Cross-sectional N= 5,092	• Aged ≥65 years • Population of Cache County, Utah	NA	• Female sex a risk factor for AD only in those with *APOE- ε4* (OR=1.58, P=0.02)
Neu (Neu, Pa et al. 2017)	Meta-Analysis N= ≈58 000	• 27 independent research studies • White Participants only mainly non-Hispanic • Ages 55-85 years excluded all patients with a clinical history of, or comorbidity with any other known neurological disease	Maximum follow up 10 years	• No different between sex for *APOE- ε4 -AD* risk in whole group (OR=3.09 men and OR=3.31 women, P=0.53) • Female *E3/E4* had a higher risk of AD between the ages of 65 and 75 years (OR=3.14 men and OR=4.37, P=0.002) • Female *E3/E4* had higher a risk of MCI between the ages of 55 and 70 years (OR=1.07 men and OR=1.43, P=0.05) • See figure 1.1
Fleisher (Fleisher, Grundman et al. 2005)	Cross-sectional study N= 193	• Aged 55 to 90 years • Took part in structural brain MRI • MCI at initial assessment • Good general health	NA	• Women with 1 or 2 *APOE- ε4* alleles were found to have significantly reduced hippocampal volume • Men only showed a significant reduction in hippocampal volume when carrying 2 *APOE- ε4* alleles. • Performance on delayed word recall task mirrored this trend
Alvarez (Alvarez, Aleixandre et al. 2014)	Cross-sectional study N=362	• Recruited Healthy, MCI and AD patients • Excluded all patients with any other health conditions • not taking various neurological medications	NA	• Female *APOE- ε4* carriers showed lower BDNF levels ($p<0.01$) and MMSE scores ($p<0.01$) than non-*APOE- ε4* carriers • Males did not

Table 1.2 Continued

Li (Li, Shofer et al. 2017)	Cross-sectional study N=331	• Aged 21 to 100 • medically stable • no evidence or history of cognitive or functional decline	NA	• CSF total tau and p-tau181 had no gender differences • CSF Aβ42 had age×gender×*APOE* genotype interaction, p=0.047 • Male *APOE*- ε*4*, average CSF Aβ42 decreased gradually with age up to midlife and then levelled off. • Female *APOE*- ε*4*, average CSF Aβ42 remained relatively high through to age 50 and then had a rapid decline after

Abbreviations: MCI, Mild cognitive impairment; Aβ, Amyloid beta; CSF Cerebrospinal fluid; MRI, Magnetic resonance imaging; FDG-PET; Fludeoxyglucose positron emission topography; MRI, Magnetic resonance imaging; BDNF, Brain-derived neurotrophic factor; MMSE, Mini Mental state examination

Female sex exacerbates the neurocognitive impact of an *APOE*- ε4 genotype

Limited human cognitive and biomarker data supports the sexual dimorphism evident in epidemiological (incident disease) studies, indicating earlier onset and more extensive pathology in female *APOE4* carriers. Differences in cerebrospinal fluid (CSF) TAU and $A\beta_{42}$ load (Altmann, Tian et al. 2014, Li, Shofer et al. 2017) along with aberrant Aβ/secretase profiles in autopsy samples (Nyarko, Quartey et al. 2018) and brain hypometabolism and cortical thinning (Sampedro, Vilaplana et al. 2015) have been observed between female and male *APOE4.* Fleisher et al. observed reduced hippocampal volume and memory performance in female relative to male *APOE4* carriers, in whom significant pathological changes only occurred when in possession of two *APOE4* alleles (Fleisher, Grundman et al. 2005). Analysis of >5000 brain samples of varying ages found that women carrying the *APOE- ε4* gene had more extensive neurofibrillary tangles and senile plaques, with onset of pathology beginning considerably earlier (Corder, Ghebremedhin et al. 2004). Neuroprotective immune cell (Aβ-specific CD4+ T cell) decline was found to occur 10-15 years earlier in female carriers compared to that of male carriers (Begum, Cunha et al. 2014). Finally, levels of brain-derived neurotrophic factor (BDNF), an important modulator of neuron survival and growth in areas associated with memory, have been found to be significantly reduced in *APOE4* females relative to age matched *APOE4* males, and to correlate to poorer MMSE scores (Alvarez, Aleixandre et al. 2014).

Rodent studies also highlight such a trend. It is widely accepted that female *APOE4* targeted replacement (TR) mice present a more extreme phenotype (Raber, Wong et al. 1998, Rodriguez, Burns et al. 2013), with greater cognitive decline evident from their poorer performance on a battery of behavioural tests (Grootendorst, Bour et al. 2005, Bour, Grootendorst et al. 2008, Rodriguez, Burns et al. 2013, Heneka, Carson et al. 2015), and a greater extent of neurodegeneration (Rijpma, Jansen et al. 2013, Koutseff, Mittelhaeuser et al. 2014). For example Bour et al., demonstrated that 15-month female *APOE4*-TR mice had significantly greater deficits in spatial learning and memory compared with male *APOE4* mice (Bour, Grootendorst et al. 2008). This is consistent with findings of Rijpma et al. who found middle aged *APOE4*–TR female mice had decreased presynaptic density within the hippocampus, which was not found in *APOE4*-TR males (Rijpma, Jansen et al. 2013). Wang et al, reported learning and memory impairment, occurring at a much younger age in *APOE4*/3xTg (sporadic and familial genes) females, which coincided with higher Aβ proteins and β-site amyloid precursor

protein cleavage enzyme (BACE1) when compared to female non-Tg, female 3xTg and male *APOE4* /3xTg mice (Wang, Hou et al. 2016).

The influence of sex on *APOE* associated DHA dysregulation is currently limited, however our labs recent findings (Martinsen, Tejera et al. 2019), in which a female dependant drop in DHA in *APOE4*-TR mice was observed, supports the notion and warrants further investigation. This may potentially result from the known influence of sex on liver and systemic fatty acid synthesis and metabolism, and the differential impact of *APOE4* on brain DHA uptake and status (addressed below in section 'DHA availability and transport to the brain').

Menopause and sex hormone dysregulation as a potential explanation for sex-*APOE4* associations in AD

The process of menopause may offer an explanation for the greater AD susceptibility exhibited by females (Li, Cui et al. 2014). It may also help to explain why females appear to be predisposed to the effects of *APOE4* carrier status (Yun, Park et al. 2007, Moser and Pike 2016, Mosconi, Berti et al. 2017, Zokaei, Giehl et al. 2017). Earlier onset of menopause correlates with poorer cognition later in life (Ryan, Scali et al. 2014). The abrupt hormone dysregulation caused by the menopausal process, is likely to have significant implications on brain processes and cognition with the neuroprotective importance of oestrogens and progesterone well documented in the literature (reviewed extensively (Vest and Pike 2013, Depypere, Vierin et al. 2016)). A recent study using multimodal brain-imaging techniques highlighted the impact of menopause in AD development, with indicators of AD, such as hypo-metabolism, increased Aβ deposition and reduced grey and white matter brain volumes all evident to a greater extent as a result of menopause (menopause > perimenopause > no menopause) even after controlling for age and education (Mosconi, Berti et al. 2017). Interestingly, *APOE4* carrier status exacerbated menopausal Aβ deposition relative to other groups, which indicates an *APOE4* -menopause interaction (Mosconi, Berti et al. 2017).

One would speculate that re-establishing the hormone profile altered in menopause would ameliorate these deleterious effects. However, inconsistent effects of HRT have been reported in both the general population and *APOE4* carriers. These discrepancies are likely attributable to the heterogeneity in the initiation, treatment period, dose and combination of hormones used. Although currently inconclusive and sparse, the limited research available, addressing the impact of *APOE* genotype on the efficacy of hormone

therapy appears to complicate this paradigm further with reports of both improvements (Yun, Park et al. 2007, Jacobs, Kroenke et al. 2013, Kunzler, Youmans et al. 2014), and no effect (Yaffe, Haan et al. 2000, Kang and Grodstein 2012, Kunzler, Youmans et al. 2014), in relation to cognition for carriers of *APOE4*. Although oestrogens and activation of their receptors are known to alter *APOE* expression (Corbo, Gambina et al. 2006, Wang, Irwin et al. 2006), the molecular aetiology of possible *APOE* genotype-hormone-cognition interactions is not known.

Further research is needed to establish the benefits of hormone intervention during menopause and whether the effects are *APOE* genotype dependent, thereby warranting stratified approaches.

Docosahexaenoic acid (DHA): A dietary component implicated in AD risk

The main dietary n-3 fatty acids are alpha-linolenic acid (α-LNA) mainly from plant sources, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), with oily fish and fish oil supplements being the main dietary source of EPA and DHA (Innes and Calder 2020).

DHA, is a 22-carboxylic fatty acid consisting of 6 C=C double bonds (22:6n-3). An important constituent of the CNS, DHA can be synthesised to a limited extent from α-LNA or obtained directly from the diet.

DHA in the brain

DHA is particularly concentrated in the brain (Arterburn, Hall et al. 2006), and accounts for 15% of the total fatty acids in the cerebral cortex. This dwarfs the levels found in the peripheral tissue such as the heart and liver, where DHA contributes about 2% (Arterburn, Hall et al. 2006). DHA is distinctly associated with the grey matter (Bradbury 2011), and is highly concentrated within metabolically active neuronal regions (Bradbury 2011, Crawford, Broadhurst et al. 2013), including synaptic membranes, synaptic vesicles and mitochondria (Neuringer, Anderson et al. 1988). DHA concentration varies across phospholipid species, and is abundant within phosphatidylserine (PS) and phosphatidylethanolamine (PE), where DHA makes up around one third the total fatty acid composition (Neuringer, Anderson et al. 1988, Lauritzen, Hansen et al. 2001).

Synthesis of DHA in the brain occurs to a very limited extent (Igarashi, DeMar et al. 2007), failing to increase even in times of n-3 PUFA deprivation (Igarashi, DeMar et al. 2007). Brain DHA is supplied from the systemic circulation, with the DHA provided from the diet or synthesised mainly in the liver from its shorter chain precursor a-linoleic acid (α-LNA) (Domenichiello, Kitson et al. 2015), through the actions of desaturase and elongase enzymes (Igarashi, Ma et al. 2006).

Upon entry into the brain, DHA is activated and esterified to the cell membrane phospholipids at the sn2 position, where it is believed to have a range of beneficial structural and functional roles. The unique highly polyunsaturated, kinked structure of DHA increases membrane fluidity, regulating the properties of the membrane and therefore affecting a range of properties including membrane protein function. DHA is also associated with promoting antioxidant processes, and altering gene expression profiles (Hashimoto, Hossain et al. 2016). This occurs through the release of DHA from the membrane under the action of phospholipase A2. Although most is quickly re-esterified and conserved, release from the membrane allows DHA to partake in multiple signalling and regulatory processes, the most documented of which is DHA's role in neuroinflammation. The metabolism of DHA via enzymatic processes (e.g. cyclooxygenase, or lipoxygenase) or free radical oxidation, transforms DHA into a plethora of bioactive lipid metabolites such as resolvins, protectins and maresins (Kuda 2017, Martinsen, Tejera et al. 2019), known collectively as specialised pro-resolving mediators (SPMs), which are renowned for ability to resolve inflammation.

Does DHA intake and status affect dementia risk?

Prospective epidemiological studies, summarised by a number of meta-analyses, indicate that a higher consumption of fish and oily fish, the almost exclusive dietary source of DHA is associated with reduced dementia and AD risk (Table 1.2) (Kalmijn, Launer et al. 1997, Barberger-Gateau, Letenneur et al. 2002, Morris, Evans et al. 2003, Barberger-Gateau, Raffaitin et al. 2007, Giri, Zhang et al. 2016). To put this beneficial effect into perspective, the meta-analyses conducted by Wu and colleagues reported an 11% risk reduction in AD with each 100g increment of fish consumption per week (Wu, Ding et al. 2015), with Zhang et al. observing that a 0.1g increment in DHA per day was associated with 14% and 37% lower risks of dementia and AD respectively (Zhang, Chen et al. 2016). This is particularly relevant since the current UK recommended intake of

EPA+DHA is >450mg per day provided as two portions (each 140g) of fish per week one of which should be oily

Higher circulating DHA concentrations have been associated with improved cognition and reduced dementia and AD risk. Conquer et al. reported that AD patients have lower plasma DHA levels, in the total phospholipid, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions (Conquer, Tierney et al. 2000). This was reiterated in the Framingham study where those in the top quartile of plasma PC-DHA had a 47% lower dementia risk relative to the bottom quartile (Schaefer, Bongard et al. 2006), which is consistent with the *Rancho Bernado* study (Lopez, Kritz-Silverstein et al. 2011). Interestingly those in the Framingham study in the lowest RBC DHA quartile also had lower total brain volume and performed poorer on a range of cognitive tests including visual memory, executive function and abstract thinking tasks (Tan, Harris et al. 2012). Further to this Yassine et al reported an association between serum DHA and cerebral amyloidosis and brain volumes (particularly those areas affected by AD) (Yassine, Feng et al. 2016). Although not fully consistent, post mortem brain analysis also indicate that DHA levels are reduced in AD (Söderberg, Edlund et al. 1991, Fraser, Tayler et al. 2010). This beneficial association appears to also continue into later life. For example, Yassine et al. looked specifically at elderly individuals and found participants within the lowest serum DHA quartile to have significantly more cerebral amyloidosis (Yassine, Feng et al. 2016), However it appears there is little research examining the effects of DHA within the oldest old (85+ years), an area which warrants investigation.

A high oily fish and DHA consumption is associated with a higher socio-economic status, with such subgroups typically being more educated, engaging in higher physical activity, having lower prevalence of smoking and higher consumption of plant based foods, all of which have been shown to be neuroprotective. Although data analysis approaches correct for the impact of such co-variates, not all analyses are fully comprehensive and there may be other associated behavioural attributes which have not been considered. Therefore, a proportion of the reported benefits of fish/DHA may in certain instances be attributable to the residual confounding effect of these associated factors.

Although collectively the observational data demonstrate that a high, habitual oily fish and DHA intake, and DHA status, are associated with a reduction in AD risk, these findings have not been consistently supported by randomised controlled trials (RCTs). RCTs have yielded conflicting results; some displaying promise (Vedin, Cederholm et al. 2008, Yurko-Mauro, McCarthy et al. 2010, Lee, Shahar et al. 2013, Stonehouse, Conlon

et al. 2013), whilst others have failed to establish any beneficial effects (Quinn, Raman et al. 2010, Quinn, Rema et al. 2010, Phillips, Childs et al. 2015). Stonehouse et al. observed improved memory and reaction time in healthy young adults supplemented with 1.16g DHA for 6 months (Stonehouse, Conlon et al. 2013). In the *Alzheimer's Disease Cooperative Study* (ADCS) which recruited those with mild to moderate AD, no cognitive benefits were evident following the consumption of 2g DHA daily for 18m (Quinn, Raman et al. 2010). RCTs to date have been predominately in individuals with existing MCI or AD who will have already experienced significant neuronal loss, thereby potentially missing the 'window' of preventive and therapeutic intervention. It is increasingly understood that the initial neuropathology which ultimately manifests in clinical AD, typically occurs some 20-30 years before any noticeable and measurable effects on cognition (Braak and Del Tredici 2011). Therefore, interventions, such as DHA, should target pre-clinical at-risk and prodromal groups, in order to maximise the individual and population benefits.

Apparent inconsistencies in RCT findings are also likely to be due to other heterogeneous elements of experimental design, such as DHA dose, habitual DHA status of trial participants with likely greater benefit in those with a low baseline status, typical of western populations (Stonehouse, Conlon et al. 2013) and length of intervention. Brain DHA half-life is estimated to be up to 2.5 years, with mechanisms in place to conserve DHA in times of deprivation (Rao, Ertley et al. 2007, Rapoport, Rao et al. 2007, Umhau, Zhou et al. 2009). Many RCT are therefore too short to result in appreciable differences in brain DHA with any observed benefits reliant on changes in systemic process such as improved cardiovascular health

Pre-clinical and *in vitro* studies suggest DHA benefit

Interestingly, a number of cellular and pre-clinical studies demonstrate that DHA can ameliorate deleterious biological processes associated with AD, including some of those linked to an *APOE* genotype, as described above. Supplementing AD mouse models with a 0.6% DHA diet significantly reduced Aβ plaque formation (Lim, Calon et al. 2005, Teng, Taylor et al. 2015) and affected amyloid precursor protein processing in Tg2576 mice (Lim, Calon et al. 2005). In addition, DHA was also observed to modulate Aβ aggregation by stabilising oligomers in APP/PS1 mice (Teng, Taylor et al. 2015). This is consistent with *in vitro* findings (Hashimoto, Shahdat et al. 2009). Similarly, AD rodent

models have revealed that DHA supplementation reduces aberrant phosphorylation of TAU resulting in improved cognitive performance in 3xTg-AD mice (Arsenault, Julien et al. 2011). With DHA known to modulate cellular inflammation by multiple mechanisms (Calder 2017), it is of no surprise that DHA supplementation reduces neuroinflammation. Combined EPA and DHA supplementation prevented cytokine expression and alterations in astrocyte morphology in aged wild type C57BL/6 mice (Labrousse VF 2012). Hopperton and Thomas reported an effect of DHA supplementation on microglia activation (Hopperton, Trepanier et al. 2016) and inflammatory precursor expression (Thomas, Garg et al. 2013) in the same model. It should be noted however that such rodent studies tend to employ supra-physiological doses of DHA (human equivalent of greater than 2g) (Arsenault, Julien et al. 2011, Chouinard-Watkins, Vandal et al. 2017) for a substantial proportion of the mouse lifespan (Lim, Calon et al. 2005, Arsenault, Julien et al. 2011, Teng, Taylor et al. 2015). These strategies are utilised in the attempt to maximise the chance of observing cognitive benefit. However due to the large doses used, and relatively long intervention periods (as a proportion of total lifespan) translation to humans is often unrealistic. Despite their importance in the research of complex disease, current rodent models of AD have recognised limitations (McGowan, Eriksen et al. 2006), associated with dissimilar human-to-mouse lifespan and environmental conditions. As a result pathological protein profiles may differ chemically and morphologically, and human AD pathology is not fully recapitulated (Richardson and Burns 2002). Such inconsistencies in regard to plaque pathology may produce ‘effect sizes’ in mouse models which cannot be reached in humans, which need to be appreciated when interpreting rodent data. In addition, a wide range of rodent AD models exist, each with unique ageing profiles and pathological progressions, strengths and limitations. These need to be taken into account when collectively interpreting the results from rodent experimentation (Tai, Youmans et al. 2011).

Table 1.3 Prospective cohort studies reporting on fish and DHA intakes and dementia risk

Study	Study type	Basic participant criteria	Follow up length	n-3 PUFA intake or status	Outcome (OR/RR/HR)
Barberger-Gateau (Barberger-Gateau, Raffaitin et al. 2007)	Prospective cohort N=8085	• ≥65 years • Dementia free at baseline • Male and Female	4 years	1. Weekly consumption of fish 2. Regular n-3 rich oil intake	1. Reduced AD risk (HR 0.65, 95% CI 0.43 to 0.994) 2. Reduced all cause dementia risk (HR 0.60, 95% CI 0.40 to 0.90) in *APOE- ε4* non carriers only 3. Reduced dementia risk (HR 0.46, 95% CI 0.19 to 1.11)
Morris (Morris, Evans et al. 2003)	Prospective cohort N=815	• 65–94 years • Dementia free at baseline • Male and Female	Average 3.9 years	1. Weekly consumption of at least one portion of fish 2. Total intake of n-3 PUFA	1. Reduced AD risk (RR, 0.4; 95% CI 0.2 to 0.9) 2. Reduced AD risk with increasing n-3 PUFA intake (highest quintile RR, 0.3; 95% CI 0.1 to 0.9)
Schaefer (Schaefer, Bongard et al. 2006)	Prospective cohort N=899	• 55-88 years • Dementia free at baseline • Male and Female	9.1 years	1. Highest quartile plasma PC-DHA levels – equating to 3 servings of fish per week	1. Reduced all cause Dementia risk (RR 0.53, 95% CI 0.29-0.97) and AD risk (RR 0.61, 95% CI 0.31 to1.18)

Table 1.3 Continued

Huang (Huang, Zandi et al. 2005)	Prospective cohort N=2233	• ≥65 years • Dementia free at baseline • Male and Female	0.1 to 8.4 years	1. Fatty fish consumption ≥ times per week	1. Nominal but not significant Reduction in dementia risk (HR 0.79; CI 0.53 to 1.20) in *APOE*- ε*4* non carriers only 2. Nominal but not significant Reduction in AD risk (HR 0.69; 0.91 to 1.22) In *APOE*- ε*4* non carriers only
Devore (Devore, Grodstein et al. 2009)	Prospective cohort N=5395	• ≥55 years • Dementia free at baseline • reported dietary information at baseline • Male and Female	Average 9.6 years	1. High fish intake 2. n-3 PUFA intake	1. Those who had a high fish intake (hazard ratio: 0.95; 95% CI: 0.76, 1.19) and those consuming fatty fish (HR: 0.98; 95% CI: 0.77 to 1.24) had a similar dementia risk when compared to those who typically ate no fish. 2. Participants in the lowest tertile of long-chain N-3 intake, had similar risk of dementia to those in the highest tertile (HR: 0.97; 95% CI: 0.77 to 1.21)
Wu (Wu, Ding et al. 2015)	Meta-analysis of prospective cohort studies N=22402	• Prospective cohort studies	Minimum one year follow up	1. Higher intake of fish 2. Higher long-chain n-3 fatty acid intake 3. Increasing 100g per week increment of fish	1. 36% reduced AD risk compared with lowest intake group (95% CI 8 to 56%). This increased further if using only studies with longer follow up. 2. No statistically significant association with long-chain omega-3 fatty acids intake and AD risk reduction (RR = 0.89, 95% CI 0.74–1.08). 3. 11% reduced AD risk (RR = 0.89, 95% CI 0.79 to 0.99).

Table 1.3 Continued

Zhang (Zhang, Chen et al. 2016)	Meta-analysis of prospective cohort studies N= 181580	• Prospective cohort studies	2.1-21 ears	1. 1 serving of fish per week 2. 0.1-g per day increment of DHA	1. Lower dementia (RR: 0.95; 95% CI: 0.90 to 0.99), and AD risk (RR: 0.93; 95% CI: 0.90 to 0.95) 2. Lower dementia (RR: 0.86; 95% CI 0.76 to 0.96) and AD risk (RR: 0.63; 95% CI 0.51 to 0.76)

AD, Alzheimer's disease; CI, confidence interval; HR, hazard ratio; OR, odds ratio; PUFA, polyunsaturated fatty acid; PC-DHA, phosphatidylcholine-docosahexaenoic acid; RR, relative risk.

DHA availability and transport to the brain

Transport to the Brain

Effective circulatory transport and BBB uptake of DHA, is crucial for maintaining brain supply. DHA derived from the diet, synthesised in the liver or released from reserves in adipose tissue is packaged into various plasma pools for transportation (Lefkowitz, Lim et al. 2005), either in lipoproteins (esterified as triglycerides (TAG), diacylglycerol, phospholipids (PL) or cholesteryl esters or bound to albumin as non-esterified DHA or esterified to lyso-phosphatidylcholine (LPC) (Lagarde, Bernoud et al. 2001). The importance of each pool in supplying brain DHA is still widely debated; however, current evidence indicates that the albumin bound fractions are the predominant sources, with the non-esterified DHA pool being quantitatively the major physiological pool despite LPC-DHA being preferentially incorporated after a single dose (Chen, Kitson et al. 2015). The observation of adequate brain DHA status in LDL and VLDL receptor knockouts, indicates that direct lipoprotein uptake of DHA is not a significant source (Chen, Ma et al. 2008, Rahman, Taha et al. 2010). A more complete understanding of the ability of DHA pools and dietary sources to target the brain is of high therapeutic relevance, potentially leading to more effective use of DHA intervention to support healthy brain ageing.

DHA transport across the blood brain barrier

The movement of DHA across the BBB may be passive, transporter-mediated or a combination of both processes (Figure 1.2). Rodent studies and model membranes have demonstrated that un-esterified DHA can freely cross the BBB via simple diffusion (yellow arrow) (Ouellet, Emond et al. 2009), supporting the notion, that diffusion alone is adequate. Cluster of differentiation 36 (CD36) was previously believed to be a transporter of un-esterified DHA, however CD36 knockout mice do not display DHA deficits in the brain (3 – red arrow) (Song, Elbert et al. 2010). Similarly, the importance of lipoprotein mediated DHA uptake has been questioned; with LRP receptor knockout mice exhibiting normal DHA brain levels and supply (4 – red arrow) (Chen, Ma et al. 2008, Rahman, Taha et al. 2010). Instead, it is now proposed that under the action of lipoprotein lipase at the BBB, lipoprotein DHA is released as free fatty acid enabling simple diffusion across the BBB (5) (Sovic, Panzenboeck et al. 2005, Chen and Subbaiah 2013).

In contrast, major facilitator superfamily domain-containing protein 2 (MFSD2A) provides evidence that transporter mediated routes are indeed necessary for sufficient DHA transport to the brain. Expressed in the BBB endothelium, MFSD2A appears to be an integral part of DHA transport across the BBB and specifically transports LPC esterified DHA (6 – green arrow) (Nguyen, Ma et al. 2014). *Mfsd2a*'s importance is evident from mice lacking the transporter. These knockout mice exhibit 58.8% reduced brain DHA levels, accompanied by hippocampal and cerebellum neurodegeneration, which manifests as significant cognitive impairment (Nguyen, Ma et al. 2014, Wong, Chan et al. 2016).

In addition to MFSD2A, various fatty acid binding proteins (FABP) have been implicated in DHA uptake and retention. Pan et al. discovered a role for the carrier protein FABP5, which is the most expressed FABP at the BBB (Lee, Kappler et al. 2015). Confirming that FABP5 binds un-esterified DHA with high affinity, the group optimised an in *situ* trans-cardiac perfusion technique and deduced that transport of ^{14}C-DHA, was reduced by 36% in *Fabp5* deficient mice (Pan, Scanlon et al. 2015). They recently continued this line of research, describing how *Fabp5* knockout mice have a 14% reduction in cortical DHA levels. Loss of FABP5 and subsequent reduction in DHA transport translates to significant cognitive deficits, illustrating the importance of both FABP5 and DHA in cognitive health (Pan, Short et al. 2016). Interestingly endothelial cell uptake of DHA was significantly impaired (-48%) whilst cell transport was unaffected (Pan, Short et al. 2016), providing evidence that FABP5 influences uptake, and not endothelial cell transport, as originally thought (7 – green arrow and 8 – red arrow). Finally, evidence has emerged that supports a role for fatty acid transport protein 1 (FATP1) in DHA supply to the brain (Ochiai, Uchida et al. 2016). FATP1 appears to be a basal membrane transporter with initial cell studies suggesting it contributes to DHA transport across the BBB (9 – green arrow) (Ochiai, Uchida et al. 2016). However, *in vivo* follow up experimentation, is required to confirm these initial findings.

In summary, although DHA has been observed to cross the BBB via simple diffusion, there appears to be protein mediated processers in place to ensure an adequate supply of this essential molecule, as evidenced by *Mfsd2a* and *Fabp5* knockout rodent model data. Further characterisation of these transporters in aged mice is required to confirm their importance in adult, non-developmental related DHA brain transport. Overcoming DHA transport deficits may enhance DHA supply to the brain, subsequently improving cognitive health. Potential methods to achieve this include: supplying DHA in a highly bioavailable pool, altering transporter BBB kinetics to favour DHA uptake, or increasing

overall blood DHA status. These methods could be utilised to compensate for the reduced delivery efficiency associated with BBB transport deficits e.g. associated with ageing (Graf, Duchateau et al. 2010) or *APOE* genotype.

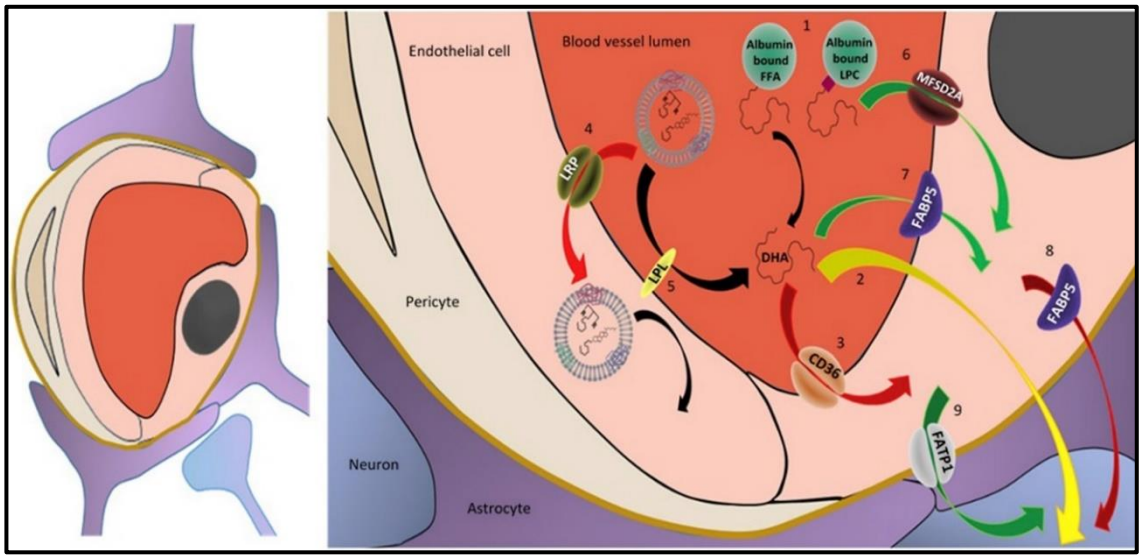

Figure 1.2 DHA - BBB transport mechanisms

Plasma DHA is associated with lipoproteins or albumin depending on esterification status (1). Un-esterified DHA can cross the BBB via simple diffusion (2). The uptake of DHA via CD36 (3) and LRP (4) mediated processes are now considered insignificant. Lipoproteins may still contribute as a DHA pool under the action of lipoprotein lipase at the BBB (5). Mfsd2a specifically aids the uptake of LPC-DHA into the endothelium (6). FABP5 is implicated in the transport of DHA across the BBB; it is thought that this is achieved via endothelial uptake (7) and not cellular transport (8). FATP1 may enhance transport of DHA across the basal membrane (9). Green arrow: Potential transport process Yellow arrow: Simple diffusion Red arrow: Disproved transport process. Adapted from Bazinet et al (Bazinet and Laye 2014)

The influence of *APOE* genotype on DHA metabolism and status

Human epidemiological evidence along with RCT data indicate that *APOE4* carriers are less responsive to the neuroprotective benefits of higher DHA intakes (Huang, Zandi et al. 2005, Quinn, Raman et al. 2010, Yassine, Braskie et al. 2017). Such findings are in agreement with results demonstrating that *APOE4 TR* mice have significantly lower brain DHA concentrations (9% reduction in cortex compared to *APOE2*) (Vandal, Alata et al. 2014), and more recent insights suggesting this may occur to a greater extent in females (Martinsen, Tejera et al. 2019), therefore indicating impaired DHA transport and/or

metabolism associated with an *APOE4* genotype. Uptake of DHA into the brain has been shown to be significantly reduced in *APOE4 TR* mice and to progressively decline with age (Vandal, Alata et al. 2014), which may be suggestive of BBB transport impairment. This potential transport impairment in *APOE4* rodents is consistent with findings from the Alzheimer's Disease Cooperative Study (ADCS) trial in humans where lower DHA levels in the cerebrospinal fluid in *APOE4* carriers following DHA supplementation was observed (Yassine, Rawat et al. 2016, Tomaszewski, He et al. 2020). The molecular aetiology of the proposed reduced BBB transport of DHA in *APOE4,* and in particular any effect on the protein mediated transport mechanisms described above, is currently unknown.

A recently conducted PET study in humans, exploring the incorporation of ^{11}C-DHA surprisingly reported that young *APOE4* carriers have increased DHA brain delivery and incorporation rates (Yassine, Croteau et al. 2017). Although these findings need to be confirmed in a larger study, this apparent increase, contradicts the suggested *APOE4* transport deficits. This may represent a compensatory mechanism to overcome DHA loss via altered brain metabolism. The progression to further transport/metabolism deficits observed in aged *APOE4* carriers may overwhelm this compensatory process leading to eventual reduced brain DHA concentrations and predisposing carriers to AD. Further investigation is needed to fully establish the effect of *APOE4* on DHA transport and brain delivery and metabolism and how they are affected by age.

An altered systemic DHA metabolism, associated with lower overall DHA availability in *APOE4* carriers may also underlie a lower brain DHA status. Chouinard-Watkins et al, observed (using radiolabelled ^{13}C DHA) a reduced DHA plasma incorporation (31% less), and significantly increased DHA β-oxidation (80%) in elderly *APOE4* carriers (Chouinard-Watkins, Rioux-Perreault et al. 2013) relative to *APOE4* non-carriers. The greater hepatic uptake and β-oxidation of DHA potentially explains the lower plasma response observed in *APOE4* carriers with high-BMI (Chouinard-Watkins, Conway et al. 2015). Altered DHA metabolism is apparent in the liver of *APOE4 TR* mice, which express *APOE* under murine *Apoe* promoter producing a physiologically relevant *APOE* model (Tai, Youmans et al. 2011). In these mice, PUFA homeostasis is significantly dysregulated, potentially as a result of altered fatty acid transporter expression, specifically FABP1 (Chouinard-Watkins, Pincon et al. 2016). Interestingly supplementation with a relatively large dose (3g/day) of DHA restored these deficits observed in the liver (Chouinard-Watkins, Pincon et al. 2016). In light of this observations, supplementation with larger doses of DHA (up to 3 g/day) in *APOE4*

carriers may present a plausible route to overcome the transport and metabolism deficits associated with *APOE4* (Chouinard-Watkins, Vandal et al. 2017). Chouinard-Watkins and colleagues showed such supplementation prevented memory decline in *APOE4* rodent model with *APOE4* TR mice performing similarly to both *APOE3* TR mice fed a normal diet and the diet highly enriched with DHA (Chouinard-Watkins, Vandal et al. 2017, Yassine, Braskie et al. 2017). Given the apparent deficits in DHA transport and metabolism in those with an *APOE4*, alternative intervention strategies should also be considered.

To our knowledge, the impact of sex and menopausal status on DHA status and BBB transport and its interaction with *APOE* genotype is currently completely unknown. It is proposed as a high priority research area to provide insight into the mechanistic basis for the greater effect of the *APOE4 allele* in females.

Literature review conclusions and research gaps

The implications of the *APOE4* genotype in cognition and AD risk are far-reaching and complex, detrimentally influencing a range of AD mechanisms from Aβ deposition to DHA metabolism. This makes the process of developing a mitigating intervention challenging. The extent of *APOE4's* influence appears to be modulated to some extent by a triad of factors namely sex, age (with impact on menopausal status in women) and DHA status. Targeted nutrition strategies may overcome the negative influence of these factors, attenuating genetic vulnerability and 'tipping the balance' in favour of AD prevention, particularly if such an intervention is implemented early in the disease process. From current evidence, advocating the consumption of oily fish and DHA appears a robust approach to reduce AD risk and in *APOE4* carrier's higher doses may be warranted to achieve the benefit associated with non-carriers, due to impairment in transport and metabolism. This may also be true for the elderly who display an age dependant transport deficit, and females where the effects of the *APOE4* genotype are exacerbated.

Characterisation of the impact of sex and menopausal status on, brain DHA uptake and metabolism and responsiveness to DHA intervention, independent of, and according to *APOE* genotype status are identified as research priority area. Dose-response studies in sex by *APOE* genotype groups are needed to identify effective DHA intake doses and develop stratified nutrition intervention approaches.

Research gaps

With no current disease modifying treatments currently available, Alzheimer's disease poses an ever-increasing health burden globally (Nichols, Szoeke et al. 2019). To date, potential AD therapeutics have failed to show significant clinical effectiveness or safety required for therapeutic use. In addition, the multifactorial nature of AD may render specific drug targets redundant for a proportion of the AD/at risk population. Therefore, alternative strategies are warranted.

DHA and EPA are pleiotropic in their neuroprotective actions and are known to modify multiple AD related mechanistic pathways. Furthermore, regular consumption of long chain n-3 PUFA (Barberger-Gateau, Raffaitin et al. 2007, Zhang, Chen et al. 2016), and greater n-3 PUFAs status are associated with reduced AD risk and improved cognition. As a result, n-3 PUFA supplementation has been proposed as a potential intervention strategy to overcome *APOE4* mediated AD risk. Reports however suggest, that cognitive responsiveness to DHA intake is lower in *APOE4* individuals (Metherel, Armstrong et al. 2009, Kofler, Miles et al. 2012, Childs, Kew et al. 2014, Walker, Browning et al. 2014, Minihane 2016, Davis, Hecht et al. 2017, Slim, Vauzour et al. 2017), and therefore establishing an optimal dosage for *APOE4* carriers is warranted. In addition, the pathological impact of *APOE4* carrier status appears to be modified by sex, with female carriers found to have increased MCI or AD risk between the ages of 55 and 70 years compared to their male counterparts (Farrer, Cupples et al. 1997, Neu, Pa et al. 2017). This may suggest a possible role of menopausal transition, however research exploring the impact of menopause on *APOE4* genotype is limited.

If validated, then those with an *APOE4* genotype, particularly peri/post-menopausal females, are a large 'at-risk' population group who could be targeted for preventive intervention, such as LC n-3 PUFA supplementation. Even strategies capable of delaying disease onset by as little as two years would have profound implications on current disease burden (Brookmeyer, Gray et al. 1998).

Hypothesis and study overview

Research Question and Overview

The overall aim of this PhD project is to investigate the relationship between *APOE* genotype, menopause and n-3 PUFA status on cognitive outcomes. We aim to establish if a menopause **APOE4* interaction exists for circulating and brain n-3 PUFA status and brain DHA transport proteins. Furthermore, we explore whether n-3 PUFA supplementation can ameliorate *APOE4* and menopausal related deficits in cognition, along with their underlying molecular mechanisms. To achieve this we fulfil the following objectives: 1) Explore the relationship between *APOE* genotype, hormone replacement therapy use, and fish oil intake on n-3 PUFA status and cognition utilising data obtained from the CANN study (NCT02525198. (Irvine, Scholey et al. 2018)), 2) Establish an *APOE*-TR model of menopause, 3) Study the combined impact of menopause and *APOE4* on cognition and identify contributing mechanisms. 4) Determine the effectiveness of two clinically relevant doses of n-3 PUFA fish oil supplement in ameliorating deficits in cognition and neurophysiology associated with *APOE* genotype and menopause.

Hypothesis

We hypothesise that:

1. *APOE* genotype leads to dysregulated n-3 PUFA status and subsequent cognitive decline.
2. Menopause will exacerbate the pathological mechanisms associated with *APOE4*
3. n-3 PUFA supplementation will ameliorate these deficits in a dose dependant manner

Chapter 2 : Influence of *APOE* carrier-status, oily fish intake and HRT on PUFA status and cognition (A CANN study analysis)

Introduction

The global incidence of dementia stands at 50 million, and is set to triple by 2050 (Patterson 2018), with concerns that there are no effective disease modifying treatments. Studies in 'at risk' humans (e.g. SMI and MCI) are an effective method to identify risk factors, and disease markers present at the population level, which can inform us of associated mechanisms. This may in turn provide insight from which novel intervention strategies can be developed.

The *APOE4* genotype is a well-established genetic determinant of Alzheimer's disease (AD). Possession of just one *APOE4* allele increases AD risk by 2- to 4-fold, and reduces disease onset by ~12 years (Corder, Saunders et al. 1993, Roses 1996, Farrer, Cupples et al. 1997). Despite this, a clear aetiological basis for *APOE4* mediated AD risk remains unclear. This is largely a result of the complex, pleiotropic nature of *APOE*, with its interaction with non-genetic determinants such as diet and sex, making additional contributions. The ability of non-genetic determinants to modulate *APOE4* mediated AD risk, supports the prospect of disease modifying intervention.

Incorporation of greater n-3 PUFAs such as DHA into the diet, achieved with greater intake of oily fish or fish oil supplementation, is associated with improved cognitive performance in older adults (Del Brutto, Mera et al. 2016), and a general reduction in mild cognitive impairment (MCI) and AD risk (Barberger-Gateau, Raffaitin et al. 2007, Zhang, Chen et al. 2016, Zhao, Xiong et al. 2018, Zhang, Han et al. 2019). It has been suggested that carriers of the *APOE4* allele are more sensitive to n-3 PUFA deficiencies (Nock, Chouinard-Watkins et al. 2017), and potentially respond less effectively to supplementation (Chouinard-Watkins, Rioux-Perreault et al. 2013). This may result from altered metabolism and/or transport of n-3 PUFAs in *APOE4* carriers (Chouinard-Watkins, Rioux-Perreault et al. 2013, Chouinard-Watkins and Plourde 2014, Vandal, Alata et al. 2014, Chen and Bazinet 2015, Yassine, Rawat et al. 2016). In fact, specific *APOE4* associated disturbances in DHA:AA ratios within blood phospholipids have been used to effectively identify individuals with MCI/AD (Abdullah, Evans et al. 2017). Interestingly, such deficits appear to be overcome by utilising larger doses of DHA when supplementing *APOE4* carriers (Chouinard-Watkins et al., 2016; Chouinard-Watkins et al., 2017; Tomaszewski, He et al. 2020)).

In addition to this, and adding further complexity, a degree of uncertainty remains as to which plasma DHA pools are the main sources of DHA, for transfer across the blood brain barriers and therefore having the greatest influence on brain DHA maintenance (Lacombe, Chouinard-Watkins et al. 2018). Within blood plasma, DHA can be found in various forms, esterified to phospholipids (PL), lysophospholipids (LPC), cholesteryl esters (CE), or triacylglycerols (TAG) within lipoproteins. Furthermore, DHA may also be complexed with albumin as a non-esterified (NE) free FA or esterified to LPC. Red blood cell (RBC) erythrocytes and platelets also carry DHA esterified to PLs within their plasma membranes (Lacombe, Chouinard-Watkins et al. 2018). Of these pools, the plasma NE-FA, and LPC fractions appear to be particularly efficient at targeting the brain (Chouinard-Watkins, Lacombe et al. 2018), however PL plasma fractions likely contribute to some extent (Chouinard-Watkins, Lacombe et al. 2019). With that said, the erythrocyte contribution should not be entirely ignored, as evidence supporting or ruling out its role is limited, despite extensive evidence linking improved cognitive outcomes and lower AD risk to those with higher n-3 PUFA erythrocyte status (Hooper, De Souto Barreto et al. 2018). This remains an active research area, requiring further investigation to unravel the complexity of the process.

Sex is ever-increasingly acknowledged for its influence on the impact of *APOE4* on AD risk (Belloy, Napolioni et al. 2019). Compared to males, female *APOE4* carriers have increased MCI and AD risk between 55-70 years and 65-75 years respectively (Neu, Pa et al. 2017), which may be suggestive of a menopausal link. Interestingly, estrogen/sex hormone dysregulation has already been implicated in the pathophysiology of AD (Pines 2016) with such risk highlighted by the greater cognitive decline associated with premature menopause (Ryan, Scali et al. 2014). One would therefore hypothesise that hormone replacement therapy (HRT), which aims to restore female sex hormone profiles, may effectively overcome this predisposition. However, overall results of HRT in AD prevention have been mixed (O'Brien, Jackson et al. 2014, Imtiaz, Tuppurainen et al. 2017). This may in part be a result of the so called ‘window of opportunity’ implying that efficacy is dependent on when HRT is administered (Davey 2013, Bean, Kumar et al. 2015). Similarly, variations in HRT combinations and doses are also likely to contribute to the variation in effectiveness. In addition, specific genetic differences may alter a person’s response to HRT. The relationship of HRT and the *APOE* genotype remains vastly understudied but given the established impact of sex and the possibility of a menopausal interaction, it is plausible that altering the sex hormone profile may differentially effect those carrying an *APOE4* allele. Currently, only a handful of studies have explored the *APOE* genotype in HRT, in the context of dementia risk or cognition, the results of which have been mixed (Yaffe, Haan et al. 2000,

Burkhardt, Foster et al. 2004, Yue, Hu et al. 2007, Ryan, Carrière et al. 2009). A reflection of the surprising lack of research, exploring the influence of sex on *APOE* genotype (Michaelson 2014).

This study utilises data obtained from the CANN (Cognitive Ageing, Nutrition and Neurogenesis; NCT02525198) study in which I was actively involved in the clinical visits and in particular the biological sample processing and storage for the Norwich cohort, along with the data analysis plan and conduct included here. The primary objective of CANN was to 'evaluate whether daily supplementation with a combined flavonoid/DHA rich-fatty acid supplement over a 12-month period improved cognitive performance in older adults (≥ 55 years) with Mild Cognitive Impairment (MCI) or Subjective Memory Impairment (SMI)'. The analysis conducted as part of my thesis utilises predominantly pre-treatment, baseline data and focuses on female participants within the cohort. Here, we analyse the impact of a number of factors including *APOE4* carrier status and HRT on erythrocyte PUFA status and follow this up with further analysis of these factors on cognition.

Materials and methods

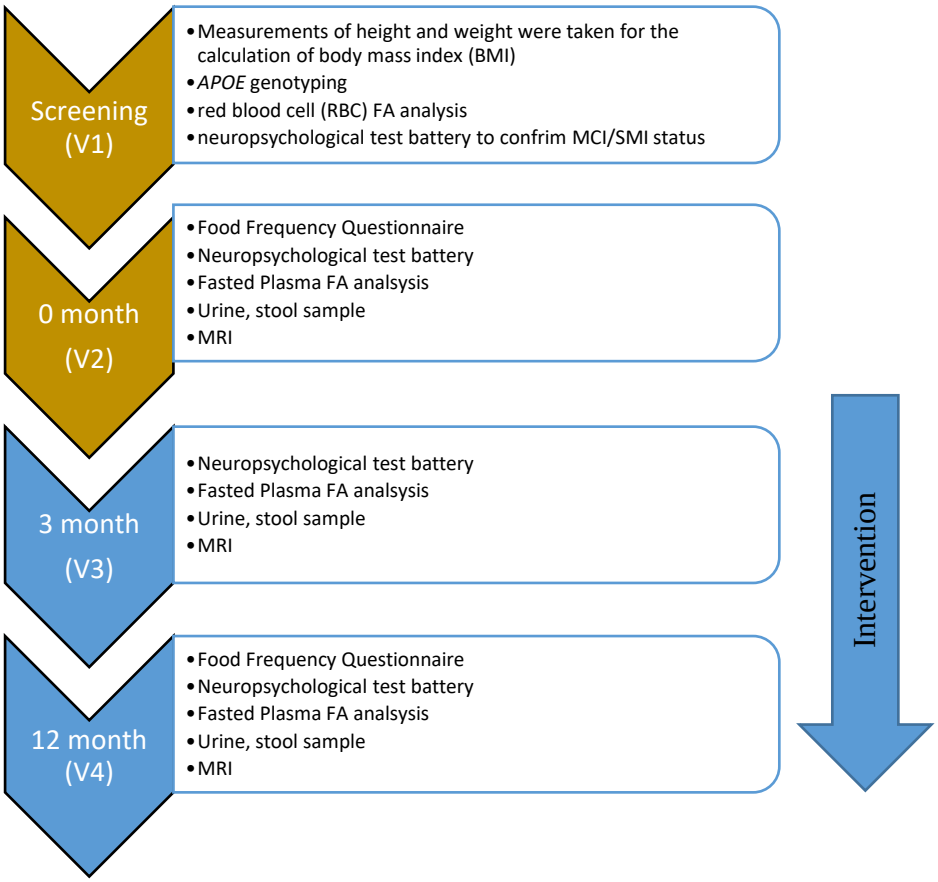

Figure 2.1 Overview of CANN timeline.

This project focuses on pre-intervention generated data obtained at screening (V1) and Baseline (V2) data (highlighted in gold) which were ~3 weeks apart

CANN study design

The CANN study is a multi-centre, 12-month double-blinded, placebo-controlled parallel intervention trial in which participants were randomised onto one of two intervention arms (1.5 g docosahexaenoic acid + eicosapentaenoic acid and 500 mg total flavanols from cocoa daily or control macronutrient matched placebo), and was completed in 2019. The trial was conducted over two study centres; University of East Anglia (Norwich, UK) and the Swinburne University of Technology (Melbourne, Australia). 259 individuals with mild cognitive impairment or subjective memory impairment, but with no indication of dementia (as assessed by the Montreal Cognitive Assessment (MoCA) score ≥18) were actively recruited into the study, in which the cognitive benefits of a combined flavonoid/fatty acid supplementation were investigated along with the underlying mechanisms of action (Irvine, Scholey et al. 2018). As outlined in Fig.1 the trial consisted of a screening visit followed by 3 clinical visits (0, 3 and 12 months) in which biological samples were collected and a battery of cognitive tests were performed. In addition to general health related criteria; participants were also excluded from the study based upon a dietary related criteria, which included a high n-3 FA status (determined by red blood cell [RBC] n-3 FA index > 6% [EPA+DHA as a % of total FAs), and taking fish oils or other n-3 FA-rich supplements. The full inclusion/exclusion criteria is given in Table 2.1.

Table 2.1: CANN study Full inclusion/exclusion criteria

(adapted from (Irvine, Scholey et al. 2018))

Inclusion	
Type	*Criterion*
General	Males and females, aged 55 years and above
	Diagnosis of MCI or SMI with no indication of clinical dementia or depression
	Willing and able to provide written informed consent, and verbal informed consent for the telephone eligibility screen, and to comply with all study procedures
	Fluent in written and spoken English
	In good general health, including blood biochemical, hematological and urinalysis results being within the normal range at V1
	Normal or corrected-to-normal vision and hearing
	Stable use of any prescribed medication for at least 4 weeks prior to V2
MRI (50% of cohort)	Aged from 55 to 85 years
Exclusion	
Type	*Criterion*
General and health-related	Diagnosis of AD or other form of dementia, or significant neurological disorder
	Parent or sibling who developed dementia before 60 years of age

Table 2.1 Continued

	Past history/MRI evidence of brain damage, including significant trauma, stroke, or other serious neurological disorder, including loss of consciousness for > 24 h
	Clinically diagnosed psychiatric disorder likely to affect the cognitive measures
	Existing gastrointestinal disorder likely to impact on the absorption of flavonoids and FAs (e.g. celiac, Crohn's, irritable bowel syndrome)
	Major cardiovascular event (e.g. myocardial infarction or stroke) in the last 12 months
	Carotid stents or severe stenosis
	1 Any other existing medical condition likely to affect the study measures (as judged by the trial's clinical advisor)
	Uncontrolled hypertension (SBP > 160 mmHg, DBP > 100 mmHg)
	BMI ≥ 40 kg/m^2
	2 Current or ex-smoker who stopped < 6 months ago
	Prescribed medications likely to influence the study measures (as judged by the clinical advisor)
	3 Participated in any other study involving an investigational product in the last 4 weeks
4 Drugs, alcohol, smoking	History of alcohol or drug dependency within the last 2 years
	Taking antipsychotics, high-dose antidepressants (as judged by the clinical advisor), cholinesterase inhibitors, benzodiazepines, anti-convulsants, illicit drugs/other drugs that can influence psychometric test results. Statins and NSAIDs are not exclusionary but use must be stable for at ≥ 2 months prior to V2
	Subjective reporting of sedation from any prescribed medication e.g. night sedation (Z drugs)/pain relief medication
5 Dietary-related	Known allergy to fish or any other component of the intervention products
	Taking flavonoid-containing supplements
	6 Taking fish oils or other n-3 FA-rich supplements
	High n-3 FA status (determined by red blood cell [RBC] n-3 FA index > 6% [EPA+DHA as a % of total FAs)
	High flavonoid intake (> 15 portions of flavonoid-rich foods per day)
	Hypersensitivity to fish products
7 MRI (50% of cohort)	Presence of metal implants
	Claustrophobia

AD, Alzheimer's disease; BMI, body mass index; DBP, diastolic blood pressure; FA, fatty acid; MRI, magnetic resonance imaging; NSAIDs, non-steroidal anti-inflammatory drugs; SBP, systolic blood pressure

Fatty Acid analysis

Plasma and erythrocyte total lipids were extracted using the Folch extraction method (Folch, Lees et al. 1957), using chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. Total lipid fractions were subjected to acid-catalysed transmethylation for 16 h at 50 ºC, using 1 ml of toluene and 2 ml of 1% sulphuric acid (v/v) in methanol (Christie and Han 2010). The resultant erythrocyte and plasma fatty acid methyl esters (FAME) were purified by SPE silica cartridges (Clean-up 203 Cusil 156, UCT) and thin layer chromatography (TLC) respectively. Plasma FAMEs were visualised under 1% iodine in chloroform spray. After elution, FAME were separated and quantified by gas-liquid

chromatography. Individual methyl esters were identified by reference to authentic standards and to well-characterized fish oil (PUFA-3 from menhaden oil, SUPELCO, Supelco Park, Bellefonte, USA). Data was collected and processed using the GC Chemstation software (version B04-02). Erythrocyte fatty acid analysis was conducted at the University of Stirling for UK samples and HealthScope (Melbourne). Plasma fatty acid analysis for both centres were performed at the University of Stirling. n-3 index is the combined value of DHA and EPA. Total n-3 PUFA is the total of: 18:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. Whilst total n-6 is the total of: 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6.

Genotyping

For *APOE* genotyping, blood was collected into an Cell Preparation Tube (CPT) (BD Biosciences, San Diego, CA, USA) to provide a buffy layer and used for genomic DNA extraction using a DNA extraction kit (Qiagen, Hildenberg, Germany), as per manufacturer's instructions. DNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted using MilliQ water (Millipore, Billerica, MA, USA) to achieve a concentration in the range of 1–10 ng. *APOE* genotype was determined by two real-time reverse transcription polymerase chain reaction (RT-PCR) single nucleotide polymorphism (SNP) genotyping assays, to determine the 112 T/C (rs429358) *APOE4* polymorphism and 158 C/T (rs7412) *APOE2* polymorphism per the Applied Biosystems (Foster City, CA, USA) TaqMan SNP Genotyping Assays protocol (2010) (Biosystems 2010). The analyses were carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems).

Cognitive assessment

A neuropsychological test battery was performed at screening and baseline to determine cognitive performance. Data was obtained from the cognitive tests outlined below, as reported in the CANN methodology paper (Irvine, Scholey et al. 2018).

The Montreal Cognitive Assessment (MoCA): A rapid screening instrument for mild cognitive dysfunction (Nasreddine, Phillips et al. 2005). The assessment assesses different cognitive domains: attention and concentration, executive functions, memory, language, visio-constructional skills, conceptual thinking, calculations, and orientation. The total possible score is 30 points; a score of 26 or above is considered normal and a score of less than 17 indicates dementia.

Logical Memory I and II: Taken from the WMS-R, Logical Memory (Wechsler 1987) is a validated clinical measure frequently used to assess verbal memory in patients with MCI and AD. Participants are read a short passage, and after hearing the story are asked to retell it

from memory. Recall is measured immediately (Logical Memory I) and after a 30-minute delay (Logical Memory II).

California Verbal Learning Test-II: The CVLT-II (Delis 2000) is a reliable and well-validated test of verbal learning and memory. Participants are read a list of 16 words, belonging to four distinct groups (e.g. animals), and must recall them across a series of immediate and delayed trials (including an interference trial). The CVLT-II is sensitive to subtle episodic memory impairments in patients with MCI (Rabin, Pare et al. 2009).

Boston Naming Test: The BNT (Kaplan 1983) is a confrontation naming task consisting of 60 black line drawings of common objects, which participants are asked to identify (identification difficulty differs among the objects). The CANN study uses a 30-item version of this task (Saxton, Ratcliff et al. 2000).

Figure Copy: Taken from the RBANS, the Figure Copy task (Randolph, Tierney et al. 1998) assesses visuospatial function. The RBANS is a well-validated neuropsychological test battery for identifying and characterizing abnormal cognitive decline in older adults that has been shown to be sensitive to MCI due to AD (Karantzoulis, Novitski et al. 2013). Participants copy a modified Rey-Figure comprising 10 elements and receive 2 points (1 for accuracy and 1 for placement) for correct responses on each of the 10 elements, with a highest total possible score of 20.

Digit Span task (Forward and Backward): The Digit Span task, a subtest from WAIS III (Wechsler 1997) that assesses attention and short-term memory, comprises the Digits Forward and Digits Backward subtests. For each Digits Forward item, a series of digits (e.g. '8, 3, 4') is read out and participants must immediately repeat them back in order. If they do this successfully, they are given a longer list of digits (e.g. '9, 2, 4, 0'). For Digits Backward, the participant is required to repeat back a series of digits in the reverse order. The total score on this task is given by the sum score for Digits Forward plus Digits Backward.

Trail Making Test – A and B: The TMT (Reitan 1955) provides a measure of executive function. Participants are required to connect numbered circles on a sheet of A4 paper. There are two versions of the test: Version A, in which targets are all numbers in consecutive order (1,2,3…25), and Version B, in which the participant alternates between numbers and letters (1, A, 2, B…etc.). The participant must finish the test as quickly as possible, without making errors or removing the pen from the paper, and the time taken to complete the test indexes performance.

Test of Premorbid Functioning: The ToPF (Wechsler 2009) estimates premorbid intelligence. Participants read aloud a list of 70 irregularly spelled words, such as *gnat*, *lieu* and *subtle*, and the total score is the number of words pronounced correctly. Unlike many other domains of intelligence and memory abilities, reading recognition is relatively stable in the presence of the cognitive decline associated with normal aging or brain injury. The ToPF provides a reliable estimate of intelligence in clinical and research settings.

Cognitive Drug Research test battery: The CDR Computerized Cognitive Assessment System (Simpson, Surmon et al. 1991) will be used to measure the effects of the intervention on cognition. The battery is sensitive to cognitive changes, including in trials of MCI, dementia and SMI. It has been used in 1,200 clinical trials across 3,000 sites in more than 60 countries. Tasks are presented via color monitors on laptops; with the exception of the written Word Recall tasks, performed with pen and paper, with all responses are recorded via a two-button (YES/NO) response box. The entire battery takes approximately 25–30 minutes to complete.

For the CANN study, the following tasks are completed, in order: Bond-Lader Visual Analogue Scale of Mood and Alertness, Leeds Sleep Evaluation Questionnaire, Word Presentation, Immediate Word Recall, Picture Presentation, Simple Reaction Time, Digit Vigilance, Choice Reaction Time, Spatial Working Memory, Numeric Working Memory, Delayed Word Recall, Word Recognition and PRT (Bond 1974). At V1, the CDR is completed twice in succession so that participants are well familiarized prior to the clinical visits (V2-4).

iPosition task: The iPosition task (Watson, Voss et al. 2013) assesses relational memory function, which is critically reliant on the hippocampus. Participants must spatially reconstruct an array of five abstract shapes. In a typical trial, participants view on a computer screen, and must then memorize, the location of each shape. Following the stimulus phase, there is a brief delay (4 s), during which the objects disappear and then reappear in a line at the top of the screen; the participant is then asked to move them back to their original location. This task has demonstrated high sensitivity to hippocampal integrity and can detect deficits in relational memory in patients with mild hippocampal damage (Watson, Voss et al. 2013).

Verbal Fluency Test: The D-KEFS Verbal Fluency Test is composed of three conditions: letter fluency (participant says as many words beginning with a specific letter as possible); category fluency (participant says words belonging to a designated semantic category), and category switching (participant alternates between saying words belonging to two different

semantic categories). The category-switching task can strongly discriminate between healthy controls and MCI and AD patients (Nutter-Upham, Saykin et al. 2008).

Statistical analysis

For the purpose of my thesis, analysis was focused towards female participants. Participants with the *APOE2*/*APOE4* allele combination were excluded from the analysis to maintain mutually exclusive carrier groups n =136. Data is shown as the mean value ± standard error of mean. Extreme outliers were identified in SPSS via visual inspection of frequency distribution plot (Q-Q plots) and box plots, those deemed to be significant (1.5 X Inter Quartile Range), were omitted from further analyses. All data was screened prior to analysis for normality, via visual inspection and Shapiro-Wilk test. If data did not follow normal distribution it was subsequently transformed via log or square root transformation. Age, BMI, centre, smoker status, hormone therapy, MCI/SMI, *APOE4* carrier status, sex, education, IQ and fish intake were considered as covariates in the analysis and were chosen based upon their potential influence on fatty acid status and cognitive performance.

Student's t-test and Pearson's chi squared test were performed on baseline characteristics data $p < 0.05$ was considered significant.

Analysis of covariance (ANCOVA) was performed on female participant screening (V1) erythrocyte, and Baseline (V2) plasma values to assess the impact of *APOE* genotype, age, BMI, hormone therapy and fish intake (determined via food frequency diaries and questionnaires) on fatty acid composition. FA profiles of erythrocyte, total plasma, and the PC fraction of the plasma were analysed. For the PC fractions 36:5 and 39.6, 40:6 were focused on as they are known carriers of EPA and DHA respectively (Chalil, Kitson et al. 2018, Lopes, Melo et al. 2019). Data was available for the LPC fraction, however levels were undetectable in the majority of participants, and further analysis was deemed unnecessary. Analysis was first performed unadjusted with a single explanatory value (model M1), after which covariates age, BMI, centre, smoker status, and HRT use were subsequently added into the multivariate analysis (If not the subject of analysis) (model M2). Effects and interactions where $P < 0.05$ were considered significant.

Analysis of covariance (ANCOVA) was also performed on screening (V1) and baseline cognitive assessment data (V2), to assess the impact of *APOE4* carrier status, HRT, and oily fish intake have on cognition. Analysis was performed fully adjusted (model M2) as factors such as age and IQ can highly influence cognitive testing. Covariates considered for use in this analysis were age, BMI, centre, smoking status, HRT use, MCI/SMI status, *APOE4*

carrier status, sex, education, IQ and oily fish intake (if not the subject of the analysis). Effects and interactions where $P < 0.05$ were considered significant.

All statistical analysis was performed using IBM SPSS statistics version 25 software

Results

Study population characteristics

The full study population including both female and male participants has been reported previously (Irvine, Scholey et al. 2018). For the purpose of this study, a total of 136 female participants, 75 in the Norwich cohort and 61 in the Melbourne cohort were used in the analysis. *APOE* genotype frequencies in this study population, reflected the general population as reported by others (Lyall, Ward et al. 2016), with *APOE3*/*E3* the most common allele combination accounting for ~60 % of the population, whilst rarer combinations *APOE2/E2* and *APOE4/E4* accounted for just ~2% each. SMI and MCI were split evenly within carrier status subgroups and did not significantly differ as result of *APOE4* carrier status ($p > 0.05$). The average age of female participants was ~65 years ± 0.5, with the E4 carrier subgroup nominally but not significantly younger than non-carriers (63.7 ± 0.9 and 65.3 ± 0.7 years respectively $p > 0.05$). Participants were generally slightly overweight, with an average BMI of ~27kg/m^2 ± 0.4 regardless of carrier status ($p > 0.05$).

Table 2.2 Baseline characteristics of female from the CANN study population utilised for analysis within this study.

	Overall (n=136)	**Non - ε4 Carrier (n= 97)**	**ε4 Carrier (n=39)**	**P value**
Genotype Breakdown (n)		• E2/E2 = 3 (2.2%) • E2/E3 = 10 (7.4%) • E3/E3 = 84 (61.8%)	• E3/E4 = 36 (26.5%) • E4/E4 = 3 (2.2%)	
Age (y)	64.9 ± 0.5	65.3 ± 0.7	63.7 ± 0.9	0.172
BMI (kg/m2)	26.9 ± 0.4	26.9 ± 0.5	26.8 ± 0.9	0.842
SMI	71 (52.2%)	51 (37.5 %)	20 (14.7 %)	0.891
MCI	65 (47.8%)	46 (33.8 %)	19 (14.0 %)	

All values are mean ± SEM. Brackets represent % of study population. Student's t-test was used to test for differences in age and BMI resulting from APOE4 carrier status. Pearson's Chi squared test was used to test for differences in frequency distributions of SMI and MCI

between E4 carriers and non-carriers. BMI, body mass index, SMI subjective memory impairment, MCI, Mild cognitive impairment.

Factors influencing n-3 PUFA status

Altered erythrocyte and plasma n-3 PUFA status has been widely associated with health, disease and cognitive function (Conquer, Tierney et al. 2000, Calder and Yaqoob 2009). We therefore explored how n-3 PUFA status is influenced by a range of potential determinants which are described in each section. Taking both erythrocyte and plasma fractions provide us with a more detailed understanding of n-3 PUFA status. RBC provides a greater long-term assessment, due to the greater stability of RBC n-3 PUFAs (mainly esterified in the PLs membrane), ~3month to alter. Whereas the more dynamic plasma n-3 PUFAs, mainly lipoprotein associated or free fatty acid, is influenced by recent intake and is sensitive to day by day changes (Harris and Thomas 2010).

Greater oily fish intake increases n-3 PUFA status, whilst rising BMI reduces n-3 PUFA status

As expected, incremental intake in oily fish portions (None, 1-3 per month, one or more portions a week) a rich source of long chain n-3 PUFA led to an increase in n-3 PUFA status, with erythrocyte, DHA, n-3 index and total n-3 increasing whilst AA and AA:DHA ratio reduced ($p < 0.05$). Although initially significant (model M1), when fully adjusted EPA did not reach significance ($p = 0.094$). Interestingly, significance was restricted to erythrocytes, although both plasma and particularly PC fractions showed strong trends in response to fish consumption. Conversely, increasing BMI had the opposite effect, significantly reducing erythrocyte DHA and n-3 index ($p<0.05$) whilst EPA was trending towards significance ($p = 0.072$). Once again, these changes were only significant in the erythrocyte fraction, although strong trends were observed in the PC fractions.

Table 2.3 Baseline Erythrocyte and plasma FA profile according to Fish intake and Body mass index.

	FI T1 (Never)	FI T2 (1-3 /month)	FI T3 (1+ per/ week)	FI M1	FI M2	BMI < 25 kg/m^2	BMI 25-30 kg/m^2	BMI ≥30 kg/m^2	BMI M1	BMI M2M2
RBC (%)										
DHA	3.42 ± 0.14 a	3.87 ± 0.10 b	3.94 ± 0.10 b	**0.002**	**0.004**	4.02 ± 0.10	3.77 ± 0.10	3.53 ± 0.14	**0.022**	**0.045**
EPA	0.85 ± 0.05	1.00 ± 0.03	0.97 ± 0.04	**0.006**	0.094	1.01 ± 0.03	0.93 ± 0.04	0.94 ± 0.06	0.076	0.072
AA	13.70 ± 0.3	13.30 ± 0.2	12.90 ± 0.2	0.114	**0.034**	13.20 ± 0.2	13.50 ± 0.2	13.00 ± 0.3	0.321	0.579
n-3 index	4.27 ± 0.15 a	4.92 ± 0.10 b	4.95 ± 0.12 b	**0.0001**	**0.002**	5.04 ± 0.10	4.77 ± 0.11	4.49 ± 0.16	**0.008**	**0.027**
Total n-3 PUFA	7.10 ± 0.17 a	7.55 ± 0.13 b	7.56 ± 0.15 b	**0.008**	**0.077**	7.69 ± 0.13	7.45 ± 0.15	7.19 ± 0.20	**0.044**	0.090
Total n-6 PUFA	30.50 ± 0.3 a	29.30 ± 0.2 b	29.50 ± 0.2 b	0.099	**0.028**	29.50 ± 0.26	29.63 ± 0.22	29.80 ± 0.34	0.486	0.656
AA:DHA	4.16 ± 0.16 a	3.56 ± 0.10 b	3.35 ± 0.10 b	**0.0001**	**0.0001**	3.35 ± 0.10	3.69 ± 0.10	3.84 ± 0.17	**0.032**	0.087
Plasma (µg/ml)										
DHA	1.26 ± 0.13	1.62 ± 0.14	1.40 ± 0.09	0.181	0.216	1.53 ± 0.09	1.44 ± 0.09	1.35 ± 0.14	0.236	0.321
EPA	0.24 ± 0.03	0.29 ± 0.02	0.25 ± 0.02	0.322	0.415	0.26 ± 0.18	0.28 ± 0.02	0.24 ± 0.03	0.398	0.605
AA	1.71 ± 0.14	1.80 ± 0.09	1.67 ± 0.14	0.508	0.582	1.62 ± 0.09	1.80 ± 0.14	1.76 ± 0.18	0.705	0.842
Plasma PC fractions (µg/ml)										
PC 36:5 EPA containing	47.88 ± 2.52	57.12 ± 2.73	56.28 ± 2.94	0.231	0.525	58.80 ± 2.73	55.23 ± 2.94	48.93 ± 2.94	0.246	0.364
PC 38:6 DHA containing	63.00 ± 3.15	72.45 ± 2.10	74.34 ± 2.31	0.068	0.197	75.60 ± 2.52	69.30 ± 2.10	66.15 ± 2.94	0.061	0.177
PC 40:6 DHA containing	16.80 ± 1.05	18.90 ± 0.63	18.90 ± 0.84	0.338	0.470	19.10 ± 0.84	18.48 ± 0.63	18.48 ± 1.05	0.734	0.797

All values are mean ± SEM. Fish intake groups: Never (n = 27), 1-3 portions per month (n = 53), 1 portion or more per week (n = 53). BMI groups < 25 kg/m^2 (n = 55), 25-30 kg/m^2 (n = 50), ≥30 kg/m^2 (n = 28). Model one (M1) ANOVA main-effects model (unadjusted) including only fish intake or BMI as an explanatory variable. Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates age, centre, carrier status, smoking status, HRT and either oily fish intake, or BMI. Group differences were determined using unadjusted ANOVA with Tukey post hoc. Bold font indicates significant p value ($p < 0.05$) different letters indicate different mean. FI, Fish intake; BMI, body mass index; RBC, Red blood cell; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; AA, Arachidonic acid; AA:DHA, Arachidonic acid: Docosahexaenoic acid ratio, PC, phosphatidylcholine

***APOE4* carrier status alters plasma and erythrocyte FA profile in females**

APOE4 genotype is believed to alter n-3 PUFA transport and metabolism to and within the brain. Altered blood brain barrier integrity leading to reduced transport across the blood brain barrier has been proposed as a potential mechanism (Yassine, Braskie et al. 2017, Tomaszewski, He et al. 2020). Baseline FA composition analysis revealed a significant genotype difference in PUFA profiles, with *APOE4* carriers displaying elevated n-3 PUFA levels, evident from the ~5% higher n-3 index ($p < 0.05$ table 2.4). Interestingly, EPA levels were particularly influenced by genotype, with both erythrocyte and PC fractions significantly 15 ± 0.04 % and 12 ± 2.73% higher respectively in *APOE4* carriers ($p < 0.05$). Plasma EPA levels followed a similar trend however did not reach significance ($p = 0.076$). Although not significant, DHA showed a similar nominal increase in the erythrocyte fraction of *APOE4* carriers. Conversely, total n-6 PUFA levels were significantly lower in *APOE4* carriers, as was LA, whilst SFA and MUFA remained constant regardless of carrier status.

Table 2.4 Baseline Erythrocyte and plasma FA profile according to E4 carrier status.

	Non - ε4 Carrier	ε4 carrier	M1	M2
RBC (%)				
DHA	3.77 ± 0.08	3.90 ± 0.11	0.246	0.186
EPA	0.92 ± 0.03	1.06 ± 0.03	**0.005**	**0.0001**
DPA	2.24 ± 0.04	2.28 ± 0.04	0.555	0.708
LA	13.16 ± 0.3	12.29 ± 0.4	0.045	**0.014**
AA	13.31 ± 0.2	13.14 ± 0.2	0.869	0.919
ALA	0.41 ± 0.03	0.33 ± 0.04	0.218	0.308
n-3 index	4.74 ± 0.09	4.96 ± 0.12	0.063	**0.027**
Total n-3	7.43 ± 0.11	7.63 ± 0.14	0.139	0.090
Total n-6	29.93 ± 0.18	28.96 ± 0.25	**0.002**	**0.002**
AA:DHA	3.65 ± 0.08	3.47 ± 0.11	0.259	0.212
Total SFA	39.54 ± 0.13	39.89 ± 0.22	0.096	0.115
Total MUFA	20.29 ± 0.26	20.10 ± 0.37	0.725	0.378
Plasma (µg/ml)				

Table 2.4 continued

DHA	1.35 ± 0.16	1.58 ± 0.12	0.245	0.294
EPA	0.23 ± 0.014	0.27 ± 0.018	0.068	0.076
AA	1.71 ± 0.081	1.71 ± 0.12	0.872	0.865
Plasma PC Fractions (%)				
PC 36:5 EPA containing	53.13 ± 2.1	59.85 ± 2.73	**0.009**	**0.005**
PC 38:6 DHA containing	70.35 ± 1.68	73.29 ± 2.73	0.418	0.354
PC 40:6 DHA containing	18.27 ± 0.63	19.11 ± 0.84	0.306	0.267

All values are mean ± SEM. *APOE* carrier groups: *APOE4* non-carrier (n = 97), *APOE4* carrier (n = 39). Model one (M1) ANOVA main-effects model (unadjusted) including only *APOE4* carrier status as an explanatory variable. Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates age, BMI, centre, smoking status, HRT status and oily fish intake. Bold font indicates significance ($p < 0.05$). RBC, Red blood cell; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; LA linoleic acid; AA, Arachidonic acid; ALA, Alpha linoleic acid; AA:DHA, Arachidonic acid: Docosahexaenoic acid ratio; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid, PC, phosphatidylcholine.

HRT*_APOE4_ status interaction influences baseline n-3 PUFA

With AD/MCI risk in female *APOE4* carriers considerably heightened between the ages of 55-70 years (Neu, Pa et al. 2017) relative to the wild-type *APOE3/E3* genotype, there is evidence that *APOE4* carriers are particularly sensitive to the impact of menopause on cognitive ageing. The ability of HRT to mitigate the effect of age on cognition in females remains controversial with response to HRT according to *APOE* genotype relatively unknown (Depypere, Vierin et al. 2016). Given the age of the study population, we can assume that the majority of participants will be peri-menopausal if not already post-menopausal. To explore the impact sex hormones on the participant FA profiles, HRT use was incorporated into the analysis. HRT status alone had no apparent impact on FA profile. However, an HRT**APOE4* status interaction indicated a potential interactive impact on DHA concentrations. Although of borderline significance, both erythrocyte DHA ($p = 0.06$) and PC 38:6 the DHA plasma fraction ($p= 0.078$) were 17 ± 0.34% and 15 ± 4.16 lower in *APOE4* carriers receiving HRT. The PC 40:6 DHA fraction ($p= 0.03$) was significantly altered due to HRT**APOE4* carrier interaction with 26 ± 1.69% lower concentrations in HRT *APOE4* individuals relative to non-users. Similarly, erythrocyte n-3 index was significantly altered by HRT**APOE4* carrier-status and was reduced by 14 ± 0.25% in *APOE4* carriers receiving HRT. Interestingly, this is in contrast to the *APOE4* effect, in which n-3 PUFA levels were elevated, however EPA was not affected by

HRT*_APOE4_ carrier-status. All HRT*_APOE4_ carrier-status analysis is exploratory and should be evaluated with caution due to low n numbers within the _APOE4_ carrier HRT group.

Table 2.5 Baseline Erythrocyte and plasma FA profile according to HRT and HRT*carrier-status interaction.

		No - HRT	HRT	HRT M1	HRT M2	HRT*CS M1	HRT*CS M2
RBC (µg/ml)							
DHA	Non-carrier	3.75 ± 0.08	3.91 ± 0.21	0.239	0.479	0.068	0.060
	ε4 carrier	3.96 ± 0.11	3.28 ± 0.34				
EPA	Non-carrier	0.91 ± 0.03	1.07 ± 0.10	0.747	0.943	0.592	0.137
	ε4 carrier	1.06 ± 0.03	1.03 ± 0.12				
DPA	Non-carrier	2.25 ± 0.04	2.22 ± 0.13	0.442	0.566	0.452	0.630
	ε4 carrier	2.28 ± 0.05	2.30 ± 0.15				
LA	Non-carrier	13.1 ± 0.3	13.3 ± 0.9	0.073	0.462	0.175	0.460
	ε4 carrier	12.1 ± 0.4	14.4 ± 1.3				
AA	Non-carrier	13.3 ± 0.2	13.5 ± 0.5	0.532	0.868	0.563	0.849
	ε4 carrier	13.2 ± 0.2	12.7 ± 0.2				
ALA	Non-carrier	0.41 ± 0.03	0.47 ± 0.10	0.061	0.516	0.416	0.730
	ε4 carrier	0.31 ± 0.03	0.55 ± 0.23				
n-3 index	Non-carrier	4.71 ± 0.09	4.97 ± 0.26	0.343	0.542	0.091	**0.048**
	ε4 carrier	5.02 ± 0.12	4.31 ± 0.25				
Total n-3	Non-carrier	7.40 ± 0.12	7.70 ± 0.32	0.548	0.494	0.253	0.102
	ε4 carrier	7.67 ± 0.14	7.17 ± 0.41				
Total n-6	Non-carrier	29.9 ± 0.2	30.1 ± 0.6	0.227	0.414	0.505	0.669

Table 2.5 continued

	ε4 carrier	28.9 ± 0.3	29.9 ± 1.0				
AA:DHA	Non-carrier	3.66 ± 0.09	3.57 ± 0.25	0.425	0.482	0.162	0.099
	ε4 carrier	3.43 ± 0.12	3.95 ± 0.44				
Total SFA	Non-carrier	39.52 ± 0.13	39.7 ± 0.4	0.811	0.932	0.663	0.874
	ε4 carrier	39.9 ± 0.2	39.7 ± 1.3				
Total MUFA	Non-carrier	20.3 ± 0.3	20.3 ± 0.7	0.205	0.657	0.115	0.102
	ε4 carrier	19.9 ± 0.4	22.2 ± 1.8				
Plasma (µg/ml)							
DHA	Non-carrier	1.35 ± 0.09	1.8 ± 0.36	0.085	0.125	0.729	0.496
	ε4 carrier	1.53 ± 1.35	2.02 ± 0.45				
EPA	Non-carrier	0.25 ± 0.018	0.27 ± 0.045	0.208	0.109	0.973	0.644
	ε4 carrier	0.29 ± 0.023	0.23 ± 0.09				
AA	Non-carrier	1.71 ± 0.09	1.94 ± 0.36	0.372	0.300	0.717	0.470
	ε4 carrier	1.71 ± 0.14	1.85 ± 0.18				
Plasma PC Fractions (µg/ml)							
PC 36:5 EPA containing	Non-carrier	52.92 ± 2.31	55.44 ± 7.14	0.192	0.603	0.143	0.294
	ε4 carrier	61.32 ± 2.73	43.89 ± 6.72				
PC 38:6 DHA containing	Non-carrier	69.3 ± 1.68	79.8 ± 5.04	0.962	0.833	0.057	**0.078**
	ε4 carrier	72.45 ± 2.94	61.53 ± 2.94				
PC 40:6 DHA containing	Non-carrier	18.27 ± 0.63	19.95 ± 1.47	0.490	0.337	0.050	**0.033**
	ε4 carrier	19.74 ± 0.84	14.49 ± 1.47				

All values are mean ± SEM. HRT Groups: Non-carrier and no HRT (n = 86), Non-carrier and HRT (n = 11), *APOE4* carrier and no HRT (n = 36), *APOE4* carrier and HRT (n = 3), Total no HRT (n = 122), Total HRT (n = 14). Model one (M1) ANOVA main-effects model (unadjusted) including only HRT or HRT*carrier-status as an explanatory variable. Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates age, BMI, centre, smoking status and oily fish intake. Bold font indicates significance ($p < 0.05$) HRT, hormone therapy, CS, carrier-status, RBC, Red blood cell, DHA, Docosahexaenoic acid, EPA, eicosapentaenoic acid, DPA, docosapentaenoic acid, LA linoleic acid, AA, Arachidonic acid, ALA, Alpha linoleic acid, AA:DHA, Arachidonic

acid: Docosahexaenoic acid ratio, SFA, saturated fatty acid, MUFA, monounsaturated fatty acid, PC, phosphatidylcholine.

Age does not influence PUFA profile in female study participants

To determine if the aforementioned association between *APOE4* carrier status and PUFAs was influenced by age, we analysed baseline PUFA levels across distinct age tertiles (≤ 59, 60-69 and ≥ 70 years). Although PUFA concentration in circulation appeared to increase significantly with age this effect was not significant. An age*genotype interaction was not detected in this analysis, suggesting that *APOE4's* impact on PUFA status is not influenced by ageing

Table 2.6 Baseline Erythrocyte and plasma FA profile according to age and age*carrier-status interaction.

		T1 (≤ 59)	T2 (60-69)	T3 (≥ 70)	Age M1	Age M2	CS*age M1	CS*age M2
RBC (%)								
DHA	Non-carrier	3.80 ± 0.14	3.60 ± 0.12	3.99 ± 0.13	0.680	0.525	0.909	0.992
	ε4 carrier	4.02 ± 0.25	3.84 ± 0.13	4.13 ± 0.36				
EPA	Non-carrier	0.88 ± 0.06	0.93 ± 0.04	0.95 ± 0.05	0.154	0.381	0.581	0.795
	ε4 carrier	0.96 ± 0.08	1.10 ± 0.04	1.11 ± 0.06				
AA	Non-carrier	13.55 ± 0.35	13.2 ± 0.19	13.31 ± 0.39	0.498	0.784	0.206	0.270
	ε4 carrier	13.54 ± 0.40	12.85 ± 0.18	14.38 ± 1.2				
n-3 Index	Non-carrier	4.68 ± 0.18	4.67 ± 0.14	4.95 ± 0.12	0.590	0.410	0.929	0.993
	ε4 carrier	4.98 ± 0.29	4.93 ± 0.14	5.24 ± 0.39				
Total n-3	Non-carrier	7.44 ± 0.23	7.34 ± 0.17	7.63 ± 0.15	0.432	0.356	0.934	0.855
	ε4 carrier	7.66 ± 0.29	7.57 ± 0.17	8.09 ± 0.18				
Total n-6	Non-carrier	30.9 ± 0.3	30.0 ± 0.2	29.0 ± 0.4	0.965	0.854	**0.029**	**0.013**
	ε4 carrier	28.7 ± 0.3	29.0 ± 0.3	30.1 ± 0.7				
AA:DHA	Non-carrier	3.65 ± 0.15	3.77 ± 0.13	3.39 ± 0.14	0.888	0.675	0.530	0.636
	ε4 carrier	3.5 ± 0.30	3.45 ± 0.13	3.51 ± 0.15				
Plasma (µg/ml)								
DHA	Non-carrier	1.30 ± 0.14	1.35 ± 0.09	1.58 ± 0.18	0.646	0.826	0.247	0.312

Table 2.6 continued

	ε4 carrier	1.70 ± 0.23	1.62 ± 0.14	1.04 ± 0.23				
EPA	Non-carrier	0.23 ± 0.02	0.24 ± 0.02	0.28 ± 0.04	0.561	0.836	0.780	0.699
	ε4 carrier	0.32 ± 0.05	0.28 ± 0.02	0.27 ± 0.09				
AA	Non-carrier	1.89 ± 0.77	1.67 ± 0.14	1.71 ± 0.14	0.733	0.727	0.658	0.336
	ε4 carrier	1.80 ± 0.23	1.76 ± 0.16	1.35 ± 0.26				
Plasma PC fraction (µg/ml)								
PC 36:5 EPA containing	Non-carrier	47.46 ± 2.31	53.13 ± 3.15	58.59 ± 4.62	0.160	0.273	0.237	0.260
	ε4 carrier	70.98 ± 11.76	79.38 ± 8.61	119.28 ± 8.40				
PC 38:6 DHA containing	Non-carrier	68.46 ± 2.73	68.67 ± 2.52	75.81 ± 3.36	0.692	0.702	0.220	0.336
	ε4 carrier	80.22 ± 7.77	97.65 ± 8.61	134.61 ± 10.05				
PC 40:6 DHA containing	Non-carrier	18.06 ± 1.05	17.64 ± 0.84	20.16 ± 1.26	0.130	0.110	0.742	0.756
	ε4 carrier	21.00 ± 2.31	28.35 ± 3.15	41.16 ± 6.30				

All values are mean ± SEM. Groups: Non-carrier T1 (n = 21), Non carrier T2 (n = 32), Non carrier T3 (n = 24), *APOE4* carrier T1 (n = 10), *APOE4* carrier T2 (n = 26), *APOE4* carrier T3 (n = 3), Total T1 (n = 31), Total T2 (n = 78), Total T3 (n = 27). Model one (M1) ANOVA main-effects model (unadjusted) including only age or age*carrier-status as an explanatory variable. Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates BMI, centre, smoking status, HRT status and oily fish intake. Bold font indicates significance ($p < 0.05$). CS, carrier-status, RBC, Red blood cell, DHA, Docosahexaenoic acid, EPA, eicosapentaenoic acid, AA, Arachidonic acid, AA:DHA, Arachidonic acid: Docosahexaenoic acid ratio, PC, phosphatidylcholine.

Factors influencing cognition

Participants of the CANN study underwent comprehensive cognitive testing. Given that both *APOE4* carrier status and HRT have been associated with cognitive decline and were both linked to altered n-3 PUFA status in the earlier analysis, we continued to explore how these factors may influence cognition utilising the CANN cognitive data.

APOE4 carrier status is associated with a reduction in verbal fluency, memory speed and executive function

In general, *APOE4* carriers appeared to perform worse than their non-carrier counterparts on the battery of cognitive tests. Notably, verbal fluency was 10% lower (Non – carrier: 12.4 ± 0.3, *APOE4* carrier: 11.1 ± 0.4, $p < 0.05$, Table 2.7), speed of memory with 8%

lower speed (Non – carrier: 4207 ± 103s, *APOE4* carrier: 4584 ± 205s, $p < 0.05$, Table 2.7) and executive function was 6% lower performance (Non – carrier: 118 ± 2, *APOE4* carrier: 111 ± 3, $p < 0.01$, Table 2.7).

Table 2.7 Baseline cognitive test performance according to *E4* carrier status

Cognitive test	Non - carrier	ε4 Carrier	M2 P Value
MoCA	26.9 ± 0.2	26.4 ± 0.4	0.096
Verbal Fluency	12.4 ± 0.3	11.1 ± 0.4	**0.033**
Verbal Fluency responses	12.3 ± 0.4	12.3 ± 0.4	0.936
Original Misplacement	247 ± 8	245 ± 10	0.507
Accuracy single item placement	2.40 ± 0.07	2.34 ± 0.1	0.251
Power of Attention (ms)	1350 ± 15	1315 ± 20	0.748
Continuity of Attention	91.8 ± 0.3	91.8 ± 0.5	0.607
Response Variability (%)	50.9 ± 1.0	52.3 ± 2.1	0.755
Cognitive reaction time (ms)	219 ± 7	202 ± 11	0.145
Quality of working Memory (SI)	1.72 ± 0.04	1.71 ± 0.05	0.264
Quality of working episodic memory	182 ± 5	179 ± 10	0.260
Speed of Memory (ms)	4261 ± 91	4583 ± 205	**0.028**
Quality of Memory	353 ± 7	349 ± 11.0	0.112
Executive Function	117 ± 2	110 ± 3	**0.009**
Picture Recognition	86.3 ± 1.3	84.8 ± 2.5	0.299

All values are mean ± SEM. *APOE* carrier groups: *APOE4* non-carrier (n = 97), *APOE4* carrier (n = 39). (M2) ANOVA mixed effects model (adjusted) including covariates MCI/SMI, Education, IQ, age, BMI, centre, smoking status, HRT status and oily fish intake. Bold font indicates significance ($p < 0.05$)

HRT impacts on cognitive performance and is influenced by *APOE4* carrier status

Incorporating HRT status into the analysis revealed that both HRT mediated and HRT**APOE4* carrier effects. In general, receiving HRT was associated with improved cognitive performance. MoCA, cognitive reaction time, speed of memory and executive function tasks were significantly improved in those receiving HRT. Interestingly, a number of cognitive tests were significantly influenced by HRT**APOE4* carrier status interaction including the picture recognition test which was dramatically improved by 15 ± 3.11% in the *APOE4**HRT group compared to non-HRT users. Again, as with the FA analysis, all HRT**APOE4* carrier-status analysis is exploratory and should be evaluated with caution due to low n numbers within the *APOE4* carrier in the HRT group. However, the highly significant result in this section are encouraging.

Table 2.8 Baseline cognitive test performance according to HRT and HRT*carrier-status interaction.

Cognitive test	***APOE4* carrier**	**Non - HRT**	**HRT**	**HRT M2**	**CS*HRT M2**
MoCA	Non-carrier	26.8 ± 0.2	27.7 ± 0.6	**0.029**	0.314
	ε4 carrier	26.2 ± 0.4	28.0 ± 1.5		
Verbal Fluency	Non-carrier	12.3 ± 0.4	13.2 ± 1.0	0.714	0.799
	ε4 carrier	11.1 ± 0.4	11.3 ± 1.8		
Verbal Fluency responses	Non-carrier	12.3 ± 0.4	12.8 ± 1.0	0.547	0.801
	ε4 carrier	12.2 ± 0.6	13.3 ± 0.7		
Original Misplacement	Non-carrier	246.2 ± 8.7	257.2 ± 22.8	0.624	0.295
	ε4 carrier	248.7 ± 11.2	212.1 ± 42.2		
Accuracy single item placement	Non-carrier	2.4 ± 0.07	2.1 ± 0.2	0.963	0.224
	ε4 carrier	2.3 ± 0.1	2.6 ± 0.3		
Power of Attention	Non-carrier	1354.0 ± 15.7	1326.0 ± 51.5	0.766	0.866

Table 2.8 continued

	ε4 carrier	1314.6 ± 19.4	1323.0 ± 152.2		
Continuity of Attention	Non-carrier	91.8 ± 0.3	91.6 ± 0.7	0.448	0.222
	ε4 carrier	91.6 ± 0.5	93.3 ± 1.2		
Response Variability	Non-carrier	50.7 ± 1.0	52.4 ± 3.6	0.748	0.875
	ε4 carrier	52.2 ± 2.3	53.6 ± 8.4		
Cognitive reaction time	Non-carrier	220.8 ± 8.1	212.4 ± 17.3	**0.000**	**0.001**
	ε4 carrier	210.7 ± 10.3	97.3 ± 15.7		
Quality of working Memory	Non-carrier	1.70 ± 0.04	1.8 ± 0.05	0.614	0.191
	ε4 carrier	1.72 ± 0.05	1.5 ± 0.31		
Quality of working episodic memory	Non-carrier	182.5 ± 5.7	182.3 ± 17.8	0.101	0.063
	ε4 carrier	174.0 ± 9.8	239.4 ± 17.8		
Speed of Memory	Non-carrier	4267.6 ± 95.7	4209.3 ± 306.5	**0.029**	**0.036**
	ε4 carrier	4674.3 ± 211.8	3527.0 ± 604.9		
Quality of Memory	Non-carrier	352.1 ± 8.5	366.2 ±17.0	0.136	0.151
	ε4 carrier	345.3 ± 11.2	391.9 ± 46.8		
Executive Function	Non-carrier	116.7 ± 2.2	124.4 ± 8.8	**0.014**	0.147
	ε4 carrier	108.4 ± 3.1	135.8 ± 11.8		
Picture Recognition	Non-carrier	86.9 ± 1.3	81.8 ± 4.7	0.473	**0.031**
	ε4 carrier	83.9 ± 2.6	96.7 ± 1.7		

All values are mean ± SEM. Groups: Non-carrier and no HRT (n = 86), Non-carrier and HRT (n = 11), *APOE4* carrier and no HRT (n = 36), *APOE4* carrier and HRT (n = 3), Total no HRT (n = 122), Total HRT (n = 14). Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates MCI/SMI, Education, IQ, age, BMI, centre, smoking status, and oily fish intake Bold font indicates significant p values ($p < 0.05$) HRT, hormone therapy, CS, carrier-status.

Erythrocyte n-3 index has little impact on cognitive performance

Higher RBC n-3 index has been associated with improved cognitive performance and reduced AD risk. Similarly, n-3 index was found to be altered in *APOE4* individuals in our earlier analysis. However, in this study n-3 index was not associated with cognitive performance. We found no evidence of a significant n-3 index or n-3 index*carrier-status on cognitive performance across n-3 index tertiles. A similar result was attained when focusing solely on erythrocyte DHA (data not shown).

Table 2.9 Influence of n-3 Index on cognitive performance

Cognitive test	**APOE4 carrier**	**T1 n-3 Index**	**T2 n-3 Index**	**T3 n-3 Index**	n-3 index M2	CS*n-3 Index M2
MoCA	Non-carrier	27.1 ± 0.4	27.1 ± 0.5	26.5 ± 0.4	0.413	0.612
	ε4 carrier	26.4 ± 0.6	26.6 ± 0.5	26.1 ± 0.7		
Verbal Fluency	Non-carrier	12.3 ± 0.6	13.4 ± 0.4	11.7 ± 0.6	0.663	0.689
	ε4 carrier	11.6 ± 0.8	11.2 ± 0.7	10.6 ± 0.5		
Verbal Fluency responses	Non-carrier	11.8 ± 0.6	12.7 ± 0.7	12.5 ± 0.7	0.230	0.289
	ε4 carrier	12.7 ± 0.7	11.4 ± 1.1	12.8 ± 0.9		
Original Misplacement	Non-carrier	244.0 ± 14.2	236.0 ± 13.7	260.0 ± 15.0	0.782	0.788
	ε4 carrier	233.2 ± 18.0	244.5 ± 18.0	257.8 ± 20.3		
Accuracy single item placement	Non-carrier	2.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	0.816	0.540
	ε4 carrier	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2		
Power of Attention	Non-carrier	1322.1 ± 27.6	1334.4 ± 26.3	1400.8 ± 24.6	0.280	0.270
	ε4 carrier	1302.0 ± 46.7	1320.2 ± 39.4	1320.6 ± 22.1		
Continuity of Attention	Non-carrier	91.3 ± 0.7	92.2 ± 0.5	91.8 ± 0.5	0.397	0.270
	ε4 carrier	91.6 ± 0.6	90.8 ± 1.1	92.9 ± 0.6		
Response Variability	Non-carrier	51.2 ± 1.6	51.1 ± 1.8	50.2 ± 1.6	0.276	0.274
	ε4 carrier	50.5 ± 3.6	57.3 ± 4.4	48.9 ± 2.7		
Cognitive reaction time	Non-carrier	214.5 ± 11.8	214.6 ± 11.4	229.8 ± 16.1	0.479	0.281
	ε4 carrier	205.4 ± 26.7	202.9 ± 18.7	198.4 ± 12.7		
Quality of working Memory	Non-carrier	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	0.065	0.783
	ε4 carrier	1.6 ± 0.1	1.7 ± 0.1	1.8 ± 0.1		
Quality of working episodic memory	Non-carrier	186.7 ± 9.3	192.7 ± 8.7	173.2 ± 10.3	0.783	0.239
	ε4 carrier	170.2 ± 20.4	179.0 ± 19.8	185.7 ± 10.0		
Speed of Memory	Non-carrier	4328.2 ± 184.9	4079.3 ± 137.1	4346.8 ± 156.5	0.661	0.329
	ε4 carrier	4890.2 ± 695.7	4551.0 ± 214.5	4397.4 ± 179.1		
Quality of Memory	Non-carrier	347.6 ± 15.1	367.1 ± 12.8	349.7 ± 13.3	0.533	0.545
	ε4 carrier	329.2 ± 21.3	346.4 ± 21.4	365.6 ± 13.9		
Executive Function	Non-carrier	115.7 ± 4.1	121.3 ± 3.5	115.3 ± 4.1	0.312	0.615
	ε4 carrier	117.5 ± 7.6	109.9 ± 4.5	106.2 ± 5.0		

Table 2.9 continued

Picture Recognition	Non-carrier	89.0 ± 2.2	83.9 ± 2.6	86.6 ± 1.3	0.868	0.382
	ε4 carrier	84.5 ± 5.1	82.5 ± 4.7	87.5 ± 3.2		

All values are mean ± SEM. Groups: Non-carrier and T1 (2.8 - 4.49) n-3 index (n = 31), Non-carrier and T2 (4.5-5.19) n-3 Index (n = 31), Non-Carrier and T3 n-3 (5.20-6.1) Index (n = 32), *APOE4* carrier and T1 (2.8 - 4.49) n-3 Index (n = 13), *APOE4* carrier and T2 (4.5-5.19) n-3 Index (n = 13), *APOE4* carrier and T3 (5.20-6.1) Index (n= 13) Total T1 (2.8 - 4.49) n-3 index (n = 44), Total T2 (4.5-5.19) n-3 Index (n = 45), Total T3 (5.20-6.1) n-3 Index (n = 45). Model 2 (M2) ANOVA mixed effects model (adjusted) including covariates MCI/SMI, Education, IQ, age, BMI, centre and smoking status. Bold font indicates significant p values ($p < 0.05$) HRT, hormone therapy, CS, carrier-status.

Discussion

Intake of oily fish, the predominant source of the long chain n-3 PUFAs, EPA and DHA in the human diet has been associated with improved cognition in older adults (Witte, Kerti et al. 2014, Del Brutto, Mera et al. 2016), and reduced AD risk (Wu, Ding et al. 2015). The transport and/or metabolism of n-3 PUFAs such as DHA, may be altered by the *APOE4* genotype, increasing carrier susceptibility to n-3 PUFA deficiency, and cognitive decline (Nock, Chouinard-Watkins et al. 2017). In addition, AD and MCI risk associated with *APOE4* appears to be exacerbated in females between the ages of 55 – 70 years (Neu, Pa et al. 2017), suggesting a potential menopausal involvement. Here we perform a set of exploratory analyses on data obtained from the CANN study (Irvine, Scholey et al. 2018), to investigate the impact of *APOE* carrier-status, oily fish intake and HRT on PUFA status and cognition, accounting for multiple confounding variables. Our analysis reveals that *APOE* carrier-status and its interaction with HRT are associated with both altered circulating n-3 PUFA status and cognitive performance. Furthermore, we report that *APOE4* influenced specific cognitive functions, particularly verbal and executive functions, whilst generally HRT was more wide-spread evident from improvement in MoCA test performance. Our results also provide evidence to suggest that HRT is neuroprotective, and that these effects are more extensive in *APOE4* carriers. Despite fish intake significantly increasing n-3 PUFA status, cognitive performance was not improved in those with a higher erythrocyte n-3 index.

Blood PUFA status was influenced by oily fish intake, BMI, *APOE*-carrier status and *APOE*-carrier status*HRT interaction. As expected, incremental consumption of oily fish portions was found to increase n-3 PUFA status, whilst reducing n-6 PUFA levels. This PUFA change was restricted to erythrocytes, and absent in the plasma/PC fractions, and

coincides with reports advocating erythrocytes as a biomarker of long-term oily fish intake due to their slower turn-over rate (half-life 120 days) (Sun, Ma et al. 2007). Conversely, BMI had the opposite impact, with erythrocyte DHA and n-3 PUFA status following an inverse relationship with BMI. Interestingly, this is in line with Howe and colleagues (Howe, Buckley et al. 2014), who reported that this effect was gender specific, and may indicate that adiposity in females is regulated by DHA status, or vice versa.

Generally, *APOE4* carrier status led to an overall increase in n-3 PUFA status, most apparent in the erythrocyte, with EPA markedly increased in most fractions. This increase was also found to some extent by Fisk and colleagues (Fisk, Irvine et al. 2018), however the effect in this study was limited to males, and the erythrocyte fraction was not analysed. Although somewhat paradoxical given both low n-3 PUFA status and *APOE4* genotype are associated with greater AD risk, such a phenomenon has been reported as a result of increasing age (Walker, Browning et al. 2014, Fisk, Irvine et al. 2018), and may reflect reduced tissue uptake. n-3 PUFA transport deficits and reduced brain uptake have been reported in *APOE4* mouse models (Vandal, Alata et al. 2014, Nock, Chouinard-Watkins et al. 2017), and may contribute to the elevated blood levels with less entering the brain. The present analysis, which found a substantial link between EPA levels and *APOE4* carrier status in females, warrant further exploration.

Age was assessed to determine its influence on PUFA status and to distinguish a distinct age group in which *APOE4* carrier status may have the greatest impact. Although age mediated increases in n-3 PUFA status have been previously reported (Walker, Browning et al. 2014, Fisk, Irvine et al. 2018), we did not observe a significant difference across our age tertiles despite a nominal increase in a number of n-3 PUFAs. This may reflect our exclusive use of females, in which the impact of age may be less pronounced, and also a relatively limited age range compared to the Fisk et al., analysis who included individuals between 20-70 years. Similarly, an age*carrier-status interaction was not observed indicating that the impact of *APOE4* on n-PUFA status in females was not age specific, although this may be explained by the modest number of participants in the oldest tertiles.

HRT alone had no effect on the FA profile. However, continuation of this analysis addressing the HRT*carrier-status interaction revealed effects on the n-3 index and DHA profiles across erythrocyte and PC fractions. HRT appeared to mitigate the elevated n-3 PUFA levels identified in *APOE4* carriers. HRT has been previously shown to increase circulating levels of DHA and EPA in postmenopausal women (Sumino, Ichikawa et al. 2003), which appeared to occur to some extent in our *APOE3* participants. To our

knowledge this is the first analysis to investigate such parameters across genotypes. With that said this remains an exploratory analysis and needs to be validated in a study with larger numbers of *APOE4* HRT individuals. Furthermore, our analysis provided no consideration for the type of HRT prescribed nor the years or commencement or length of usage which are all likely to influence the impact of HRT on PUFA status.

Overall, cognitive performance was influenced by *APOE4* carrier-status, HRT and *APOE4* carrier status*HRT, whilst surprisingly, erythrocyte n-3 index and DHA levels did not influence cognitive performance. *APOE4* carrier status was associated with reduced performance on a subset of specific cognitive tests. The tests significantly impaired by possession of an *APOE4* allele were verbal fluency, speed of memory and executive function tasks. These tasks are indicative of poorer language function, executive function and semantic memory (Bertola, Mota et al. 2014), and are in line with a recent longitudinal study in which similar deficits were apparent in non-AD, healthy ageing *APOE4* carriers (Li, Qiu et al. 2019). This is promising given previous studies have yielded mixed results (Small, Rosnick et al. 2004), highlighting the necessity of multi-domain cognitive testing and the importance of controlling for confounding variables. Vulnerable brain networks associated with *APOE4* genotype have been established in mouse models (Badea, Wu et al. 2019), and may account for the specific impairment in cognition. As suggested by Li et al (Li, Qiu et al. 2019), knowledge of the specific cognitive domains impaired, may be useful in the future for predicting an *APOE4* carriers risk of developing MCI or dementia.

Interestingly, HRT alone led to a general improvement in cognition evident from the significant increase in MoCA test score, which is in line with a recent randomised control trial in which HRT intervention was assessed in females with MCI (Yoon, Chin et al. 2018). Although still controversial these results provide evidence that if the correct timing, form and dosage of HRT is established it may offer an effective method of intervention to reduce MCI and AD risk. This may be un-surprising given the neuroprotective, neurotrophic, neurogenerative, anti-oxidative and anti-inflammatory actions that estrogens impart upon the brain (Li, Cui et al. 2014). Speed of memory, and executive function tasks, which declined in *APOE4* carriers were conversely improved by HRT. This may indicate that those with both *APOE4* carrier status and menopause receive a ‘double hit’ of deleterious factors leaving the associated brain regions particularly vulnerable.

In addition to the general impact of HRT and in line with previous reports (Rippon, Tang et al. 2006, Ryan, Carrière et al. 2009), we also observed a HRT*_APOE_-carrier interaction, which was suggestive of an increased benefit of HRT in carriers of *APOE4* genotype. Of the cognitive tests significantly altered were reaction time and speed of memory, which were dramatically improved in *APOE4* carriers receiving HRT. These tasks are indicators of cognitive processing speed and the dramatic improvement is potentially indicative of alleviating the aforementioned 'double hit'. In addition, the picture recognition task saw *APOE4* carriers receiving HRT improve performance by 15 ± 3.11%. Deficits in recognition memory have been previously linked to ovarian failure and menopause (Hara, Park et al. 2012, Bastos, Pereira et al. 2015), but interestingly here it appears to be particularly associated with *APOE* genotype. Estrogens are known to interact with *APOE*, with experimental models reporting that estrogen may modulate APOE expression (Safieh, Korczyn et al. 2019), and may in part explain the *APOE* genotype specific responses to HRT.

Finally, increased n-3 index was not associated with improved cognitive function in this study. This is in contrast to a number of previous reports in which this association has been identified (Lukaschek, von Schacky et al. 2016, Hooper, De Souto Barreto et al. 2018). This is likely due to the exclusion criteria in which participants with high n-3 index were excluded from the study. Therefore, a subset of the population with high n-3 index (probably consuming greater amounts of oily fish) are missing, potentially explaining the lack of association in this study cohort. Furthermore, it is becoming increasingly evident, that a higher n-3 index and DHA status in the plasma may indicate defective brain DHA uptake (particularly in *APOE4* carriers) which would negate the expected positive associations between DHA status in the circulation and cognition.

Conclusion

APOE ε4 is associated with alterations in erythrocyte and PC PUFA status and poorer performance on cognitive tasks specifically associated with language and executive functions. Incorporating HRT data revealed a significant improvement in cognitive performance in response to treatment, which appeared to be more extensive in *APOE4* carriers. Interestingly, as well as improving cognitive function, HRT appeared to reverse the elevated n-3 PUFA status associated with *APOE4* carrier status. Further research with a larger cohort of *APOE4* individuals with granular HRT data such as the Millions

Women's Study or the UK Biobank is required to validate this exploratory analysis. Future studies exploring HRT should control for *APOE* genotype in light of our findings.

Chapter 3 : *APOE4* genotype exacerbates the impact of menopause on cognition and synaptic plasticity in *APOE*-TR mice

This chapter has been prepared in the form of a research paper

Title: APOE4 exacerbates the impact of a menopause mimic on cognition and synaptic plasticity in the APOE-TR mouse model

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Introduction

The development of sporadic late-onset Alzheimer's disease (AD) is complex and multifactorial, with more than 20 susceptibility loci including Apolipoprotein E (*APOE*) (Giri et al., 2016). *APOE4* is the strongest prevalent genetic risk factor with an allele frequency of 14% in the general population and concentrated in AD (allele frequency ~ 40% in Caucasians) (Bang, Kwak et al. 2003). The *APOE4* gene increases AD risk in a gene-dose dependent manner, rising 2–3 fold for heterozygous *APOE4* carriers and 12-fold in homozygotes (Corder, D. E. Schmechel et al. 1993, Farrer, Cupples et al. 1997), with age of onset considerably reduced in *APOE4* carriers (Bertram, McQueen et al. 2007, Davidson, Gibbons et al. 2007). However, a large proportion of *APOE4* carriers do not develop AD, indicating that this genotype is predictive rather than prognostic (Henderson, Easteal et al. 1995), with its affect influenced by environmental and biological variables. There is accumulating evidence that the penetrance of *APOE4* genotype is influenced by sex (Farrer, Cupples et al. 1997, Snyder, Asthana et al. 2016), which may underlie the finding that almost two thirds of AD cases are females (Podcasy and Epperson 2016). In a large meta-analysis Neu and colleagues reported increased MCI and AD risk in females versus males *APOE4* carriers between 55-70 years and 65-75 years respectively (Neu, Pa et al. 2017). Despite this observation, little is known of the aetiology underlying this susceptibility. However, given the age at which females are at increased risk (Neu, Pa et al. 2017) and the fact that earlier onset of menopause correlates with poorer cognition in later life (Ryan, Scali et al. 2014), it is plausible that menopause acts as a mediating factor.

Within the central nervous system APOE is the main lipid transporter (Holtzman, Herz et al. 2012). In comparison to other organs, the brain is highly enriched with the n-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) accounting for ~15% of

total brain lipids (Bradbury 2011). We have recently reported a strong association between age, female sex, and an *APOE4* genotype, with decreased cortical DHA and a number of specialized pro-resolving mediators in *APOE4*, which together may contribute to the development of cognitive decline and AD pathology (Martinsen, Tejera et al. 2019). DHA is specifically concentrated within the synaptosome (Neuringer, Anderson et al. 1988) (which in turn is estrogen responsive) thus it is unsurprising that reduced DHA status is consistently linked to poorer cognitive outcome and increased AD risk (Weiser, Butt et al. 2016). Synaptic loss and dysfunction, which has been characterised in both menopause and *APOE4* individuals, is directly associated with cognitive decline and occurs in the initial stages of AD (Kim, Yoon et al. 2014).

With an aetiological link accounting for the greater female *APOE4* susceptibility distinctly lacking (Pontifex, Vauzour et al. 2018), we posit menopause as a contributing factor, given the neuroprotective properties of estrogen (Li, Cui et al. 2014), and greater cognitive decline associated with early menopause (Ryan, Scali et al. 2014). Here, in a transgenic mouse model we assessed the combined impact of *APOE* genotype and ovarian failure on cognitive performance hypothesising that *APOE4* animals will be more susceptible to estrogen loss. Furthermore, we determine if changes in cognitive performance relate to alterations in brain fatty acid profiles and synaptic plasticity. Our model system combines the humanised targeted replacement (*APOE*-TR) mouse model with 4-Vinylcyclohexene diepoxide (VCD) treatment. VCD injection is an established method used to induce ovarian failure in rodents (Kappeler and Hoyer 2012) and credited for its ability to establish an intermediary human like 'perimenopause' phase, whilst maintaining ovarian tissue integrity.

Material and methods

Study approval

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) and were conducted within the provisions of the Home Office Animals (Scientific Procedures) Act 1986.

Animal model and experimental design

Forty eight female humanised *APOE3* (B6.129P2-Apoe$^{tm2(APOE*3)Mae}$ N8) and *APOE4* (B6.129P2-Apoe$^{tm2(APOE*4)Mae}$ N8) targeted replacement mice homozygous for the human *APOE3* or *APOE4* gene (Taconic, Germantown, NY, US) were used in these experiments (Sullivan, Mezdour et al. 1997, Knouff, Hinsdale et al. 1999, Martinsen, Tejera et al. 2019). Mice were maintained in a controlled environment (21 ± 2°C; 12-h light–dark cycle; light from 07:00 hours) and fed *ad libitum* on a standard chow diet (RM3-P, Special Diet Services, Essex, UK) until the age of 4 months, ensuring normal development. Following this run-in period, mice were switched to a semi-purified high fat diet (45 kCal% fat) (D17080301, Research diets, New Brunswick, NJ, USA) for the remaining duration of the experiment (See Table S1 for full dietary composition).

At 8 months of age and to assess the impact of menopause, mice from each genotype received intra peritoneal (i.p.) injections of either VCD (160mg/kg body weight) diluted in sesame oil, or sesame oil vehicle (sham) for a total of 14 injections over 3 weeks. 8 months was selected as it is roughly midlife for the animals (when human menopause occurs) and before natural ovarian failure is known to occur in C57BL/6 mice (Gosden, Laing et al. 1983, Chen, Perez et al. 2014). Following completion of the final behavioural test, 12-month aged animals were sedated with isoflurane (1.5%) in a mixture of nitrous oxide (70%) and oxygen (30%) and transcardially perfused with an ice-cold PBS containing protease (SIGMAFAST^{TM} Protease inhibitor, Sigma, Devon, UK) and phosphatase (1 mM sodium pyrophosphate and 50 mM sodium fluoride, Sigma, Devon, UK) inhibitors. Sera were isolated via centrifugation at 2000 x g for 10 min. Brains were rapidly removed, halved, snap frozen and stored at -80°C until biochemical analysis. Ovaries were rapidly removed and processed for histology.

Behavioural assessment

All behavioural tests were performed when mice were 12 months of age and immediately prior to sacrifice. A visual placing test was performed on each animal on the first day of testing, to ensure animals were not visually impaired (Pinto and Enroth-Cugell 2000).

Spatial learning and memory was evaluated with the Barnes Maze as previously described (Patil, Sunyer et al. 2009). Briefly, the maze consisted of a brightly illuminated (800 lux lighting) circular platform (92cm diameter), with 20 evenly distributed holes located around the circumference and visual cues (4 simple shapes) placed at the periphery. The

experiment was conducted over a 5-days period, with each mouse tested/trained on ability to locate the escape box 4 times per day during days 1-4. On day 5, a probe test was conducted, the maze was rotated 90°, the escape box was removed and mice were placed in the centre of the maze in which they were free to navigate for one minute. Percentage time in correct quadrant was determined using the Smart 3.0 tracking software (Panlab, Kent, UK).

The novel object recognition (NOR), a measure of recognition memory, was performed as described previously (Davis, Eacott et al. 2013, Leger, Quiedeville et al. 2013), with slight modifications. Briefly, on day 1 mice were habituated in grey 50x50x50cm apparatus illuminated with low lux (100 lux) lighting, mice were placed into the empty maze and allowed to move freely for 10 minutes. On day 2, mice were conditioned to a single object for a 10-minute period. On day 3, mice were placed into the same experimental area in the presence of 2 identical objects for 15 minutes, after which they were returned to their respective cages and an inter-trial interval of one hour was observed. One familiar object was replaced with a novel object. Mice were placed back within the testing area for a final 10 minutes. Videos were analysed for a 5-minute period, after which if an accumulative total of 15 seconds with both objects failed to be reached, analysis continued for the full 10 min or until 15s was achieved. Those not achieving 15s were excluded from the analysis (Denninger, Smith et al. 2018). A discrimination index was calculated as follows: $DI = (TN-TF)/(TN+TF)$, where TN is the time spent exploring the novel object and TF is the time spent exploring the familiar object.

Y-maze spontaneous alternation test, a measure of spatial working memory was performed on the final day of behavioural testing as previously described (Thomas, Morris et al. 2017). Briefly, the Y-maze apparatus comprised of white Plexiglas in the following dimensions (38.5 × 8 × 13 cm, spaced 120° apart) and was illuminated with low lux (100 lux) lighting. Mice were placed in the maze and allowed to explore freely for 7 minutes whilst tracking software recorded zone transitioning and locomotor activity (Smart 3.0 tracking software, Panlab, Kent, UK).

Histological and biochemical analyses

Ovaries were trimmed of fat and fixed in 10% formalin for 24h before being paraffin embedded, and processed for haematoxylin and eosin (H&E) staining as described previously (Chen, Kang et al. 2015). Follicle stimulating hormone (FSH) concentrations were determined by ELISA (Abnova KA2330) in sera samples as per manufacturer's instructions.

Fatty acid profile in the brain

Total lipids were extracted from sub-cortical brain tissues (n=5/6 per group) using the Folch extraction method (Folch, Lees et al. 1957). Fatty acid methyl esters (FAME) were obtained using acid-catalysed transesterification (Christie and Han 2010). FAME were evaporated under oxygen free nitrogen, and re-suspended in 500 µl of iso-hexane, SPE silica cartridges (Clean-up 203 Cusil 156, UCT) were used for purification purposes. The purified FAMEs were separated by gas chromatography using a Trace 1300 series Gas Chromatograph (thermo scientific, Stafford House, Boundary Way, Hemel Hempstead. HP2 7GE, U.K.) equipped with a PTV injector modified for on-column injection, and a flame ionisation detector. A fused silica capillary column (TraceGOLD™ TG-5MS Guard GC Column with SafeGuard column, 30 m × 0.32 mm × 0.25 µm; Thermo Fisher Scientific, Hemel, Hempstead, UK) was employed, and hydrogen was used as carrier gas. The temperature gradient was from 50 to 150°C at 40°C/min and then to 200°C at 2°C/min followed by 214°C at 1°C/min and finally to 220°C at 40°C/min, where it was held for 5 min. Individual methyl esters were identified by comparison to known standards (Marine oil FAME mix RESTEK #35066). Data were collected and processed using the Chromeleon software package (version 7.2).

Immunoblotting

Cortices were homogenised in lysis buffer (CelLytic™ MT, Sigma, UK) containing protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, UK) and phosphatase (PhosSTOP™, Roche, UK) inhibitors. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher, UK). Protein electrophoresis was conducted under denaturing conditions as previously described (Vauzour, Corsini et al. 2018). The following antibodies were used: anti p-Akt Ser473, (1:1000; Cell Signalling, UK), anti-total-Akt, (1:1000; Cell Signalling, UK), anti BDNF (1:500; Santa Cruz Biotechnology, USA), anti APOE (1:1000; Cell Signalling, UK), anti GAPDH

(1:2500, Cell Signalling, UK), anti-beta-actin (1:2500, Cell Signalling, UK) and anti-Rabbit IgG (H + L) DyLight™ 680 Conjugate (1:10,000, Cell Signalling, UK). Bands were revealed by fluorescence using an Odyssey 9120 Infrared imaging system (LI-COR Biosciences, Ltd, UK). Relative band intensities were quantified using the ImageJ software (Schneider, Rasband et al. 2012).

RNA isolation and qRT-PCR

RNA isolation, cDNA synthesis and qRT-PCR were carried out as previously described (Vauzour, Rodriguez-Ramiro et al. 2018). Briefly, total RNA was isolated from the brain samples using the Qiazol reagent (Qiagen, UK). One μg of total RNA was treated with DNase I (Invitrogen, UK) and used for cDNA synthesis using Invitrogen™ Oligo (dT) primers and M-MMLV reverse transcriptase. Quantitative real-time PCR (qRT-PCR) reactions were performed using SYBR green detection technology on the Roche light cycler 480 (Roche Life Science, UK). Results are expressed as relative quantity scaled to the average across all samples per target gene and normalised to the reference genes TATA-box binding protein (*Tbp*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which were identified as optimal housekeeping selection/combination using the Normfinder software (Day, Chambers et al. 2018). The primer sequences are given in Supplementary Table S2.

Statistical Analysis

All data is presented as mean ± SEM. Data analysis was performed in GraphPad Prism version 8 (GraphPad Software, CA, USA). After checking for normality/equal variances and performing log transformation if necessary, comparisons among groups were performed on normally distributed data using two-way ANOVA, followed by post-hoc Sidak test. P-values of less than 0.05 were considered statistically significant.

Results

Repeated injections of VCD result in ovarian failure independently of *APOE* genotype

Repeated injections of VCD in *APOE3-TR* and *APOE4-TR* mice resulted in a considerable loss of ovarian follicles (Fig. 1A) and a 5-fold increase in serum follicle stimulating hormone (FSH) levels (VCD: 2.84 ± 0.32 ng/mL; Sham: 0.52 ± 0.14 ng/mL; $p < 0.0001$ Fig. 1B), irrespective of *APOE* genotype. Neither VCD treatment nor genotype had any impact on body weight ($p > 0.05$ Fig. 1C). Concomitant with VCD injections and subsequent ovarian/hormonal changes, a 2-fold increase in brain expression of estrogen receptor (ER) *Esr2* (VCD: 1.85 ± 0.19 fold; Sham 0.87 ± 0.14 fold; $p < 0.0001$ Fig. 1D), and a nominal 1.3-fold increase in *Esr1* (VCD: 1.18 ± 0.14 fold; Sham: 0.92 ± 0.12 fold; $p > 0.05$ Fig 1E) were observed.

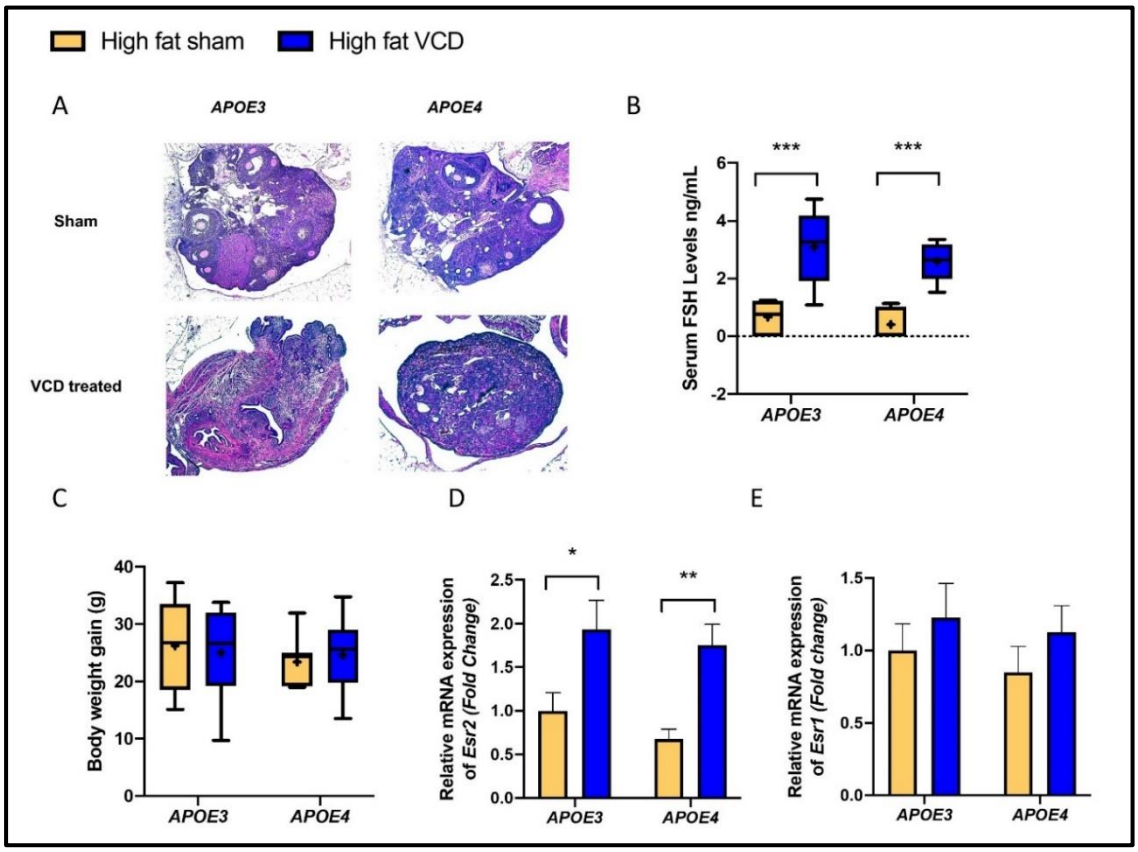

Figure 3.1VCD treatment leads to ovarian failure dependently of APOE genotype in *APOE3*-TR and *APOE4*-TR mice.

A) Representative images of *APOE3*-TR and *APOE4*-TR female mice ovaries stained with hematoxylin and eosin show important loss of ovarian follicles following repeated i.p. injections of VCD; B) VCD injected groups display elevated serum follicle stimulating hormone (FSH) levels (n=5/6); C) Body weight was unaffected by VCD treatment or genotype (n= ≥8); D-E) Brain expression of estrogen receptors *Esr1* and

particularly *Esr2* mRNA levels, are up-regulated in response to VCD injections. Data are presented as mean ± S.E.M *p< 0.05. **P < 0.01, ***P < 0.001.

Whilst *APOE4* impairs working spatial memory, VCD causes additional deficits to recognition memory

Y-maze, Barnes maze and novel object recognition were employed to establish the impact of genotype and VCD treatment on cognitive performance (Fig. 3.2). VCD had no effect on the spatial learning and memory tasks, as assessed by the Y-maze and the Barnes maze (Figure 3.2A and 3.2B). However, a genotype dependant effect was observed, with *APOE4* animals displaying ~15% lower cognitive performance than their *APOE3* counterparts as assessed by the Y-maze ($p < 0.001$ Fig. 3.2A). This was consistent with the Barnes maze, where *APOE4* animals spent ~45% less time in the correct quadrant, ($p < 0.001$ Fig. 3.2B). Representative trajectory maps are shown in Fig. 3.2C.

Unlike spatial learning and memory, recognition memory as assessed by the NOR test was influenced by menopausal status, and in a genotype dependent manner, with VCD injected *APOE4* animals losing the ability to distinguish between novel and familiar objects, ($p < 0.05$ Fig. 3.2D).

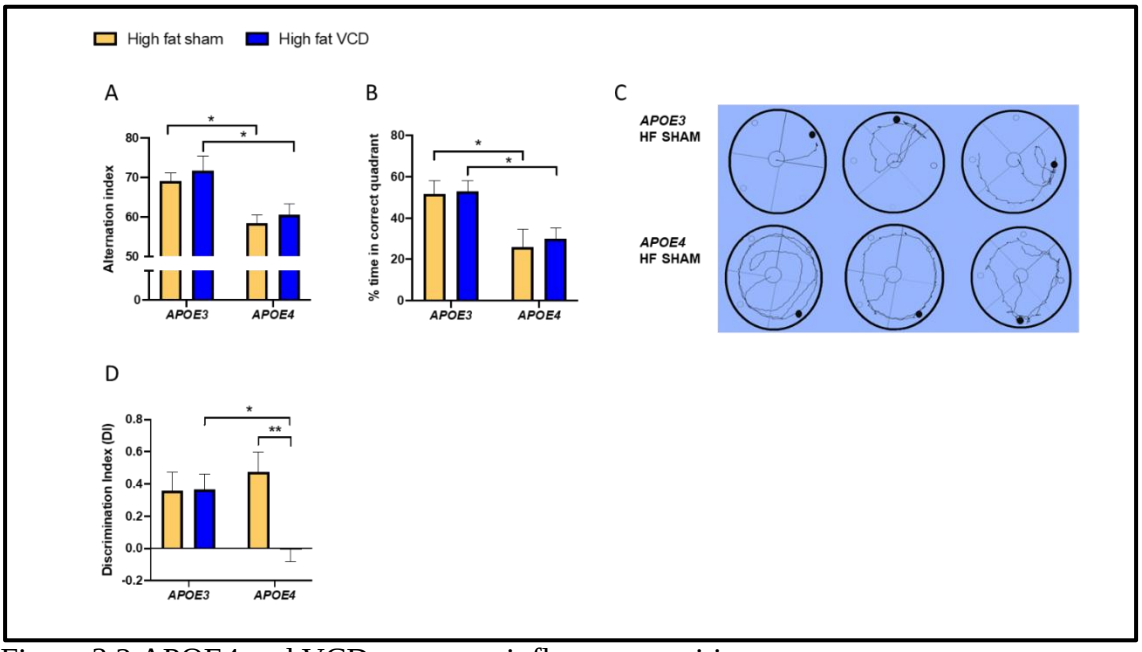

Figure 3.2 APOE4 and VCD treatment influence cognition.

A) Y-maze spontaneous alternation task (n≥8) and B) Barnes probe test (n≥8) identify deficits in spatial memory as a result of *APOE4* genotype, VCD treatment had no impact; C) Representative trajectory's from *APOE3* Sham (top) and *APOE4* sham (bottom) during the probe test, filled circle denotes former location of escape box; D) Performance

on Novel Object Recognition task (n≥8) was severely compromised in the *APOE4* VCD group. Data are presented as mean ± S.E.M *p< 0.05. **P < 0.01.

VCD injections reduce brain DHA levels independently of APOE genotype.

A significant 13% genotype independent lower brain DHA concentration was observed in VCD injected animals independent of their *APOE* genotype (VCD: 11.47 ± 0.35 %; Sham: 13.13 ± 0.16%; p < 0.001 Fig. 3.3A). Higher total mono-unsaturated fatty acids (MUFAs) was observed in *APOE3* carriers following VCD injections (p < 0.05; Table 3.1). These changes were mirrored by a decrease in total saturated fatty acids (SFAs) in *APOE3* (p < 0.05). DHA:AA ratio was 8% lower following VCD treatment (p < 0.05), in *APOE4* animals but remained constant in *APOE3* regardless of treatment.

To identify whether the observed lower DHA was related to deficits in DHA transport, gene expression profiles of key DHA transporters in the brain were assessed. *Fatp4* expression was 1.5-fold lower in *APOE4* VCD injected animals (p > 0.05 Fig. 3.3B). Similar trends were observed for *Fatp1* and *Acsl6* although it did not reach significance (Fig. 3.3c-d). No effect was observed for *Mfsd2a* and *Fabp5*.

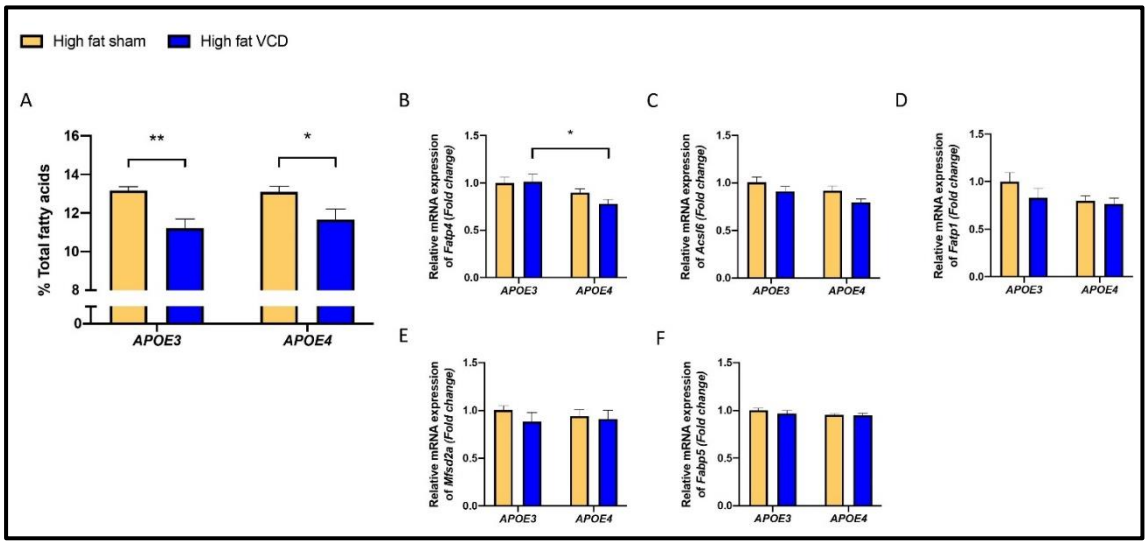

Figure 3.3 HF VCD treatment reduces brain DHA levels.

A) GC-FID analysis revealed lower brain DHA levels in VCD treated groups (n=5/6); B-F) Expression of key transporter proteins involved in DHA transport and uptake. Data are presented as the mean ± S.E.M *p< 0.05. **P < 0.01.

Table 3.1: Brain fatty acid composition of experimental animals

	APOE3		***APOE4***		***Genotype P value***	***Intervention P value***	***Interaction P value***
Fatty acid	***HF Sham***	***HF VCD***	***HF Sham***	***HF VCD***			
Total n-3 PUFA	13.30 ± 0.20 [a]	11.60 ± 0.70 [b]	13.20 ± 0.30	11.80 ± 0.50	0.964	**0.004**	0.805
20:5 n-3 (EPA)	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.01	0.03 ± 0.14	0.466	0.147	0.414
22:6 n-3 (DHA)	13.20 ± 0.20 [a]	11.20 ± 0.50 [b]	13.10 ± 0.30 [a]	11.70 ± 0.50 [b]	0.650	**0.001**	0.519
Total n-6	14.20 ± 0.20	12.60 ± 0.40	14.80 ± 0.90	13.20 ± 0.60	0.274	**0.011**	0.979
20:4 n-6 (AA)	9.37 ± 0.21	8.10 ± 0.39	8.95 ± 0.25	8.67 ± 0.50	0.860	0.058	0.221
DHA:AA	1.40 ± 0.03	1.39 ± 0.05	1.48 ± 0.01 [a]	1.36 ± 0.03 [b]	0.619	**0.049**	0.134
Total SFAs	38.70 ± 0.40 [a]	35.50 ± 0.90 [b]	36.60 ± 0.60	36.90 ± 1.20	0.689	0.100	0.054
16:0	17.60 ± 0.50 [a]	14.70 ± 0.70 b [§]	16.20 ± 0.50	17.30 ± 0.90 [§]	0.406	0.207	**0.010**
18:0	20.10 ± 0.30	18.90 ± 0.50	19.30 ± 0.20	18.30 ± 0.60	0.138	**0.025**	0.802
20:0	0.31 ± 0.02 [a]	0.41 ± 0.03 [b]	0.28 ± 0.02	0.33 ± 0.03	0.053	**0.014**	0.343
22:0	0.22 ± 0.04	0.38 ± 0.05 [§]	0.26 ± 0.01	0.19 ± 0.06 [§]	0.099	0.110	**0.030**
Total MUFAs	25.40 ± 0.40 [a]	30.50 ± 0.80 b [§]	27.30 ± 0.90	27.90 ± 1.40 [§]	0.448	**0.021**	**0.013**

Table 3.1 continued

18:1 n-9	18.20 ± 0.50 [a]	21.20 ± 0.80 [b]	19.30 ± 0.60	19.30 ± 1.20	0.684	0.091	0.084
20:1 n-9	2.03 ± 0.18 [a]	3.04 ± 0.20 [b]	2.28 ± 0.20	2.21 ± 0.21	0.166	**0.028**	**0.013**
24:1 n-9	1.37 ± 0.09 [a]	1.96 ± 0.18 [b §]	1.56 ± 0.11	1.14 ± 0.24 [§]	0.080	0.641	**0.008**

(n=5/6 per group). PUFA; Polyunsaturated fatty acid, 20:5 n-3 EPA; Eicosapentaenoic acid, 22:6 n-3 DHA; Docosahexaenoic acid, 20:4 n-6 AA; Arachidonic acid, DHA:AA; Docosahexaenoic acid to Arachidonic acid ratio, SFAs; Saturated fatty acids, 16:0; Palmitic acid, 18:0; Stearic acid, 20:0; Eicosanoic acid, 22:0; Docosanoic acid, MUFAs; Monounsaturated fatty acids, 18:1 n-9; Oleic acid, 20:1 n-9; 11-Eicosenoic acid, 24:1 n-9; Nervonic acid. Data is % of total fatty acids and mean value ± S.E.M. 2-way ANOVA. Letters a,b denote significant difference between intervention whilst § denotes significant genotype effect as analysed via post hoc. The full table can be found in supplementary data (Table S3)

VCD injections trigger a lower synaptic plasticity response in APOE4-TR mice

Lower APOE protein levels were observed in *APOE4* compared to *APOE3* animals ($p < 0.0001$; Fig. 3.4E), which was not influenced by VCD treatment. Expression of the h*APOE* transgene was constant across all groups indicating a post-transcriptional impact of genotype ($p > 0.05$ Fig. 3.4E).

VCD treatment led to a significant reduction in brain BDNF protein levels ($p < 0.05$ Fig. 3.4B), with post-hoc analysis indicating a significant, 2-fold reduction limited to *APOE4* VCD treated animals when compared to sham (*APOE4* VCD: 0.74 ± 0.01 arbitrary unit; *APOE4* Sham: 0.146 ± 0.01 arbitrary unit; $p < 0.05$). This was reflected at the gene level in which a 30% reduction in *Bdnf* expression was observed ($p < 0.05$; Fig. 3.4F), (*APOE4* VCD: 0.77 ± 0.04 fold; *APOE4* Sham: 1.11 ± 0.157 fold; $p < 0.05$). As PI3K, and in particular AKT/PKB are involved in BDNF regulation we next sought to capture their response to intervention. As with BDNF, AKT Ser473 protein was significantly altered in a genotype*VCD dependent manner ($p< 0.05$ Fig 3.4C). *Akt1* gene expression was similarly ~ 20% lower in the VCD *APOE4* group ($p <0.05$ Fig. 3.4G); however, this did not reach significance in post hoc analysis. VCD treatment also resulted in altered NMDA receptor related signalling (Fig. 3.4H-J). The transcription factors *Creb1* ($p < 0.05$; Fig. 3.4H) and *Atf4* ($p < 0.0001$; Fig. 3.4I) were downregulated in response to VCD treatment. Similarly, further along the pathway, NMDA R2b expression itself, *Grin2b* ($p < 0.05$ Fig. 3.4J) and the highly associated *Ephb2* ($p < 0.05$) were also downregulated. Post hoc analysis determined that this effect was widespread affecting both genotypes when compared to sham counterparts, with the exception for *Ephb2* in which the reduced expression was only evident in the *APOE4* VCD treated group. In addition to the VCD effect, genotype influenced expression of *Cbd1* ($p < 0.05$) independently of VCD treatment.

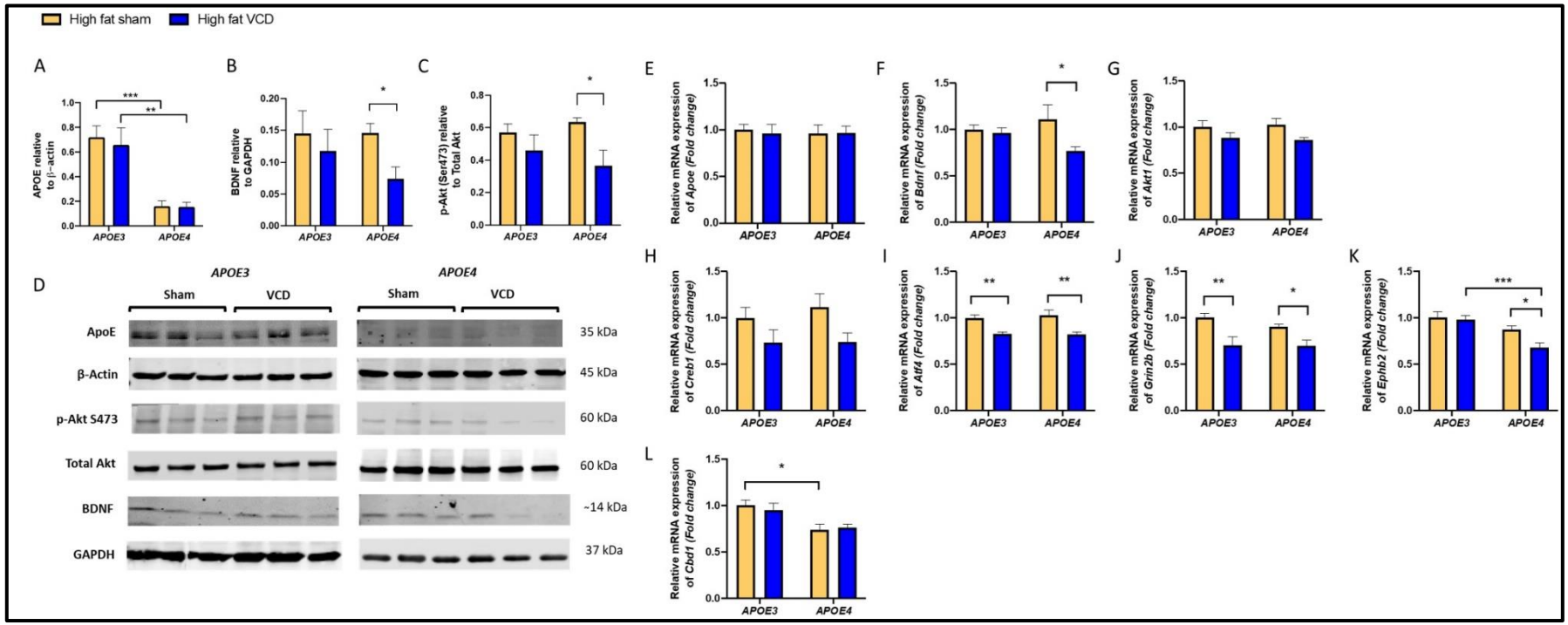

Figure 3.4 Selective impact of genotype and VCD on synaptic plasticity related genes and proteins;

A) APOE protein was lower in the cortex of *APOE4*-TR animals; B) BDNF protein was lower in *APOE4* VCD treated animals; C) AKT protein was similarly lower in *APOE4* VCD treated animals; D) representative western blots for both *APOE3* and *APOE4*, Sham and VCD treated animals (n=6); F-L) Hippocampal expression profiles relating to synaptic plasticity (n=5/6); F-G) Showing VCD mediated reductions in BDNF – AKT signalling (also observed at protein level); H-K) altered NMDA receptor signalling and L) genotype related reduction in *Cbd1* expression. Data are presented as the mean ± S.E.M *p< 0.05. **P < 0.01 ***P <0.001

Discussion

Several studies report that female carriers of the *APOE4* genotype are at a higher risk of AD (Neu, Pa et al. 2017); however little is known as to how this predisposition manifests (Pontifex, Vauzour et al. 2018). Here we sought to determine whether the cumulative *APOE4**menopause effect, contributes towards this greater risk. With VCD treatment emerging as a robust model to assess the implications of menopause in neurological disease (Marongiu 2019), we investigated for the first time the impact of VCD mediated ovarian failure in the *APOE*-TR mouse model, focusing on cognition, brain FA profiles and synaptic plasticity related signalling. Deficits in spatial learning and memory were related to the *APOE4* genotype, with an additive effect of menopause and *APOE4* carrier status on recognition memory. Furthermore, the VCD insult lowered brain DHA and synaptic plasticity signalling related genes and proteins.

Unlike previously published data by us and others reporting a strong impact of *APOE4* genotype on body weight gain in male mice (Dose, Huebbe et al. 2016, Slim, Vauzour et al. 2017), no effect of *APOE* genotype nor VCD injections was observed in our female mice. This observation is in agreement with previous reports indicating limited impact of menopause (Haas JR 2007) and *APOE4* (Jones, Watson et al. 2019) on body weight gain in female mice.

Y-maze and Barnes maze revealed *APOE4* dependant deficits in spatial learning and memory, which has previously been reported using the water-maze task (Bour, Grootendorst et al. 2008). This impairment was found to be *APOE4* female specific and was intriguingly absent in the *APOE4* male mice (Bour, Grootendorst et al. 2008). We did not detect any exacerbation of spatial deficits as a result of menopause. Conversely, recognition memory decreased in response to the menopause mimic in *APOE4* animals only. Impairment of recognition memory has been previously reported in chronic ovariectomised C57BL6 mice (Bastos, Pereira et al. 2015), and aged rhesus monkeys (Hara, Park et al. 2012), suggesting that the medial temporal lobe and surrounding cortical areas are particularly sensitive to ovarian failure. Here we report that this effect is exacerbated by *APOE4*. Together, these behavioural tests describe both genotype and genotype*VCD mediated cognitive deficits and highlight how this ‘double hit’ of factors results in a broader cognitive impairment.

Reduced DHA intake and status is associated with neuropathology, cognitive decline and higher AD risk (Tan, Harris et al. 2012, Weiser, Butt et al. 2016, Zhang, Chen et al. 2016). *APOE4* is implicated in reduced uptake of DHA into the brain (Chouinard-Watkins and Plourde 2014, Vandal, Alata et al. 2014), whilst menopause and estrogen depletion have been shown to affect DHA homeostasis (Marin and Diaz 2018). To our knowledge the impact of both menopause and *APOE4* genotype on brain DHA levels has not previously been explored. Here we show that brain DHA levels were equally diminished in both genotypes following VCD treatment. Interestingly, lower brain APOE levels were observed in both *APOE4* groups, independent of VCD insult, suggesting that DHA concentrations are uncoupled to APOE protein levels. This is in contrast to a previous report suggestive of APOE involvement in brain DHA levels (Vandal, Alata et al. 2014), however as eluded to, this effect may be more prominent in male mice. Although this is the first time brain fatty acid levels have been analysed in a VCD model, and specifically *APOE*-TR animals, previous OVX models have demonstrated similar reductions in DHA (Alessandri, Extier et al. 2011), indicating a clear role of reproductive hormones in the maintenance of brain DHA. Interestingly, despite DHA being reduced in all VCD animals, DHA:AA ratio was only reduced in the *APOE4* VCD group. Given that this ratio is likely to be an important determinant of neuro-inflammatory status, as AA and DHA are fatty acid precursors of potent pro-inflammatory eicosanoids (Zárate, El Jaber-Vazdekis et al. 2017) and specialised pro-resolving mediators respectively (Duvall and Levy 2016, Martinsen, Tejera et al. 2019), this may offer in part an explanation as why the *APOE3* animals were more resilient to the reduced brain DHA and remain unimpaired. Furthermore, in *APOE3* the VCD mediated fluctuations of higher total MUFA and lower total SFA (absent in *APOE4*) may have influenced cognition to some extent. Higher MUFA intake has previously been shown to improve brain function whilst lower MUFA brain levels are associated with AD, ageing and depression (Fernandes, Mutch et al. 2017). A number of pathways have been proposed to explain the benefits of MUFAs including the maintenance of membrane flexibility (López, Ilincheta de Boschero et al. 1995) and the actions of MUFA's as anti-inflammatory and antioxidant derivatives (Naqvi, Harty et al. 2011), which have been shown to modulate neuroinflammation in *Apoe* KO mice (Alemany, Navarro et al. 2010).

Probing key DHA transporter proteins, did not indicate any conclusive differences in expression profiles that could account for the reduced DHA observed. Of the transporters measured only *Fatp4*, showed a significant genotype related change with expression

generally lower in *APOE4* animals. The fatty acid transporters i.e. *Fatp1* and *Fatp4* have been shown to bind DHA and facilitate its transport across the endothelial cell membrane (Lo Van, Sakayori et al. 2016). This lower expression may in part explain the genotype reductions in DHA observed by other groups if this effect is exacerbated by age. In addition, although not significant, *Acsl6* was recently described from knockout studies as being a key mediator of neuroprotective DHA within the brain (Fernandez, Kim et al. 2018) and was nominally lower in both *APOE3* and *APOE4* animals in response to VCD treatment. Previous work has indicated that blood brain barrier transporter protein cell localisation and membrane shedding are influenced by *APOE* and neuropathology. This could influence the capacity to uptake DHA into the brain (Abisambra, Fiorelli et al. 2010, Bachmeier, Shackleton et al. 2014), and should be a focus of future research. On the other hand, as previously mentioned the change may be independent of transport, and instead related to metabolic disturbances which lead to greater β-oxidation of DHA (Chouinard-Watkins, Rioux-Perreault et al. 2013). This is conceivable given the role estrogens play in bioenergetic systems within the brain, such as mitochondrial function (Rettberg, Yao et al. 2014).

Given the APOE proteins known role in neurite outgrowth (Nathan, Jiang et al. 2002) and neuronal repair processes (Mahley and Huang 2012), *APOE4* specific reductions in spatial memory might be expected in light of the diminished protein levels observed in this experimentation. Further investigation may be warranted to establish if these lower ApoE4 levels are constant across sexes given that spatial memory deficits are *APOE4* female specific (Bour, Grootendorst et al. 2008). Estrogen is involved in the regulation of synaptic plasticity in key areas of the brain including the hippocampus (Arevalo, Azcoitia et al. 2015). *Esr1* (ERα) and particularly *Esr2* (ERβ) knockout mice, have identified deficits in synaptic plasticity in specific brain regions indicating the detrimental impact of ER dysregulation (Chhibber, Woody et al. 2017). The ovarian failure observed in the VCD treated *APOE*-TR mice led to an upregulation of hippocampal estrogenic receptors, although this effect is inconsistent with conflicting reports in OVX studies (Sárvári, Kalló et al. 2014, Bastos, Pereira et al. 2015, Lalert, Kruevaisayawan et al. 2018). The ERs are intrinsically linked to synaptic plasticity, acting as transcription factors or modulating key signal transduction pathways (Arevalo, Azcoitia et al. 2015). We observed that menopause dramatically altered synaptic proteins and signalling in the brain. BDNF signalling was particularly affected in *APOE4* VCD treated animals, with the effect absent in all other groups, mirroring NOR performance. This led to a reduction

in p-AKT at Ser473 most likely via the PI3K mediated signalling pathway (Autry and Monteggia 2012), known to be influenced by estrogen and ERs (Arevalo, Azcoitia et al. 2015). Reduced activation of AKT is known to inhibit neuronal cell survival and may in part explain the more extensive cognitive decline observed in the *APOE4* VCD group. VCD treatment also led to further synaptic dysfunction with all treatment groups displaying reduced hippocampal expression of *Grin2b* (NMDA R2b) in combination with reduction in CREB family transcription factors (*Creb1* and *Atf4*) all of which are integral to synaptic plasticity. Additionally, we noted that altered *Ephb2* expression, essential for NMDA receptor localisation and proper functioning (Nolt, Lin et al. 2011), was only apparent in *APOE4* VCD treatment group, which may suggest that as well as having reduced NMDA receptors seen in all VCD treated animals, the functionality is compromised as a result of *APOE4**VCD treatment. Similarly, increased *Ephb2* expression has been found to compensate for AD related NMDA receptor impairment (Archundia Herrera, Subhan et al. 2017), once again offering a possible explanation as to how *APOE3* animals may have remained unimpaired. Finally, with implications in hippocampal synaptic plasticity, adult neurogenesis and subsequent memory consolidation we assessed endocannabinoid, specifically CBD1 regulation (Scarante, Vila-Verde et al. 2017). An *APOE4* dependant reduction in *Cbd1* expression was observed, CBD1 receptor antagonism and subsequent endocannabinoid dysregulation has been associated with deficits in learning and memory (Horton, Goonawardena et al. 2019). Recently CBD1 activation has been reported to be neuroprotective conferring specific improvement in spatial memory (Patricio-Martínez, Sánchez-Zavaleta et al. 2019), therefore diminished CBD1 may contribute the spatial memory deficits we observed in *APOE4*. However, research connecting APOE and endocannabinoid signalling, particularly within the brain is limited, but warrants further investigation given these results.

Conclusion

Despite its well-established impact on late-onset AD risk, the aetiological basis of the *APOE4* genotype associated cognitive deficits and neuropathology remains elusive. Sex, and menopausal status remain overlooked factors that likely influence the progression of neurological diseases such as AD. Here we provide evidence of menopause-related risk and suggest a greater sensitivity in *APOE4* carriers, with *APOE4* animals displaying greater cognitive impairment and deficits in synaptic plasticity.

Chapter 4 : DHA-enriched fish oil ameliorates deficits in cognition and synaptic plasticity associated with the menopause in APOE4 rodents

This chapter has been prepared in the form of a research paper

Title: DHA-enriched fish oil ameliorates deficits in cognition and synaptic plasticity associated with the menopause in APOE4 rodents

Author: Matthew G Pontifex, Anneloes Martinsen, Rasha Saleh, Glenn Harden, Noemi Tejera, Michael Müller, Chris Fox, David Vauzour, Anne-Marie Minihane

Introduction

APOE4 is the strongest common genetic risk factor for the development of Alzheimer's disease (AD) (Michaelson 2014); however a fundamental understanding of its role in AD has been clouded by the pleiotropic nature of the *APOE* gene (Safieh, Korczyn et al. 2019). Evidence suggests that the AD risk associated with an *APOE4* genotype is greater in females (Farrer, Cupples et al. 1997, Snyder, Asthana et al. 2016), particularly between the ages of 55-70 years (Neu, Pa et al. 2017), which may in part account for the greater overall incidence of AD in women (Podcasy and Epperson 2016). The specific age at which this heightened risk occurs, coupled with the importance of oestrogens in cognition, and the association between menopause and cognitive decline are indicative of a potential menopausal involvement (Ryan, Scali et al. 2014, Arevalo, Azcoitia et al. 2015). From the limited evidence currently available (Pontifex et al., submitted (Porrello, Monti et al. 2006, Bojar, Stasiak et al. 2016, Karim, Koc et al. 2019)), neurological deficits associated with menopause may be exacerbated by an *APOE4* genotype.

Docosahexaenoic acid (DHA) is the primary n-3 PUFA in the brain, accounting for ~15 % of total lipids (Bradbury 2011). Epidemiological studies show that higher docosahexaenoic acid (DHA) intake and status, primarily achieved through intake of oily fish, improves cognitive performance and reduces AD risk (Zhang, Chen et al. 2016, Zhang, Zhuang et al. 2018). Although evidence from randomised control trials with DHA supplementation has been inconsistent (Jiao, Li et al. 2014, Weiser, Butt et al. 2016, Bo, Zhang et al. 2017), potentially due to study set up (e.g. length of intervention, disease progression), the benefits of DHA supplementation have been widely reported in shorter-

lived, rodent models of AD (Teng, Taylor et al. 2015, Fang, Shi et al. 2019, Takeyama, Islam et al. 2019). Such models have revealed several brain structural and functional roles for DHA (Belkouch, Hachem et al. 2016), including; neuronal signalling, survival/neurogenesis, anti-amyloidogenic and anti-inflammatory effects (Calon, Lim et al. 2005, Lim, Calon et al. 2005, Belkouch, Hachem et al. 2016). In addition, human studies utilising ^{11}C and ^{13}C labelled carbon have identified *APOE4* associated deficits in DHA metabolism and transport (Chouinard-Watkins, Rioux-Perreault et al. 2013, Yassine, Croteau et al. 2017), which may account for *APOE4* associated lower brain DHA status as we previously reported (Martinsen, Tejera et al. 2019). As a result, *APOE4* carriers may be more vulnerable to dietary n-3 PUFA deficiencies (Nock, Chouinard-Watkins et al. 2017), which may in turn impact cognition, thus providing a rationale for higher intakes (Yassine, Braskie et al. 2017). There is evidence that menopause also disrupts n-3 PUFA status (Witt, Christensen et al. 2010, Jin, Kim et al. 2016, Colangelo, Ouyang et al. 2017). Therefore, post-menopausal *APOE4* carriers may be at particular risk of DHA deficiency and likely to be responsive to intervention.

We previously reported that both *APOE4* and menopause impact cognition, synaptic plasticity and brain DHA (Chapter 3). Here we posit that supplementation with DHA-rich fish oil, may restore DHA status, and in turn ameliorate the cognitive deficits observed in the *APOE4* genotype carriers. The effects of DHA supplementation is established by administering two physiologically relevant concentrations of DHA-enriched fish oil to a VCD, menopause induced *APOE*-TR mouse model. Behavioural tests of cognition are performed and are related to brain fatty acid and synaptic plasticity profiles.

Material and methods

Study approval

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) and were conducted within the provisions of the Home Office Animals (Scientific Procedures) Act 1986.

Animal model and experimental design

Ninety six female humanised *APOE3* (B6.129P2-Apoe$^{tm2(APOE*3)Mae}$ N8) and *APOE4* (B6.129P2-Apoe$^{tm2(APOE*4)Mae}$ N8) targeted replacement mice homozygous for the human *APOE3* or *APOE4* gene (Taconic, Germantown, NY, US) were used in these experiments (Sullivan, Mezdour et al. 1997, Knouff, Hinsdale et al. 1999, Martinsen, Tejera et al. 2019). Mice were maintained in a controlled environment (21 ± 2°C; 12-h light–dark cycle; light from 07:00 hours) and fed *ad libitum* on a standard chow diet (RM3-P, Special Diet Services, Essex, UK) until the age of 4 months, ensuring normal development. Following this run-in period, mice were switched to one of the three diets all of which were on a background of a semi-purified high fat diet (45 Kcal% fat) (Research diets, New Brunswick, NJ, USA) for the remaining duration of the experiment. The three diets were as follows, 1) high fat diet (HF); 2) high fat diet with the addition of a lower fish oil dose (LFO); 3) and high fat diet with the addition of a higher fish oil dose (HFO) (See supplementary Table 1 for full dietary composition). For fish oil enriched diets, a bespoke blend of (EPAX 1050 TGN + EPAX 6000 TGN) 4:1 DHA: EPA fish oil) was added to the background diet. A 4:1 ratio (w/w) was selected based upon previous studies (Chouinard-Watkins, Vandal et al. 2017), highlighting the importance of high DHA in *APOE4* carriers, however we retained some EPA given that in humans all oily fish sources provide both EPA and DHA in variable ratios depending on species. The enriched diets provided DHA+EPA at 0.7 or 2.7 g/kg of diet. Given that mean food intake was 3.2 ± 0.02 g/day this equates to DHA+EPA human doses of 0.51 ± 0.02 g and 1.97 ± 0.02 g per day respectively, based on allometric scaling and body surface (BSA)-based calculations (Reagan-Shaw, Nihal et al. 2008, Sharma and McNeill 2009). To prevent lipid oxidation, diets were stored at −20°C until use and fresh feed was provided every 3 days.

In order to assess the impact of menopause, at 8 months of age, mice from each genotype received i.p. injections of either VCD (160mg/kg body weight) diluted in sesame oil, or sesame oil vehicle (sham) for a total of 14 injections over 3 weeks. 8 months was selected as it is roughly midlife for the animals (when human menopause occurs) and before natural ovarian failure is known to occur in C57BL/6 mice (Gosden, Laing et al. 1983). Following completion of the final behavioural test, 12-month aged animals were sedated with isoflurane (1.5%) in a mixture of nitrous oxide (70%), and oxygen (30%) and transcardially perfused with an ice-cold PBS containing protease (SIGMAFAST^{TM} Protease inhibitor, Sigma, Devon, UK) and phosphatase (1 mM sodium pyrophosphate and 50 mM sodium fluoride, Sigma, Devon, UK) inhibitors. Sera were isolated via centrifugation at 2000 x g for 10 min. Brains were rapidly removed, halved, snap frozen

and stored at -80°C until biochemical analysis. Ovaries were rapidly removed and processed for histology (see below).

Behavioural assessment

All behavioural tests were performed when mice were 12-month of age and immediately prior to sacrifice. A visual placing test was performed on each animal on the first day of testing to ensure animals were not visually impaired (Pinto and Enroth-Cugell 2000).

Spatial learning and memory was evaluated with the Barnes Maze as previously described (Patil, Sunyer et al. 2009). Briefly, the maze consisted of a brightly illuminated (800 lux lighting) circular platform (92cm diameter) with 20 evenly distributed holes located around the circumference and visual cues (4 simple shapes) placed at the periphery. The experiment was conducted over a 5-day period with each mouse tested/trained on ability to locate the escape box 4 times per day during days 1-4. On day 5, a probe test was conducted, the maze was rotated 90° and mice were placed in the centre of the maze in which they were free to navigate for one minute. Percentage time in correct quadrant was determined using the Smart 3.0 tracking software (Panlab, UK).

The novel object recognition (NOR), a measure of recognition memory was performed as described previously (Davis, Eacott et al. 2013, Leger, Quiedeville et al. 2013), with slight modifications. Briefly, on day 1 mice were habituated in grey 50x50x50cm apparatus illuminated with low lux (100 lux) lighting, mice were placed into the empty maze and allowed to move freely for 10 minutes. On day 2, mice were conditioned to a single object for a 10-minute period. On day 3, mice were placed into the same experimental area in the presence of 2 identical objects for 15 minutes, after which they were returned to their respective cages and an inter-trial interval of one hour was observed. One familiar object was replaced with a novel object. Mice were placed back within the testing area for a final 10 minutes. Videos were analysed for a 5-minute period, after which if an accumulative total of 15 seconds with both objects failed to be reached, analysis continued for the full 10 min or until 15s was achieved. Those not achieving 15s were excluded from the analysis (Denninger, Smith et al. 2018). A discrimination index was calculated as follows: $DI = (TN-TF)/(TN+TF)$, where TN is the time spent exploring the novel object and TF is the time spent exploring the familiar object.

Y maze spontaneous alternation test, a measure of spatial working memory was performed on the final day of behavioural testing as previously described (Thomas, Morris et al. 2017). Briefly, the Y-maze apparatus comprised of white Plexiglas in the following dimensions (38.5 × 8 × 13 cm, spaced 120° apart) and was illuminated with low lux (100 lux) lighting. Mice were placed in the maze and allowed to explore freely for 7 minutes whilst tracking software recorded zone transitioning and locomotor activity.

Histological and biochemical analyses

Ovaries were trimmed of fat and fixed in 10% formalin for 24h before being paraffin embedded, and processed for haematoxylin and eosin (H&E) staining as described previously (Chen, Kang et al. 2015). Follicle stimulating hormone (FSH) concentrations were determined by ELISA (Abnova KA2330, address) in sera samples as per manufacturer's instructions. Total lipid was extracted from sub-cortical brain tissues (n=5/6 per group) and erythrocyte fraction using the Folch extraction method (Folch, Lees et al. 1957) as previously reported (Martinsen, Tejera et al. 2019).

Immunoblotting

Cortices were homogenised in lysis buffer (CelLytic™ MT, Sigma, UK) containing protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, UK) and phosphatase (PhosSTOP™, Roche, UK) inhibitors. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher, UK). Protein electrophoresis was conducted under denaturing conditions as previously described (Vauzour, Corsini et al. 2018). The following antibodies were used: anti p-AKT Ser473, (1:1000; Cell Signalling, UK), anti-total-AKT, (1:1000; Cell Signalling, UK), anti BDNF (1:500; Santa Cruz Biotechnology, USA), anti p-ERK Thr202/Tyr204 (1:1000; Cell Signalling, UK), anti-total-ERK (1:1000, Cell Signalling, UK), anti GAPDH (1:2500, Cell Signalling, UK), anti and Anti-Rabbit IgG (H + L) DyLight™ 680 Conjugate (1:10,000, Cell Signalling, UK). Bands were revealed by fluorescence using an Odyssey 9120 Infrared imaging system (LI-COR Biosciences, Ltd, UK). Relative band intensities were quantified using the ImageJ software (Schneider, Rasband et al. 2012).

RNA isolation and qRT-PCR

RNA isolation, cDNA synthesis and qRT-PCR were carried out as previously described (Vauzour, Rodriguez-Ramiro et al. 2018). Briefly, total RNA was isolated from the brain samples using the Qiazol reagent (Qiagen, UK). One μg of total RNA was treated with DNase I (Invitrogen, UK) and used for cDNA synthesis using Invitrogen™ Oligo (dT) primers and M-MMLV reverse transcriptase. Quantitative real-time PCR (qRT-PCR) reactions were performed using SYBR green detection technology on the Roche light cycler 480 (Roche Life Science, UK). Results are expressed as relative quantity scaled to the average across all samples per target gene and normalised to the reference genes TATA-box binding protein (*Tbp*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which were identified as optimal housekeeping selection/combination using the Normfinder software (Day, Chambers et al. 2018). The primer sequences are given in Supplementary Table 2.

Statistical Analysis

All data are presented as mean ± SEM. Data analysis was performed in GraphPad Prism version 8 (GraphPad Software, CA, USA). After checking for normality/equal variances and performing log or box-cox transformations if necessary, comparisons among groups were performed using two-way ANOVA, followed by either post-hoc Tukey, or Sidak test for comparison across dietary groups, and genotype respectively. P-values of less than 0.05 were considered statistically significant.

Results

DHA/EPA supplementation does not protect against VCD-induced ovarian failure

Ovarian follicle loss did not occur in sham injected animals (Fig. 4.1A). Follicle loss across the VCD treated groups led to a 5-fold increase in serum FSH levels (ng/ml) ($p < 0.0001$ Fig. 4.1B), and was not influenced by genotype nor dietary interventions ($p > 0.05$). Neither genotype, VCD treatment nor dietary intervention affected body weight, although the addition of HFO nominally reduced body weight gain by 19 ± 2.3% in both *APOE* genotypes when compared to the non-supplemented groups ($p > 0.05$ Fig. 4.1C). Animals consumed on average 3.20 ± 0.02 g/day of their diet, delivering 8.64 ± 0.02 mg or 2.24 ± 0.02 mg DHA + EPA in the HFO and LFO groups respectively (equivalent to 1.97 g or 0.51 g in humans).

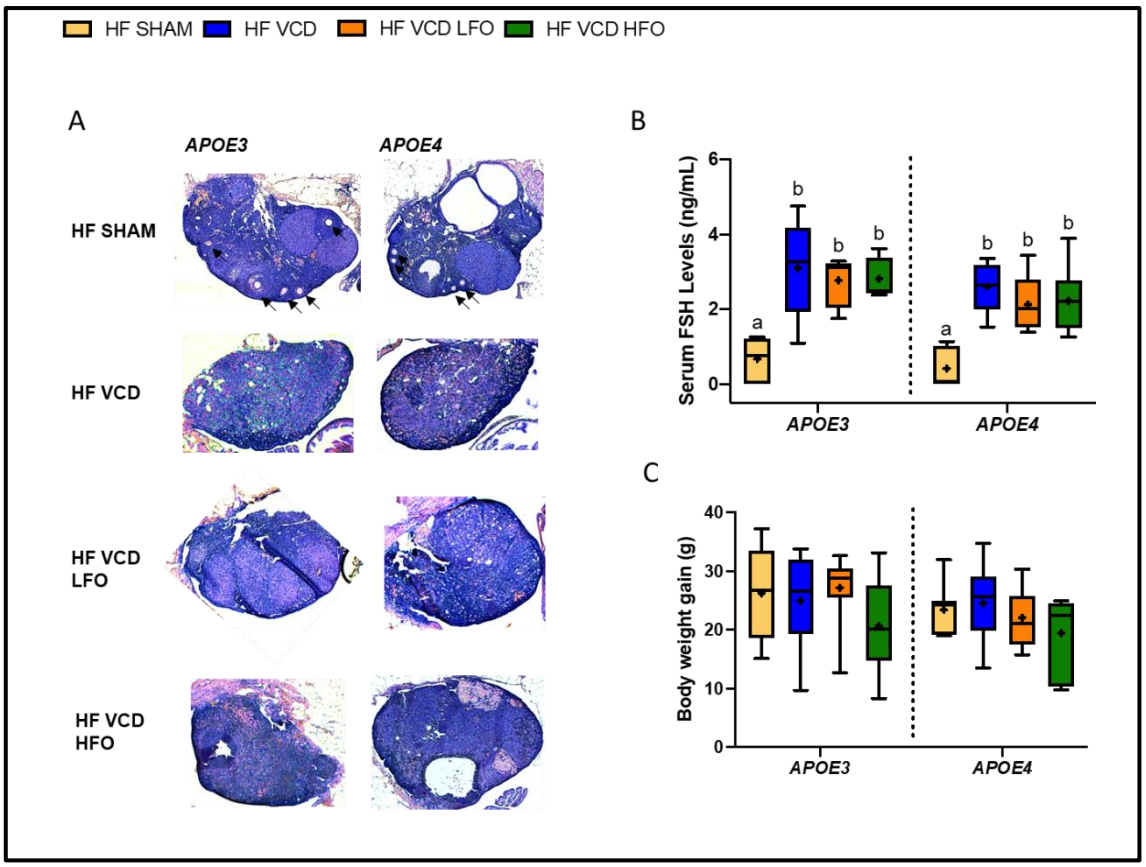

Figure 4.1**:** Impact of VCD treatment and DHA/EPA intake on ovarian failure and systemic FSH levels.

A) Representative images show follicles (arrows) in sham injected animals, which were lost following VCD treatment regardless of genotype and dietary interventions; B) Serum FSH levels were elevated in all VCD treated animal groups (n=5/6); C) Body weight was not significantly altered by VCD treatment, genotype or dietary intervention (n= ≥7). Data represent the mean ± S.E.M. Different superscripts indicate statistical differences between diet groups. FSH: follicle stimulating hormone; HF: high fat; VCD: 4-vinylcyclohexene diepoxide; LFO: low fish oil dose; HFO: high fish oil dose.

DHA rich fish oil supplementation restores *APOE4* induced impairment in recognition memory, but does not improve *APOE*-mediated spatial deficits

As previously reported, VCD treatment completely abolished recognition memory in *APOE4* animals (Chapter 3), with animals exhibiting no preference between the familiar or the novel object ($p < 0.05$; Fig 4.2A). Deficits in recognition memory were corrected by adding DHA enriched fish oil, although only HFO supplementation resulted in a significant increase when compared to sham injected animals ($p < 0.05$; Fig. 4.2A).

DHA-rich fish oil supplementation, at either dosage had no significant impact on the spatial learning and memory tasks. Barnes maze performance dropped by 2-fold in untreated *APOE4* animals compared to *APOE3* counterparts ($p < 0.05$ Fig. 4.2B). A trend towards increased performance in the *APOE4* *VCD treated HFO supplementation group was observed but did not reach significance ($p = 0.07$; Fig. 4.2B). In the Y-maze, a 1.2-fold decrease in alternation index was observed in *APOE4* animals versus *APOE3* ($p<0.05$), which was not corrected by DHA enriched fish oil intake at either dosage ($p > 0.05$; Fig. 4.2C).

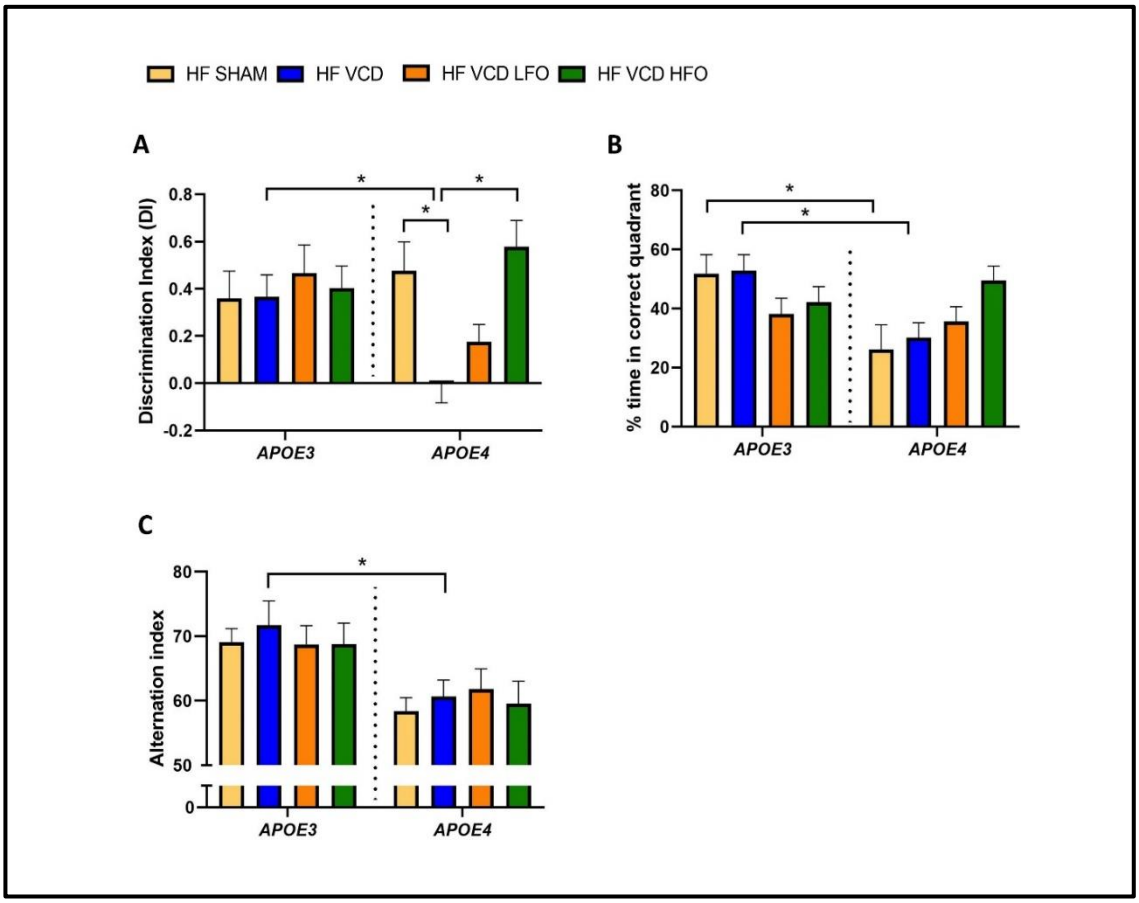

Figure 4.2: High fish oil (HFO) supplementation restores recognition memory.

A) Performance on NOR task (n=≥7) was restored to sham levels in *APOE4* VCD HFO group. Spatial memory tasks B) Barnes probe test (n=≥7), and C) Y maze spontaneous alternation task (n=≥7) were not significantly improved by fish oil supplementation ($p > 0,05$), Data represent the mean ± S.E.M. *$p< 0.05$. **$P < 0.01$. HF: high fat; VCD: 4-vinylcyclohexene diepoxide; LFO: low fish oil dose; HFO: high fish oil dose.

DHA-rich fish oil supplementation replenishes low brain DHA levels mediated by VCD treatment.

Erythrocyte DHA status was not significantly different across genotype, and only HFO supplementation resulted in a (% total fatty acids) significant ~2 and 3-fold increase in DHA levels in *APOE3* and *APOE4* animals respectively ($p < 0.05$; Fig. 4.3A and Table 4.1). The ~13% genotype independent lower (% total fatty acids) brain DHA observed in VCD injected animals was significantly restored by LFO and HFO supplementation ($p < 0.05$; Fig. 4.3B and Table 4.1). Brain DHA:AA ratio, an anti-inflammation indicator, was significantly increased by fish oil supplementation, in a dose dependant manner, with both genotypes increasing by 15 ± 0.1% and 30 ± 0.2% following LFO and HFO supplementation compared to VCD treatment alone ($p < 0.01$; Fig. 4.3C and Table 4.1). Although not significant, EPA levels increased by ~3 fold in the *APOE4* VCD HFO group when compared to HF VCD alone (Table 4.1).

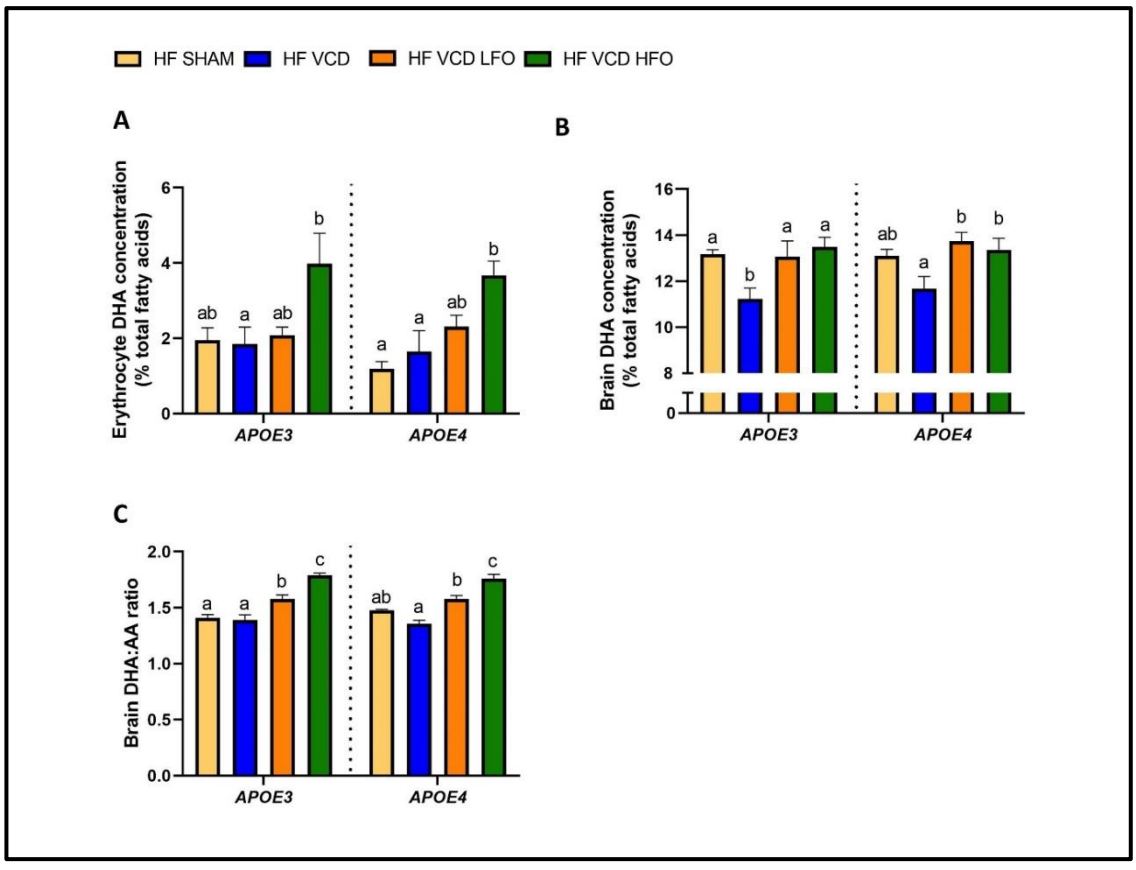

Figure 4.3**:** Fish oil supplementation restores brain DHA levels. GC-FID analysis revealed that,

A) there was no significant difference in DHA erythrocyte status across genotype, which was only significantly increased by HFO supplementation (n=5/6), B) VCD treatment resulted in lower brain DHA status which was restored by both LFO and HFO fish oil supplementation (n=5/6), C) DHA:AA status in the brain was increased in a dose dependant manner by fish oil supplementation. Different superscripts indicate statistical

differences between diet groups. HF: high fat; VCD: 4-vinylcyclohexene diepoxide; LFO: low fish oil dose; HFO: high fish oil dose.

Table 4.1 Brain and Erythrocyte fatty acid composition of experimental animals (n=5/6 per group)

	APOE3				***APOE4***				***Genotype P value***	***Intervention P value***	***Interaction P value***
Fatty acid	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**			
Erythrocyte (%)											
22:6 n-3 (DHA)	1.95 ± 0.33 ^{ab}	1.85 ± 0.44 ^{a}	2.08 ± 0.21^{ab}	3.98 ± 0.80 ^{b}	1.19 ± 0.19 ^{a}	1.65 ± 0.55 ^{a}	2.31 ± 0.30 ^{ab}	3.68 ± 0.38 ^{b}	0.333	**0.0001**	0.511
Brain (%)											
Total n-3 PUFA	13.30 ± 0.20 ^{ab}	11.60 ± 0.70 ^{a}	13.10 ± 0.70 ^{ab}	13.60 ± 0.40 ^{b}	13.20 ± 0.30 ^{ab}	11.80 ± 0.50 ^{a}	14.00 ± 0.40 ^{b}	13.50 ± 0.50 ^{ab}	0.550	**0.001**	0.696
20:5 n-3 (EPA)	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.0.01	0.03 ± 0.14	0.01 ± 0.01	0.09 ± 0.03	0.640	0.067	0.529
22:6 n-3 (DHA)	13.20 ± 0.20 ^{a}	11.20 ± 0.50 ^{b}	13.10 ± 0.70 ^{a}	13.50 ± 0.40 ^{a}	13.10 ± 0.30 ^{ab}	11.70± 0.50 ^{a}	13.70 ± 0.40 ^{b}	13.40 ± 0.50 ^{b}	0.479	**0.0002**	0.765
Total n-6 PUFA	14.20 ± 0.20 ^{a}	12.60 ± 0.40 ^{ab}	12.00 ± 0.50 ^{b}	11.10 ± 0.30 ^{b}	14.80 ± 0.90 ^{a}	13.20 ± 0.60 ^{ab}	12.70 ± 0.20 ^{b}	11.50 ± 0.40 ^{b}	0.093	**0.0001**	0.992
20:4 n-6 (AA)	9.37 ± 0.21 ^{a}	8.10 ± 0.39 ^{ab}	8.28 ± 0.39 ^{ab}	7.55 ± 0.28 ^{b}	8.95 ± 0.25	8.66 ±0.50	8.70 ± 0.22	7.88 ± 0.26	0.354	**0.002**	0.508
DHA:AA	1.40 ± 0.03 ^{a}	1.39 ± 0.05 ^{a}	1.58 ± 0.04 ^{b}	1.79 ± 0.02 ^{c}	1.48 ± 0.01 ^{ad}	1.36 ± 0.03 ^{a}	1.58 ± 0.03 ^{bd}	1.76 ± 0.03 ^{c}	0.998	**0.0001**	0.400
Total SFAs	38.70 ± 0.40	35.50 ± 0.90	36.70 ± 1.40	37.00± 1.40	36.60 ± 0.60	36.90 ± 1.20	37.70 ± 0.90	36.60± 0.80	0.961	0.409	0.216

Table 4.1 continued

Total MUFAs	25.40 ± 0.40 ^{a}	30.50 ± 0.80 ^{b}	26.50 ± 1.20 ^{ac}	30.00 ± 0.80 ^{bc}	27.30 ± 0.90 ^{ab}	27.90 ± 1.40 ^{ab}	26.50 ± 0.80 ^{a}	30.20 ± 0.90 ^{b}	0.615	**0.001**	0.054

PUFA; Polyunsaturated fatty acid, 20:5 n-3 EPA; Eicosapentaenoic acid, 22:6 n-3 DHA; Docosahexaenoic acid, 20:4 n-6 AA; Arachidonic acid, DHA:AA; Docosahexaenoic acid to Arachidonic acid ratio, SFA; Saturated fatty acid, 16:0; Palmitic acid, 18:0; Stearic acid, 20:0; Eicosanoic acid, 22:0; Docosanoic acid, MUFA; Monounsaturated fatty acid, 18:1 n-9; Oleic acid, 20:1 n-9; 11-Eicosenoic acid, 24:1 n-9; Nervonic acid. Data is % of total fatty acids and mean value ± SEM. 2-way ANOVA. Different superscripts denote significant difference between means within genotypes whilst § indicates genotype differences (Tukey or Sidak post hoc). The full table can be found in supplementary data (Table S4 and S5).

Fish oil supplementation enhances synaptic plasticity related genes and proteins

The 31 ± 0.16% reduction in brain *Bdnf* gene expression ($p < 0.05$; Fig. 4.4Ai) observed in *APOE4* VCD injected animals was restored through both HFO and LFO supplementation ($p < 0.01$; Fig 4.4Ai). At the protein level *APOE4* VCD treated animals also presented with decreased BDNF, which was only restored by the HFO intervention, which increased levels ~4-fold compared to non-supplemented VCD treated animals ($p<0.01$; Fig. 4.4Aii). A similar trend was observed for the AKT signalling pathway, a known regulator of BDNF. At the gene level the 16 ± 0.23% reduction in *Akt1* gene expression observed in the VCD treatment group was nominally but not significantly increased with both LFO and HFO ($p > 0.05$ Fig. 4.4Bi). As with BDNF, at the protein level phospho-AKT Ser473 was only significantly increased in the *APOE4* HFO supplementation group ($p < 0.01$; Fig 4.4Bii). ERK, another upstream regulator of BDNF also responded similarly. At the gene level *Mapk1* which encodes ERK2, was upregulated in only the *APOE4* HFO group ($p < 0.05$; Fig 4.4Ci). No significant differences in ERK Thr202/Tyr204 levels were detected across genotype, VCD or dietary intervention ($p > 0.05$; Fig 4.4Cii), despite a nominal increase in phospho-ERK 2 in the *APOE4* VCD HFO group. Both mRNA expression and protein levels of these targets remained constant within the *APOE3* intervention groups.

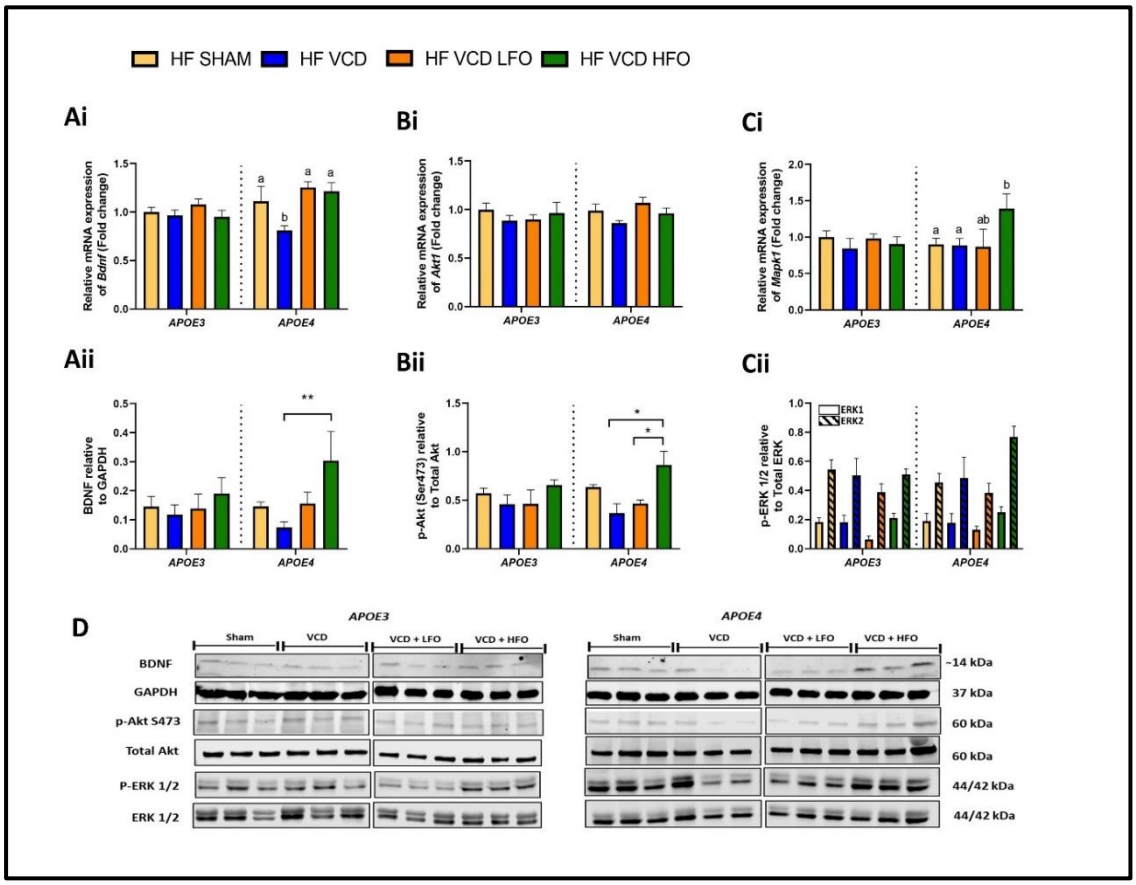

Figure 4.4: Impact of fish oil supplementation on synaptic plasticity related genes and proteins in VCD treated animals;

A-Ci) BDNF, AKT and ERK signalling (also observed at the protein level). Ai) Lower *bdnf* expression in *APOE4* VCD treated animals was restored by fish oil supplementation, Bi) Similarly in *APOE4* mice there was a trend towards increased AKT expression following both LFO and HFO, Ci) Whilst *Mapk1* expression increased significantly in only *APOE4* HFO supplemented animals. Aii) Brain BDNF protein levels were significantly increased in the *APOE4* HFO group, Bii) phospho-AKT Ser473 protein was similarly increased in the *APOE4* brain by HFO supplementation, Cii) Although not significant, a nominal increase in p-ERK protein was observed in the *APOE4* HFO group, D) Representative western blots for both *APOE3* and *APOE4*, Sham and VCD treated animals (n=4/6), E-K).

Expression of *Igf-1* which can increase neurogenesis through PI3K/AKT mediated pathways was similarly increased in only the *APOE4* HFO group ($p < 0.05$; Fig 4.5A). Although not significantly altered in *APOE3* animals (Fig. 4.5B), *Creb1* expression in *APOE4* animals was highly upregulated by 2.7 ($p < 0.05$) and 2.6 ($p = 0.068$) fold by LFO and HFO respectively. *Arc* a downstream effector of *Creb1* showed a similar expression profile with fish oil supplementation increasing gene expression amongst *APOE4* VCD treated animals, however this did not achieve significance ($p > 0.05$; Fig 4.5C). Interestingly, *Atf4*, a key regulator of learning and memory (Corona, Pasini et al. 2018), known to be upregulated via BDNF (Liu, Amar et al. 2018), had significantly reduced expression in VCD treated *APOE3* and *APOE4* animals ($p < 0.05$; Fig 4.5D) and did not improve in *APOE3* animals with fish oil supplementation ($p > 0.05$; Fig 4.5D). Conversely, HFO supplementation significantly restored *Atf4* expression to that of sham in *APOE4* mice ($p < 0.05$; 4.5D). This has been suggested to occur in a tropomyosin receptor kinase (TRKB) dependant manner (Liu, Amar et al. 2018). Interestingly, expression of *Ntrk2* which encodes TRKB was significantly increased in only the *APOE4* VCD treated HFO group when compared to sham counterparts ($p < 0.05$; Fig 4.5E). Although only significant in *APOE3* LFO treated animals there was a trend in which fish oil supplementation mitigated the educed *Grin2b* expression as a result of VCD treatment ($p < 0.05$; Fig. 4.5F). Lower *Cbd1* expression was apparent in all intervention groups apart from the LFO group and was not affected by VCD treatment ($p < 0.05$; Fig 4.5G).

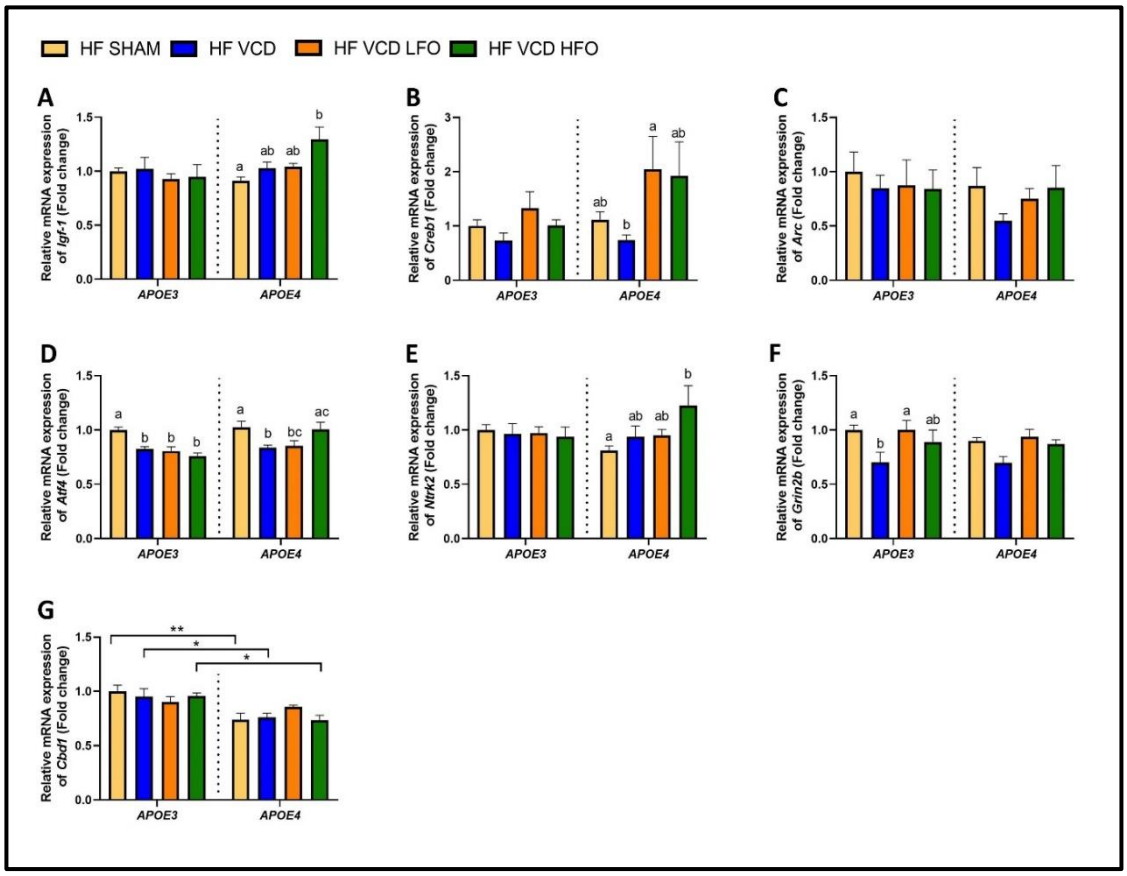

Figure 4.5 Hippocampal expression profiles relating to synaptic plasticity (n=5/6);

A) *Igf-1* expression increased in only the *APOE4* HFO group, B) *Creb1* expression was highly upregulated in response to fish oil supplementation in *APOE4* animals only, C) In *APOE4* animals *Arc* a downstream target of *Creb1* followed a similar expression profile, with VCD reduced *Arc* increasing in response to fish oil supplementation, D) *Atf4* expression reduced as a result of VCD treatment in both *APOE3* and *APOE4* animals, however only HFO supplementation in *APOE4* animals restored this deficit, E) *Ntrk2* was similarly only upregulated through HFO supplementation in *APOE4* animals. F) *Grin2b* was reduced in response to VCD treatment across both genotypes and improved with fish oil supplementation, although significance was limited to the *APOE3* LFO group, G) a genotype related reduction in *Cbd1* expression was apparent across all groups excluding LFO. Data represent the mean ± S.E.M. *p< 0.05. **P < 0.01 ***P <0.001. Different superscripts indicate statistical differences between diet groups. HF: high fat; VCD: 4-vinylcyclohexene diepoxide; LFO: low fish oil dose; HFO: high fish oil dose.

Discussion

APOE4 is pleiotropic, impacting multiple brain physiological processes and pathological cascades associated with AD (Zhong and Weisgraber 2009). The ability of DHA to modify many of these disease pathways, with its anti-inflammatory and neuroprotective properties, makes it an attractive option for use as a dietary strategy to improve cognition and potentially reduce AD risk (Frautschy and Cole 2010). This study follows on from

our previous report, in which both *APOE* genotype and VCD mediated deficits in cognition and synaptic plasticity associated pathways were established (Chapter 3). Here we show that HFO supplementation results in a reversal of *APOE4**VCD deficits in recognition memory. Conversely, supplementation had only a modest impact on genotype related spatial memory impairment. Gene expression and protein analysis supported these findings with HFO supplementation, restoring synaptic plasticity related pathways, leading to increased BDNF protein abundance and AKT phosphorylation, which were both diminished by *APOE4**VCD. Deficits in brain DHA resulting from VCD treatment and observed in both genotypes, were restored by both LFO and HFO, with the greatest DHA:AA ratio achieved by HFO.

As previously reported, body weight did not differ according to *APOE* genotype and was unaffected by VCD treatment in our female mice (Haas JR 2007, Jones, Watson et al. 2019). Although not reaching significance, body weight in both genotypes, was reduced (~20%) in response to HFO supplementation. This has been described in other rodent studies (Buckley and Howe 2010), and interestingly in humans, in which female *APOE4* carriers with the highest n-3 index, were found to have the lowest BMI and body fat, a phenomenon absent in males (Howe, Buckley et al. 2014). This may indicate a sex specific role for EPA and DHA in the regulation of metabolism/adiposity in females (Howe, Buckley et al. 2014) and more generally shows how n-3 PUFAs may have sex specific effects.

We previously identified reduced performance in Barnes and Y maze in *APOE4* animals, indicating a genotype spatial memory impairment, that is independent of VCD treatment, and which may be sex specific (Bour, Grootendorst et al. 2008). The absence of a VCD influence suggests a predisposition in middle aged *APOE4* females, which is uncoupled from menopause (Bour, Grootendorst et al. 2008), although sex hormones may still be involved. Spatial memory performance was not significantly altered by FO supplementation in *APOE4*-TR mice. Indeed, we found that Barnes probe test performance did not significantly improve, despite nominally increasing in response to HFO doses. Furthermore, during the learning phase of the Barnes we again observed a nominal but non-significant improvement in the HFO group (Figure S2). In contrast to spatial memory, HFO supplementation significantly improved recognition memory in *APOE4* VCD treated animals, indicating that FO supplementation was more effective in ameliorating the impact of *APOE4**VCD than the *APOE4* deficits. There are limited

reports evaluating the cognitive impact of n-3 PUFA supplementation in menopausal models, and to the best of our knowledge this is the first report in an *APOE*-TR menopause model. In agreement with a previous report, higher doses of DHA appear an effective strategy to curb the deleterious impact of *APOE4* on cognition. However supplementation may be unnecessary in the unimpaired *APOE3* animals where fish oils had no impact (Chouinard-Watkins, Vandal et al. 2017). This nonetheless may change in older *APOE3* animals, as they become more susceptible to cognitive decline associated with ageing. Utilising specific 'brain targeting' DHA, (i.e. in phospholipid form), which is metabolised to LPC-DHA and reportedly more efficacious at crossing the blood brain barrier (Patrick 2018), may present a way to negate the current need for high doses. Interestingly, Sugasini and colleagues reported an improvement in *APOE4* mediated spatial memory deficits when utilising LPC-DHA, supporting this notion (Sugasini, Thomas et al. 2017).

Both LFO and HFO restored the VCD mediated reduction in brain DHA observed in both genotypes. Allessandri and colleagues (Alessandri, Extier et al. 2011) observed a similar reduction in brain DHA resulting from OVX, which was restored through 17β-estradiol (E2) treatment. Interestingly, they report that 17β-estradiol treatment increased both hepatic and brain LC-PUFA synthesizing enzymes such as Δ9-, Δ6- and Δ5-desaturase, restoring or maintaining brain DHA. Supplementation with DHA-rich n-3 PUFA in this study may similarly restore (Chouinard-Watkins, Pincon et al. 2016), or compensate for the dysregulation of these synthesising enzymes, subsequently improving the brain PUFA profile. In addition, the dysregulated fatty acid profile caused by menopause reportedly leads to alterations in neuronal membrane lipid raft structure, which can in turn disrupt the signalosome (Marin and Diaz 2018). Supplementation with DHA could mitigate such detrimental effects. Furthermore, brain DHA:AA ratio improved with supplementation in line with other reports (Hashimoto, Hossain et al. 2002, Hashimoto, Tanabe et al. 2005). Given the pro-inflammatory, free radical characteristics of AA precursors (Hashimoto, Tanabe et al. 2005, Zárate, El Jaber-Vazdekis et al. 2017), and the ability of DHA to modulate inflammation via specialised pro-resolving mediators (Duvall and Levy 2016, Martinsen, Tejera et al. 2019), DHA:AA is a potential indicator of neuro-inflammatory status. The increase in DHA:AA was dose dependant, with HFO achieving the highest ratio, which may in part explain the greater cognitive benefits associated with HFO.

As with cognitive performance and brain DHA status, FO supplementation attenuated the impact of VCD treatment on synaptic plasticity related signalling. At the protein level this was again most apparent in the *APOE4* HFO group, in which the abundance of

BDNF, and phosphorylation of AKT were significantly elevated. Neurological benefits of n-3 PUFA supplementation on both AKT (Zhang, Liu et al. 2015, Shi, Fu et al. 2019), and the neurotrophic factor, BDNF (Dong, Xu et al. 2018, Sun, Simonyi et al. 2018), have previously been reported. At the gene level *Bdnf* was significantly upregulated by fish oil supplementation, whilst *Akt1* was not, suggesting that supplementation led to post-translational modification of AKT, rather than direct upregulation. The ability of HFO to increase BDNF protein and activate AKT, both established intermediates in neuronal cell survival and synaptic plasticity (Belkouch, Hachem et al. 2016), provides a mechanistic basis for the improvement in cognition observed. Furthermore, *Akt1* is a downstream target of *Ntrk2 (Wu, Chen et al. 2019),*and *Igf-1(Feng, Lu et al. 2020),* both of which were upregulated through HFO supplementation, therefore providing two potential routes in which the effects of DHA may be propagated. However further studies inhibiting components of these pathway are required for confirmation. Interestingly, DHA has previously been reported to promote cell survival by maintaining basal AKT activity under adverse conditions (Akbar, Calderon et al. 2005), offering an explanation as to why these changes were restricted to the 'challenged' *APOE4* VCD animals. Fish oil supplementation also increased the expression of the transcription factors *Creb1* and *Atf4* in *APOE4* animals, both of which are implicated in BDNF signalling (Liu, Amar et al. 2018). BDNF's activation of *Atf4* is believed to both; promote protection from oxidative/genotoxic stresses and to improve learning and memory (Corona, Pasini et al. 2018, Liu, Amar et al. 2018), whilst CREB itself is a known regulator of BDNF gene expression. CREB can also be activated through ERK (Boneva and Yamashima 2012, Sona, Kumar et al. 2018). HFO supplementation resulted in a significant increase in *Mapk1*, and a nominal, but non-significant increase in ERK phosphorylation, highlighting an additional route for the observed effects. Interestingly, the genotype reduction of *cbd1* was not significantly improved by FO supplementation. Given that *Cbd1* and endocannabinoid dysregulation have been previously reported to influence spatial memory (Patricio-Martínez, Sánchez-Zavaleta et al. 2019), the absence of recovery in response to supplementation may in part explain the subsequent lack of improvement in spatial memory performance. *Cbd1* has similarly been reported to influence AKT-BDNF signalling (Blázquez, Chiarlone et al. 2015); therefore the loss of *Cbd1* may be responsible for the reduced *Akt1* activation, subsequently impeding synaptic plasticity and neuronal cell survival.

Conclusion

Female *APOE4* carriers are at greater risk of AD, which may in part be explained by a menopause**APOE4* interaction. The results of this study suggest that FO supplementation, but particularly at higher doses, may be a viable intervention to mitigate the deleterious *APOE4**menopause interaction. Further investigation is warranted to establish if this is the case in humans.

Chapter 5 : General discussion: The Interactive impact of *APOE* genotype and oestrogen status on cognition, lipid status and synaptic signalling

Summary of results and general discussion

Main findings

AD contributes towards ~70% of dementia cases, and is a growing concern both in the UK, and internationally. Excluding familial early onset AD, the *APOE* gene is the strongest genetic risk factor for AD and is associated with the more common, genetically complex form of the disease referred to as late onset AD (LOAD) (Giri, Zhang et al. 2016). Despite this, and after a quarter century of *APOE* research, a comprehensive understanding of aetiological basis for *APOE* in AD is lacking (Belloy, Napolioni et al. 2019). This is predominantly a result of the pleiotropic nature of *APOE*, with the modulatory influence of environmental factors also contributing. There is accumulating evidence to suggest that female *APOE4* carriers are at heightened risk of AD development. According to Neu et al, female risk for MCI and AD is heightened between the ages of 55-70 and 65-75 respectively (Neu, Pa et al. 2017). This has led us and others to hypothesise a potential menopausal and subsequent sex hormone involvement (Scheyer, Rahman et al. 2018). Surprisingly, despite the reiteration of a female *APOE4* predisposition (Farrer, Cupples et al. 1997, Altmann, Tian et al. 2014, Neu, Pa et al. 2017), and a known importance of oestrogens in learning, memory and AD risk (Scheyer, Rahman et al. 2018, Lu, Sareddy et al. 2019), the impact of sex hormones on *APOE* genotype is relatively unknown and thus forms the basis for this thesis.

In chapter 2, we investigated the impact of *APOE* carrier-status, oily fish intake and HRT on PUFA status and cognition. To do this we utilised the baseline data of 136 female participants who took part in the CANN study, a multi-centre, 12-month double-blinded, placebo-controlled parallel intervention trial.

The first part of the analysis showed that oily fish intake, BMI, *APOE*-carrier status and *APOE*-carrier status*HRT impact PUFA status. As expected, incremental increases in oily fish intake from never, to 1/3 times a month, and 1 or more times a week led to a 15% and 16% greater n-3 index (EPA and DHA) respectively. Interestingly, although this increase was observed across erythrocytes, plasma and PC fractions, significance was

limited to the erythrocytes, presumably a consequence of the longer half-life of DHA in the erythrocyte fraction and therefore more reflective of longer term (months) rather than recent (days) intake. BMI had the opposite effect, with a lower DHA and n-3 index with increasing BMI. Again, there was a trend for this across all fractions however, significance was restricted to the erythrocyte fraction. *APOE4* increased n-3 index in our female participants. Most notably EPA was significantly increased in the majority of fractions analysed. This rise in n-3 index associated with *APOE4* may be explained by reduced tissue uptake.

HRT alone had no effect on the FA profile, however analysis exploring the *APOE**HRT interaction revealed that HRT reduced the elevated n-3 PUFA levels associated with *APOE4*, providing evidence of an *APOE4*, n-3 PUFA, sex hormone interaction. In the second part of the analysis we explored if these factors influenced cognition. *APOE4* carrier-status, HRT and *APOE4* carrier status*HRT, influenced cognitive performance, whilst n-3 index and DHA status did not. *APOE4* led to reduced cognitive performance in a specific subset of tasks namely verbal fluency, speed of memory and executive function. Interestingly, HRT generally led to improvements in cognition supporting the notion that sex hormone dysregulation is associated with cognitive decline. In addition, we found that HRT had specific *APOE* effects dramatically improving time and speed of memory as well as recognition memory, which has been linked to oestrogens previously. Overall chapter 2 revealed that 1) oily fish intake, BMI, *APOE4*-carrier status and *APOE4*-carrier status*HRT influence PUFA status 2) *APOE4* carrier-status, HRT and *APOE4* carrier status*HRT, but not n-3 index influence cognitive performance 3) HRT may impact *APOE4* carriers to a greater extent.

In chapter 3 we sought to investigate further, with a focus on underlying mechanisms, the effect of sex hormone status and *APOE4* on cognition in females using a biologically relevant animal model. We established a middle aged female menopausal *APOE*-TR mouse model for the first time. To achieve this we administered repeated i.p. injections of VCD, gradually depleting ovarian follicle reserves, and thus more closely mimicking human menopause, compared to other established techniques i.e. OVX.

We first wanted to determine if we were successful in establishing the *APOE*-TR menopause model. Histology clearly showed a distinct lack of ovarian follicles in VCD treated animals, which was accompanied by a significant increase in the hormone FSH, as one would expect with VCD treatment. In addition, hippocampal expression of the oestrogen receptors, particularly *Esr1*, increased substantially in response to VCD

treatment, highlighting that VCD treatment was having a neurological impact. Body weight remained constant regardless of genotype or VCD treatment. We performed behavioural tests to assess how both *APOE* genotype and VCD treatment impact the cognitive performance of the animals and discovered both genotype and VCD mediated deficits in cognition. Genotype resulted in spatial memory deficits, observed in both the Barnes and Y-maze tasks, whilst a striking reduction in recognition memory was apparent in only *APOE4* VCD treated animals, indicating an *APOE4**VCD interaction. Interestingly, as mentioned earlier, the CANN analysis revealed that HRT influenced recognition memory in *APOE4* carriers only, supporting this *APOE4**sex hormone influence on recognition memory. The main finding from the animal lipid analysis was that VCD injections led to a drop in brain DHA levels in both *APOE3* and *APOE4* animals, providing further evidence of a relationship between sex hormones and brain DHA status. A significant drop in the DHA:AA ratio, observed only in the *APOE4* VCD group, and similarly a rise in MUFA, only in the *APOE3* VCD group, may contribute to the greater cognitive deficits associated with *APOE4*. However, this requires further investigation.

Surprisingly, despite these changes in brain FA profiles we found little evidence to suggest that DHA transporter proteins were compromised at the gene level in response to VCD. This suggests that the mechanisms explaining these deficits either occur post-translationally or are more associated with altered metabolism (transport independent). With both *APOE4* and DHA implicated in the modulation of synaptic plasticity and as it represents one of the first processes to be impaired in AD we probed key synaptic plasticity related genes and proteins. Our findings in this section supported our behavioural results to some extent. Of notable mention was the significant drop in brain BDNF and phosphorylated AKT proteins which was limited to the *APOE4* VCD group. We also found that VCD alone led to decreased expression of a number of synaptic plasticity related genes, including *Atf4* and *Grin2b* suggesting that this effect was not limited to *APOE4*. This raises the question as to how the *APOE3* animals appear to be protected from these effects in synaptic plasticity metabolism. To summarise in chapter 3 we, 1) established VCD is an effective way to induce ovarian failure in the *APOE*-TR mouse model; 2) identified that a "Double hit" (menopause and *APOE4* genotype) resulted in broader cognitive impairment relative to the impact of *APOE4* or VCD alone; 3) *APOE4* and *APOE4**VCD impair both spatial and recognition memory respectively; 4) Brain DHA levels are reduced in response to VCD treatment; and 5) *APOE4* exacerbates VCD disturbances in synaptic plasticity related pathways.

After establishing an effective model and identifying mechanisms which may account for the associated deficits, we proceeded to investigate if an intervention could ameliorate the impact of *APOE4* and VCD. With n-3 PUFA dysregulation a recurring theme in previous experiments and already associated with *APOE4* and sex hormone status we hypothesised that fish oil supplementation may be a suitable strategy.

In chapter 3, utilising the same model as established and described in chapter 2, we added two further groups to the experiment, HF VCD LFO and HF VCD HFO. Both doses which provided ~2g and 0.5g per day were chosen to be at the upper and lower limits of physiologically relevant human dosage. 'Mega-doses' have been assessed previously however their translational potential is questionable. Ovarian follicle loss was also observed in all VCD treated animals, with dietary intervention having no impact on FSH levels, indicating that any effects were independent of FSH modulation. Behavioural analysis, however, revealed that HFO supplementation significantly recovered deficits in recognition memory associated with *APOE4**VCD. Although a nominal increase in spatial memory performance was observed with HFO supplementation, it did not reach significance. Fish oil supplementation regardless of dose restored the diminished brain DHA levels observed in both genotypes in response to VCD treatment; however HFO improved DHA:AA to a greater extent. Again, synaptic plasticity related protein and gene expression was largely in agreement with the behavioural analysis. Of particular interest was the significant increase of phosphorylated AKT Ser473 and BDNF protein levels (key for neuronal cell survival and neurogenesis) observed in *APOE4* animals supplemented with HFO. Supplementation also significantly increased *Creb1* and *Atf4* expression and nominally increased *Arc* and *Grin2b* in *APOE4* animals. In *APOE4* animals HFO supplementation increased *Mapk1*, *Igf-1* and *Ntrk2* expression revealing potential routes in which DHA exerts its effects. Overall in chapter 4 we show, 1) High dose n-3 PUFA supplementation ameliorates *APOE4**menopause mediated deficits in recognition memory; 2) n-3 PUFA supplementation is less effective at improving genotype related spatial memory impairment; 3) Brain DHA levels diminished by VCD treatment are restored by supplementation; 4) DHA:AA increased to a greater extent in animals receiving higher n-3 PUFA dosage; and 5) High n-3 PUFA supplementation improved *APOE4**VCD disturbances in synaptic plasticity related pathways.

Overall summary of chapter 2 to 4

The sex disparity in *APOE4* mediated AD risk has been reiterated numerous times over the past two decades (Farrer, Cupples et al. 1997, Altmann, Tian et al. 2014, Neu, Pa et al. 2017). Throughout the experiments described in this thesis, we sought to determine if there was a sex hormone/menopausal component to this risk that was linked to altered n-3 PUFA status (transport/metabolism) and therefore potentially mitigated through an n-3 PUFA intervention. CANN analysis revealed significant differences in n-3 index and cognition in those receiving HRT, which was more extensive in carriers of *APOE4,* supporting the notion that sex hormones have a disproportionate impact on *APOE4,* as previously reported (Porrello, Monti et al. 2006, Bojar, Stasiak et al. 2016, Riedel, Thompson et al. 2016, Mosconi, Rahman et al. 2018). In contrast to the CANN analysis, the *in vivo* animal study in *APOE*-TR mice did not detect any direct *APOE4* or *APOE4*VCD* specific alterations in DHA levels within the erythrocytes or brain. However, such genotype effects may only become apparent as late as 18 months of age (Martinsen, Tejera et al. 2019). VCD treatment and subsequent ovarian failure led to significant reductions in brain DHA status across both genotypes, as has been reported in OVX mice (Alessandri, Extier et al. 2011), emphasising the importance of sex hormones in maintaining brain DHA homeostasis. *APOE4**VCD on the other hand, altered brain DHA:AA ratio, similarly important as a lower brain ratio impacts neuroinflammation, oxidative stress and synaptic plasticity (Connor, Tenorio et al. 2012, Komprda 2012).

Recognition memory, assessed via the picture recognition task in the CANN study was specifically improved in *APOE4* carriers receiving HRT. This corroborates with our *APOE*-TR mouse model results, in which striking deficits in the NOR task were observed in the VCD treated *APOE4* animals only. Ovarian failure has previously been associated with recognition memory deficits (Bastos, Pereira et al. 2015), indicating a particular sensitivity of the medial temporal lobe and surrounding cortical areas to sex hormone dysregulation. However, our results indicate how this is somehow exacerbated by *APOE4*, a novel finding which warrants further investigation. Conversely, spatial memory performance was unaffected by VCD, with deficits wholly attributable to *APOE4* genotype alone. An interesting finding in itself, considering that Bour et al (Bour, Grootendorst et al. 2008), reported these spatial memory deficits to be *APOE4* female specific. Therefore, indicating a non-menopausal *APOE4* female deficit, uncoupled from menopause. Based upon our results we posit that this could be linked to the *Cbd1* receptor downregulation, also independent VCD treatment and observed in all *APOE4*-TR mice, particularly given the importance of the endocannabinoid system and cannabinoid receptors in synaptic plasticity and neuroprotection (Blázquez, Chiarlone et al. 2015,

Scarante, Vila-Verde et al. 2017, Horton, Goonawardena et al. 2019, Patricio-Martínez, Sánchez-Zavaleta et al. 2019). Menopause also had implications for synaptic plasticity related signalling in the brain. In accordance with the cognitive data, *APOE4**VCD had the greatest deleterious impact on synaptic plasticity related signalling, with BDNF and AKT signalling particularly affected. BDNF and AKT are key mediators in neuronal cell survival, neurogenesis and synaptic plasticity (Autry and Monteggia 2012), and are known to be modulated by estrogens/estrogenic receptors (Chhibber, Woody et al. 2017, Chhibber and Zhao 2017). Interestingly these deficits could possibly be attributable to the aforementioned PUFA disturbances. As reported by Marin et al (Marin and Diaz 2018), alterations of highly specific lipid raft compositions, known to occur through sex hormone dysregulation, can disrupt the signalosome, therefore reducing neuronal protection and synaptic plasticity. In addition, we also disprove the theory that these menopausal effects relate to reduced APOE protein (Riedel, Thompson et al. 2016), which remained constant regardless of VCD insult. On the other hand, levels were significantly reduced by *APOE4* genotype and may therefore likely represent a mechanistic basis for the *APOE4* effect.

In light of the alterations in n-3 index, caused by both *APOE* genotype and menopause we speculated that DHA enriched n-3 PUFA fish oil supplementation may offer a viable intervention strategy to mitigate the impact of these factors. Overall, our results suggested clear benefits of supplementation on the *APOE4**menopause interaction. Recognition memory which was modulated by *APOE4**sex hormones, in both CANN participants and in the *APOE*-TR model, was significantly improved in the *APOE4* HFO group. The improvement appeared to be consistent with the synaptic plasticity gene expression and protein analysis. The *APOE4* HFO group that had significantly improved NOR task performance, also had increased BDNF protein abundance, and AKT activation. Increases in *Igf-1*, *Mapk1* and *Ntrk2* expression, were also observed in this group providing 3 routes in which the DHA enriched supplement may be mediated. Again, these diverse signalling changes may be consistent with restoring the lipid raft profile, which in turn may strengthen the signalosome (Marin and Diaz 2018), and should be explored further. However, this may not be the full story as both LFO and HFO led to restoration of the diminished brain DHA levels, whilst synaptic plasticity and subsequent cognitive benefit were largely limited to HFO. This may be indicative of either further underlying processes which were not established in this experimentation, or perhaps reiterates the importance of the DHA:AA ratio which was increased significantly more by HFO.

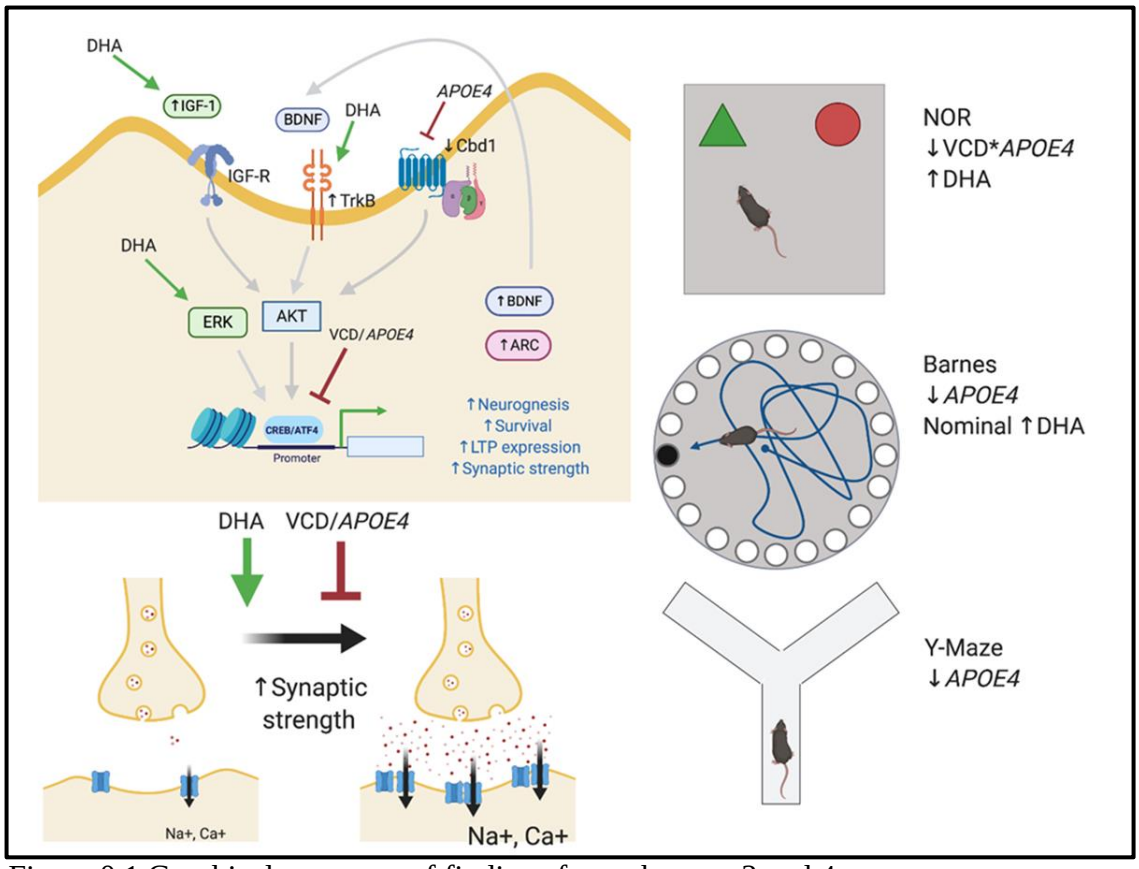

Figure 0.1 Graphical summary of findings from chapters 3 and 4

Conversely, the spatial memory deficits that were associated with *APOE4* genotype alone, did not significantly improve with FO treatment. Although, Barnes maze performance did show signs of improvement in response to HFO in both the learning and test phases, consistent with that reported by Chouinard Watkins et al (Chouinard-Watkins, Vandal et al. 2017). Similarly, analysis of the CANN data, did not detect any cognitive improvement relating to n-3 index. Together, this may therefore indicate the necessity of the higher, and potentially DHA enriched doses to overcome *APOE4* mediated deficits. Specific brain targeting forms of DHA may offer an alternative approach to these higher doses, as shown by Sugasini and colleagues (Sugasini, Thomas et al. 2017, Sugasini, Yalagala et al. 2019), who reported an improvement in the *APOE4* mediated spatial memory deficits when utilising DHA in LPC form.

Altogether, our findings suggest that *APOE4* exacerbates the detrimental impacts of menopause, whilst supplementation with high but physiologically relevant DHA enriched n-3 PUFAs may offer some protection. Therefore, such supplementation should be considered and studied further in these ‘at risk’ menopausal *APOE4* carriers.

Discussion of experimental models and methodology

Analysis of CANN study data: strengths and limitations

This PhD research project began by examining female participant data obtained from the CANN study, to explore how a number of potential determinants including: *APOE*, HRT, and n-3 PUFA intake influence PUFA status and cognition. As described in chapter 2, the CANN study was a multi-centre (Norwich and Melbourne), 12-month double-blinded, placebo-controlled parallel intervention trial (Irvine, Scholey et al. 2018). However, for the purpose of our analysis, we focused primarily on female participant baseline and screening data. Participants were prospectively recruited based upon their MCI or SMI status and age, and the study was devised to actively assess many of the determinants explored in this analysis, with the exception of HRT. This provided us with significant power to evaluate the effects of *APOE* genotype and n-3 PUFA status. Conversely, the number of *APOE4* carriers receiving HRT was quite low, as acknowledged in chapter 2 and therefore this analysis should be viewed as exploratory, with any conclusions treated with caution. However, the size of the effect changes and statistical significance was indeed promising. In addition, given that HRT use was not a major 'focus' in the RCT, information regarding the type, combination and onset of HRT use was not available and was another weakness of this analysis, given the known influence these factors can have on HRT effectiveness. One of the exclusion criteria for the CANN study was participants with high omega-3 fatty acid status. This was determined using the RBC omega-3 fatty acid index, and participants with a level greater than 6% of total fatty acids were excluded. Therefore, interpretation of this data should consider that a subset of the actual population, with a high n-3 index, and therefore likely consuming higher amounts of EPA and DHA were not represented. This may contribute to the lack of significant impact on cognition associated with an increasing n-3 index, particularly as we later indicate the necessity of higher n-3 PUFA doses. A particular strength was the comprehensive data gathered during the CANN study, allowing us to incorporate many relevant covariates into our analytical model, which are sometimes not included in other studies, improving the validity and robustness of our results. Similarly, we had access to comprehensive fatty acid analysis data, therefore giving us comprehensive overview of PUFA status both short and long term. Another strength of this analysis was the extensive cognitive test battery, which was performed during the CANN study. This enabled us to explore specific

cognitive domains, and establish subtle differences in our non-demented participants, that may have otherwise been missed.

***In vivo* human *APOE*-TR VCD menopause induced mouse model: strengths and limitations**

Model: In chapters 3 and 4, a VCD menopause induced *APOE*-TR mouse model was utilised. Such a model has extensive benefits from a research perspective. Firstly, *APOE*-TR mice, express the human form of *APOE* under the control of the murine regulatory sequence (Sullivan, Mezdour et al. 1997, Knouff, Hinsdale et al. 1999), resulting in APOE protein production in a physiological relevant manner. The use of both *APOE3*-TR and *APOE4*-TR mice enabled us to make direct behavioural and biochemical comparisons and establish isoform-specific effects. This *in vivo* approach also enables greater control of many confounding variables and full access to all tissue (e.g. brain) which would have been problematic, or unachievable when studying a human population. Although not a complete AD model, the less severe phenotype attributed to *APOE4*-TR mouse compared to related crosses (i.e *APOE4*-5xFAD aka E4FAD), enabled us to age our animals. This provided time for the dietary intervention to be delivered and have an effect, whilst simultaneously allowing us to induce menopause utilising the VCD method at a more relevant age. In addition, this also meant that the more subtle impacts of *APOE*, menopause and diet could be studied, and were not masked by an overwhelming genotype effect. The other major component of the model was the addition of menopause. To induce menopause we opted to use the VCD method (Kappeler and Hoyer 2012), which is gaining popularity for its ability to specifically target and deplete ovarian follicle reserves whilst maintaining ovarian tissue integrity (Marongiu 2019). Furthermore, the VCD model with its gradual follicle depletion, more closely resembles human menopause, in comparison to other experimental options such as OVX which result in abrupt, more widespread change. To our knowledge, this was the first time that VCD had been used in an *APOE*-TR mouse model and therefore we conducted a short pilot study prior to the main experimentation to assess efficacy and tolerance. Similarly, such methods had been previously reported in equivalent aged animals (Chen, Perez et al. 2014). One main weakness of the VCD menopause model is the requirement of ~ 14 i.p. injections; however, the alternative approach, OVX requires an invasive surgical procedure. In the attempt to minimise any issues associated with i.p., we altered the side

of injection delivery, monitored body weight/physical appearance of the animals, and included 2 recovery days (no injections) at the halfway point.

Dietary intervention: Typical western style diet is ~ 35 Kcal% from fat, for this study a slightly exaggerated version (~45 Kcal%) was provided to all of the mice, so that we remained in a physiologically relevant range whilst ensuring exacerbation of the genotype effects to accelerate cognitive decline(Moser and Pike 2017). The DHA to EPA ratio of the diets containing dietary fish oil (HFO and LFO) was 4:1 and was selected based upon the Chouinard Watkins study (Chouinard-Watkins, Vandal et al. 2017), in addition to other evidence highlighting the importance of high DHA for *APOE4* carriers. We retained some EPA however given that in humans all oily fish sources provide both EPA and DHA in variable ratios depending on species. Furthermore, EPA has been shown to be cardioprotective, which could contribute to the cognitive benefits (Bhatt, Steg et al. 2019). The HFO and LFO diet provided the human equivalent of 0.5g and 2g, EPA+DHA per day, corresponding to 1-2 portions of oil fish a week portion or 1 portion per day, and roughly respectively. These two doses were chosen as they fall within current guidelines worldwide, one representing the standard recommended intake in health adults (0.5g EPA+DHA per day) with the higher dose often recommended for the purposes of triglyceride lowering or as an anti-inflammatory (Skulas-Ray, Wilson et al. 2019). Supra-physiological doses have been utilised previously in rodent studies but their translational potential is questionable, and may also be problematic from a sustainability viewpoint. Therefore, we opted to stay within a physiological range. We did not have the financial resources to include both a low-fat diet group, and a low-fat VCD group for both the *APOE3* and *APOE4* TR mice, which would have been an appropriate approach to uncouple the effect of the HF diet. To a lesser extent, the addition of wild type C57BL/6 (genetic background for the *APOE*-TR mice), carrying murine *Apoe* may have also provided further controls.

Behavioural tests: were performed at 12 months to assess cognition in our animals. The tests chosen allowed us to measure distinct forms of memory (recognition and spatial) relevant in AD progression. All tests performed were relatively non-invasive, utilising the animal's natural behaviours, therefore minimising potentially confounding factors such as stress. Additionally, the researcher was blinded throughout behavioural experimentation and analysis to minimise potential bias.

Outcome Measures: The fatty acid composition of the erythrocytes and the brain was analysed from total extracted lipids using gas chromatography. Data was normalised to

the internal standard nonadecanoic acid (19:0) and expressed as % of total fatty acids, which is common practice. In Chapter 3 and 4, mRNA expression and protein abundance of synaptic plasticity related markers were measured using qPCR and western blot methods. The two methods provide a cost-effective quantitative and semi-quantitative overview of the molecular target, enabling us to gain a transcriptional and post translational understanding. In future studies the use of broader analytical techniques such as RNA sequencing and metabolomics may be warranted to get a greater overview of the mechanisms involved. In addition, immunohistochemistry may offer spatial interpretations of these findings.

Overall conclusion

In this PhD project, we investigated the relationships between *APOE* genotype, sex hormones, and n-3 PUFA status. We provide evidence supporting the notion that n-3 index and DHA status are dysregulated by both *APOE4* genotype and menopause. We highlight the impact of sex hormones on cognition and reveal that depletion (VCD model) and addition (HRT) appear to influence cognition in an *APOE4* dependant manner. We forward, deficits in synaptic plasticity related signalling as a potential mechanistic basis for the cognitive decline observed. Finally, we show that a DHA rich n-3 PUFA fish oil supplementation, particularly at higher dosage (2g/day human equivalent) ameliorates the deficits associated with *APOE4**menopause, improving recognition memory and restoring both brain DHA and synaptic plasticity related signalling. Therefore, DHA rich supplementation in 'at risk' peri/early menopausal *APOE4* women should be considered as an intervention strategy. Altogether, this PhD project contributes to our understanding of the potential mechanisms through which *APOE* modulates AD risk in females, offering novel insights into its interaction with sex hormone status, and n-3 PUFA supplementation. It therefore represents an important basis for future nutrigenetics and neurological disease research.

Future directions

With novel data and concepts arising from this project we would like to briefly discuss future research perspectives. Clearly, there remain many gaps and disparities in our knowledge relating to this subject area, many of which derive from the complexity of

APOE and the fundamental lack of research exploring its impact in females. We will therefore address some of the key questions and gaps, suggesting methods, which we believe, could be useful in progressing our understanding.

How do HRT combinations impact *APOE4* mediated AD risk? Can we come to a consensus on HRT usage? What are the fundamental mechanisms involved?

The general use of HRT to prevent disease is highly controversial and debate has endured over a number of decades (Cagnacci and Venier 2019). From an AD perspective, our results appear to support the pro side of the argument, with cognition improved in both carriers and non-carriers alike. Furthermore, the effects of HRT are widespread and may modify other disease processes in a deleterious manner (Cagnacci and Venier 2019), therefore a holistic, interdisciplinary approach may be needed to truly assess the efficacy and safety of HRT. Our research does however highlight how genotypes such as *APOE4* can modulate the overall effectiveness of HRT, and therefore should be carefully considered when designing or analysing future HRT research. One of the weaknesses of our study was the limited knowledge of the HRT used. Similarly, the age of menopausal onset and HRT treatment was not obtained in this study, all of which are known to influence efficacy of the treatment. Analyses incorporating this information would strengthen our understanding of HRT in AD development. Therefore, to address these issues we would envisage a need for either a large cross sectional or prospective study in which extensive information regarding HRT and *APOE* genotype and cognitive measures or incident dementia was available. This could then help inform, further clinical trials on optimal HRT delivery i.e. doses given, hormone combination, and when to intervene. Such clinical data would be required to establish the impact of HRT intervention on dementia risk. Given that the transitional 'peri-menopausal' phase appears to be a significant point in regards to female AD risk/pathology, (Mosconi, Berti et al. 2017, Mosconi, Rahman et al. 2018), any clinical trials should consider this as a potential intervention point.

Finally, our results and others clearly implicate sex hormones in cognition; however the mechanisms behind this remain somewhat unknown. One suggestions is that peri/post-menopausal states cause bioenergetics changes (Scheyer, Rahman et al. 2018), and thus further PET imaging studies may help inform this process. The link between sex hormones and n-3 PUFAs is also an interesting one, particularly in light of Alessandi's (Alessandri, Extier et al. 2011), and Marin's reports (Marin and Diaz 2018). Further

investigation perhaps utilising DHA tracers would help elucidate the fate of DHA in response to menopause.

How does DHA and menopause influence synaptic plasticity?

We observed striking changes in synaptic plasticity related signalling in *APOE4* animals in response to both menopause and HFO, however the mechanism behind this remains unknown. Taking a reductionist approach and modelling this into neuronal cell models may potentially help determine the fundamental mechanism involved. This could potentially be achieved by generating a primary neuron cell culture from *APOE*-TR mice, and exposing them to various combinations of estrogens and DHA. Electrophysiological methods (e.g. patch clamp) or Multi Electrode Arrays (MEA) could be used to monitor neuronal activity, whilst molecular techniques could be employed to monitor gene and protein changes. In addition and practically more ambitious, an *in-vivo* electrophysioloical method could be utilised in a similar VCD treated *APOE*-TR model to assess neuronal activity *in-vivo*.

Why is recognition memory susceptible?

In both CANN analysis and our mouse model we found evidence for an *APOE4**sex hormone influence on recognition memory, Highlighting this as a highly relevant area for future research. Recognition memory relies upon neuronal circuitry involving the perirhinal cortex, hippocampus, medial prefrontal cortex and retrosplenial cortex (Warburton 2018). Dissecting these specific regions, in VCD treated mice and performing a high throughput analytical method such as RNA sequencing could highlight key and novel changes in gene expression, enabling us to have a greater overview of the processes and mechanisms behind this effect.

Could brain-targeting forms of DHA reduce dosage?

Current evidence appears to recommend higher doses of DHA to benefit *APOE4* carriers. Indeed our rodent experiments found only the 2g/day human equivalent dosage provided any significant cognitive benefit, and others have described the requirement of even larger doses (Chouinard-Watkins, Vandal et al. 2017). This is problematic both translationally, and also sustainably. Therefore, strategies to increase efficacy and reduce the required dose are essential. One potential strategy may be to utilise ‘brain targeting’ forms of DHA. As shown in mice by Sugansini and colleagues (Sugasini, Thomas et al. 2017), daily gavage with 1mg LPC DHA per day, led to dramatic increase in brain DHA profiles

compared to non LPC forms. Given this ~ 3 fold increase in brain DHA observed in LPC DHA supplemented animals compared to wild type controls, it would be interesting to determine the effect of lower doses, perhaps performing a dose response experiment. Similarly, determining the optimal dose required to mitigate *APOE4* and menopausal impacts would be useful. Finally, the aforementioned 'brain targeting' forms of DHA have largely been explored in animal models and a large clinical trial would be needed to truly assess their translational potential.

Is there an involvement of the gut microbiota?

The influence of gut microbiota is an emerging factor in neurological disease, including AD. We know that *APOE4* leads to substantial changes in metabolism. Similarly, recent reports from our group have indicated that there are *APOE4* specific changes in the gut microbiota structure and function (Tran, Corsini et al. 2019). However, given the novel nature of these findings there is little known about how menopause or n-3 PUFA may influence the process. Therefore, sequencing the gut microbiota profile of VCD treated, n-3PUFA supplemented *APOE3* and *APOE4* animals, may potentially uncover a previously unexplored, novel mechanism.

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Appendix

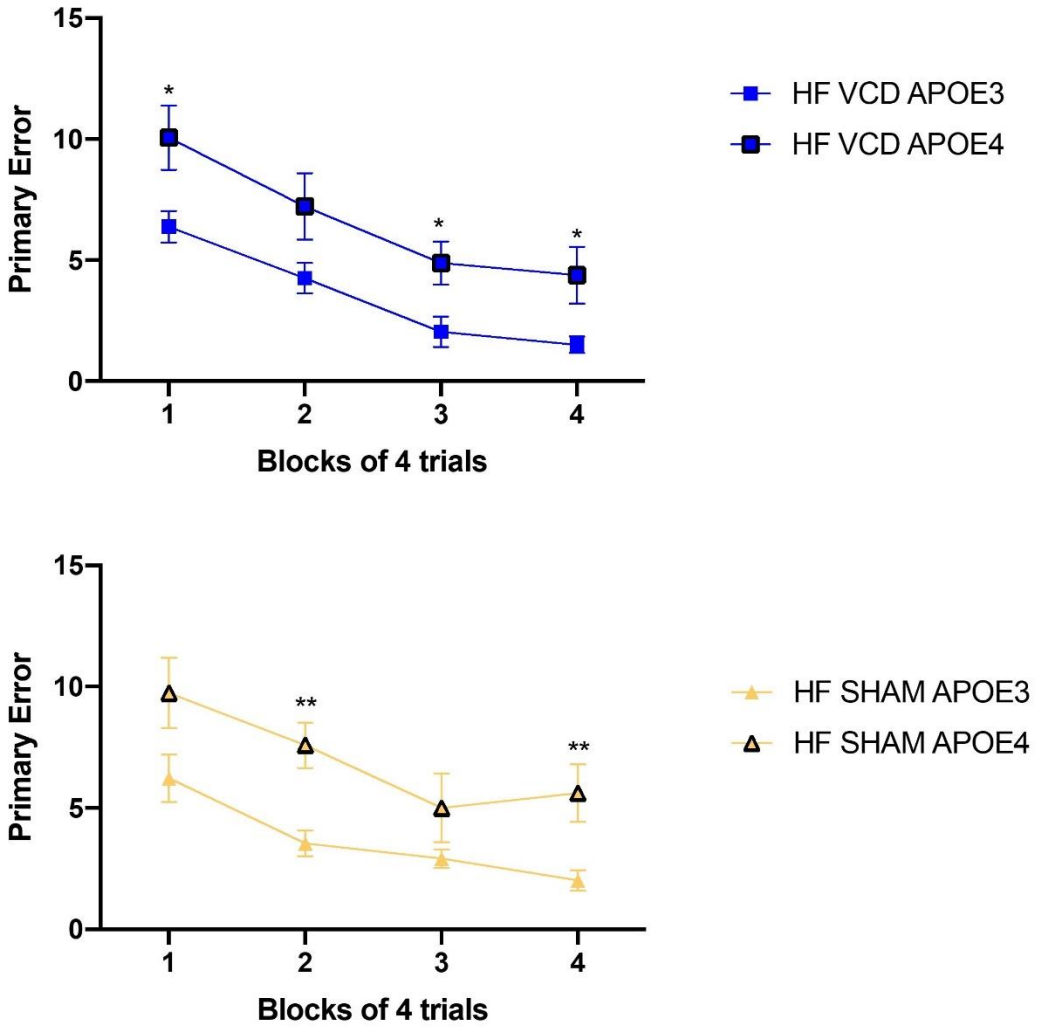

Figure S1 Barnes maze learning phase primary error trajectory.

An upwards shift in primary errors was observed in *APOE4* animals in both a) VCD treated and b) sham animals. Data represent the mean ± S.E.M *p< 0.05. **P < 0.01

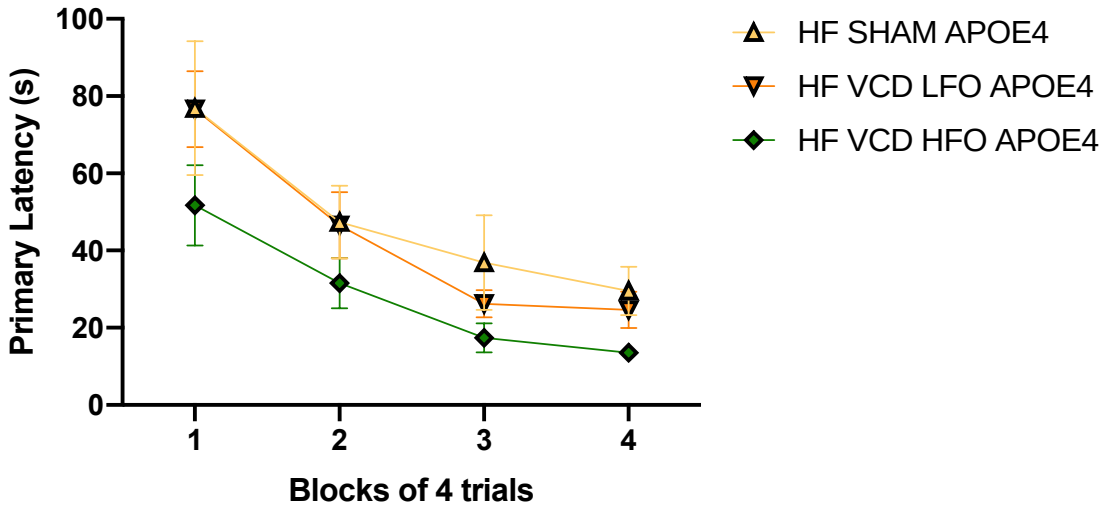

Figure S2 Barnes maze learning phase primary latency trajectory.

A nominal downwards shift in primary latency was observed in HFO supplemented *APOE4* animals compared to sham non-supplemented counterparts, however this did not reach significance Data represent the mean ± S.E.M

Table S1: Full dietary composition of diet used in experimentation

Diets	High Fat (HF)	High Fat - Low fish oil (HF LFO)	High fat - High fish oil (HF HFO)
% Composition	**Kcal**	**Kcal**	**Kcal**
Protein	20	20	20
Carbohydrate	35	35	35
Fat	45	45	45
Total	100	100	100
Diet component gm/kg	**gm/kg**	**gm/kg**	**gm/kg**
Casein	233	233	233
L-Cystine	3	3	3
Corn starch	85	85	85
Maltodextrin 10	117	117	117
Sucrose	201	201	201
Cellulose, BW200	58	58	58
Corn oil	47	47	46
Palm oil	189	188	185
EPAX oil	0	1	5

Table S2 Primer sequences used for qRT-PCR

Gene	Description	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Chapter 3			
Esr2	Estrogen Receptor Beta	GGTCCTGTGAAGGATGTAAGGC	TAACACTTGCGAAGTCGGCAGG
Esr1	Estrogen Receptor Alpha	TCTGCCAAGGAGACTCGCTACT	GGTGCATTGGTTTGTAGCTGGAC
Fatp4	Fatty acid transporter protein 4	GACTTCTCCAGCCGTTTCCACA	CAAAGGACAGGATGCGGCTATTG
Acsl6	Acyl-CoA synthetase long-chain family member 6	CAGAGGAACTCAACTACTGGACC	CCAATGTCTCCAGTGTGAAGCC
Fatp1	Fatty acid transporter protein 1	TGCCACAGATCGGCGAGTTCTA	AGTGGCTCCATCGTGTCCTCAT
Mfsd2a	Major facilitator superfamily domain-containing protein 2	GGTCTCAGAAGTTGCCAATCGC	GAAGGCACAGAGGACGTAGATG
Fabp5	Fatty acid binding protein 5	GACGACTGTGTTCTCTTGTAACC	TGTTATCGTGCTCTCCTTCCCG
APOE	Apolipoprotein E	GGGTCGCTTTTGGGATTACCTG	CAACTCCTTCATGGTCTCGTCC
Bdnf	Brain derived neurotrophic factor	GGCTGACACTTTTGAGCACGTC	CTCCAAAGGCACTTGACTGCTG
Akt1	RAC-alpha serine/threonine-protein kinase	CCTTTATTGGCTACAAGGAACGG	GAAGGTGCGCTCAATGACTG
Creb1	CAMP responsive element binding protein 1	ACCCAGGGAGGAGCAATACAG	TGGGGAGGACGCCATAACA
Atf4	Activating transcription factor 4	CTCTTGACCACGTTGGATGAC	CAACTTCACTGCCTAGCTCTAAA
Grin2b	Glutamate (NMDA) receptor subunit epsilon-2	CTGGTGACCAATGGCAAGCATG	GGCACAGAGAAGTCAACCACCT
Ephb2	Ephrin type-B receptor 2	CAACGGTGTGATCCTGGACTAC	CACCTGGAAGACATAGATGGCG
Cbd1	Cannabinoid receptor type 1	ATCGGAGTCACCAGTGTGCTGT	CCTTGCCATCTTCTGAGGTGTG
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
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Bdnf	Brain derived neurotrophic factor	GGCTGACACTTTTGAGCACGTC	CTCCAAAGGCACTTGACTGCTG

Akt1	RAC-alpha serine/threonine-protein kinase	CCTTTATTGGCTACAAGGAACGG	GAAGGTGCGCTCAATGACTG
Mapk1	Mitogen-activated protein kinase 1	GGTTGTTCCCAAATGCTGACT	CAACTTCAATCCTCTTGTGAGGG
Igf-1	Insulin-like growth factor 1 (IGF-1)	GTGGATGCTCTTCAGTTCGTGTG	TCCAGTCTCCTCAGATCACAGC
Creb1	CAMP responsive element binding protein 1	ACCCAGGGAGGAGCAATACAG	TGGGGAGGACGCCATAACA
Arc	Activity-regulated cytoskeleton-associated protein	GCTGGAAGAAGTCCATCAAGGC	ACCTCTCCAGACGGTAGAAGAC
Atf4	Activating transcription factor 4	CTCTTGACCACGTTGGATGAC	CAACTTCACTGCCTAGCTCTAAA
Ntrk2	Neurotrophic tyrosine kinase receptor type 2	CCACGGATGTTGCTGACCAAAG	GCCAAACTTGGAATGTCTCGCC
Grin2b	Glutamate (NMDA) receptor subunit epsilon-2	CTGGTGACCAATGGCAAGCATG	GGCACAGAGAAGTCAACCACCT
Cbd1	Cannabinoid receptor type 1	ATCGGAGTCACCAGTGTGCTGT	CCTTGCCATCTTCTGAGGTGTG
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tbp	TATA-binding protein	CTACCGTGAATCTTGGCTGTAAAC	AATCAACGCAGTTGTCCGTGGC

Table S3 Full brain fatty acid composition table of HF VCD and HF sham groups

	APOE3		***APOE4***		***Genotype P value***	***Intervention P value***	***Interaction P value***
Fatty acid	***HF Sham***	***HF VCD***	***HF Sham***	***HF VCD***			
Total n-3 PUFA	13.3 ± 0.2 [a]	11.6 ± 0.7 [b]	13.2 ± 0.3	11.8 ± 0.5	0.964	**0.004**	0.805
20:5 n-3 (EPA)	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.0.01	0.03 ± 0.14	0.466	0.147	0.414
22:5 n-3	0.06 ± 0.02	0.10 ± 0.05	0.05 ± 0.05	0.06 ± 0.02	0.526	0.207	0.526
22:6 n-3 (DHA)	13.2 ± 0.2 [a]	11.2 ± 0.5 [b]	13.1 ± 0.3 [a]	11.7 ± 0.5 [b]	0.650	**0.001**	0.519
Total n-6	14.2 ± 0.2	12.6 ± 0.4	14.8 ± 0.9	13.2 ± 0.6	0.274	**0.011**	0.979
18:2 n-6	0.44 ± 0.05	0.43 ± 0.07	0.44 ± 0.09	0.39 ± 0.06	0.712	0.613	0.993
20:2 n-6	0.04 ± 0.01	0.08 ± 0.03	0.04 ± 0.02	0.07 ± 0.02	0.719	0.082	0.851
20:3 n-6	0.26 ± 0.02	0.21 ± 0.02	0.23 ± 0.01	0.23 ± 0.02	0.594	0.154	0.125
20:4 n-6 (AA)	9.37 ± 0.21	8.10 ± 0.39	8.95 ± 0.25	8.67 ± 0.50	0.860	0.058	0.221
22:4 n-6	3.31 ± 0.09	3.23 ± 0.14	3.33 ± 0.06	3.07 ± 0.15	0.565	0.148	0.447
22:5 n-6	0.77 ± 0.13	0.54 ± 0.09	1.07 ± 0.26	0.77 ± 0.10	0.077	0.081	0.777
DHA:AA	1.40 ± 0.03	1.39 ± 0.05	1.48 ± 0.01 [a]	1.36 ± 0.03 [b]	0.619	**0.049**	0.134
Total SFAs	38.7 ± 0.4 [a]	35.5 ± 0.9 [b]	36.6 ± 0.6	36.9 ± 1.2	0.689	0.100	0.054
14:0	0.22 ± 0.03	0.24 ± 0.04	0.16 ± 0.04	0.18 ± 0.05	0.151	0.537	0.947
16:0	17.6 ± 0.5 a	14.7 ± 0.7 [b §]	16.2 ± 0.5	17.3 ± 0.9 [§]	0.406	0.207	**0.010**
18:0	20.1 ± 0.3	18.9 ± 0.5	19.3 ± 0.2	18.3 ± 0.6	0.138	**0.025**	0.802
20:0	0.31 ± 0.02 [a]	0.41 ± 0.03 [b]	0.28 ± 0.02	0.33 ± 0.03	0.053	**0.014**	0.343
22:0	0.22 ± 0.04	0.38 ± 0.05 [§]	0.26 ± 0.01	0.19 ± 0.06 [§]	0.099	0.110	**0.030**
24:0	0.31 ± 0.09	0.78 ± 0.18	0.38 ± 0.10	0.53 ± 0.14	0.442	**0.032**	0.277
Total MUFAs	25.4 ± 0.4 [a]	30.5 ± 0.8 [b §]	27.3 ± 0.9	27.9 ± 1.4 [§]	0.448	**0.021**	**0.013**
16:1 n-9	0.22 ± 0.05	0.22 ± 0.06	0.20 ± 0.03	0.37 ± 0.06	0.242	0.118	0.120
16:1 n-7	0.43 ± 0.03	0.42 ± 0.01	0.39 ± 0.02	0.46 ± 0.04	0.845	0.182	0.195
18:1 n-9	18.2 ± 0.5 [a]	21.2 ± 0.8 b	19.3 ± 0.6	19.3 ± 1.2	0.684	0.091	0.084
18:1 n-7	3.62 ± 0.05	3.58 ± 0.16	3.38 ± 0.04	3.46 ± 0.13	0.070	0.897	0.792
20:1 n-9	2.03 ± 0.18 [a]	3.04 ± 0.20 [b]	2.28 ± 0.20	2.21 ± 0.21	0.166	**0.028**	**0.013**
22:1 n-9	0.17 ± 0.02	0.21 ± 0.03	0.21 ± 0.01	0.18 ± 0.02	0.741	0.750	0.128
24:1 n-9	1.37 ± 0.09 [a]	1.96 ± 0.18 [b §]	1.56 ± 0.11	1.14 ± 0.24 [§]	0.080	0.641	**0.008**
Total DMA	8.52 ± 0.27 [a]	9.82 ± 0.47 [b]	9.46 ± 0.24	8.61 ± 0.44	0.711	0.549	**0.009**

(n=5/6). PUFA; Polyunsaturated fatty acid, 20:5 n-3 EPA; Eicosapentaenoic acid, 22:5 n-3; Docosapentaenoic acid, 22:6 n-3 DHA; Docosahexaenoic acid, 18:2 n-6; Linoleic acid, 20:2 n-6; Eicosadienoic acid, 20:3 n-6; Dihomo-gamma-linolenic acid, 20:4 n-6 AA; Arachidonic acid, 22:4 n-6; Adrenic Acid, 22:5 n-6 Docosapentaenoic acid, DHA:AA; Docosahexaenoic acid to Arachidonic acid ratio, SFA; Saturated fatty acid, 14:0; Myristic acid, 16:0; Palmitic acid, 18:0; Stearic acid, 20:0; Eicosanoic acid, 22:0; Docosanoic acid, 24:0; Tetracosanoic acid, MUFA; Monounsaturated fatty acid, 16:1 n-9; palmitoleic acid, 16:1 n-7; palmitoleic acid, 18:1 n-9; Oleic acid, 18:1 n-7; cis-Vaccenic acid, 20:1 n-9; 11-Eicosenoic acid, 22:1 n-9; Erucic acid 24:1 n-9; Nervonic acid. Brain fatty acid composition of experimental animals (n=5/6 per group) Data is % of total fatty acids and mean value ± SEM. 2-way ANOVA. Letters a, b denote significant intervention difference whilst § denotes significant genotype effect as analysed via post hoc.

Table S4 Full brain fatty acid composition of all experimental animal groups (n=5/6 per group)

	APOE3				***APOE4***				***Genotype P value***	***Intervention P value***	***Interaction P value***
Fatty acid	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**			
Total n-3 PUFA	2.05 ± 0.35 *a*	1.89 ± 0.45 *ab*	2.08 ± 0.21 *ab*	3.72 ± 0.38 *b*	1.19 ± 0.19 *a*	1.65 ± 0.55 *b*	2.49 ± 0.31 *ac*	3.94 ± 0.39 *c*	0.416	**0.0001**	0.286
20:5 n-3 (EPA)	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.01	0.06 ± 0.03	0.02 ± 0.01	0.03 ± 0.14	0.01 ± 0.01	0.09 ± 0.03	0.640	0.067	0.529
22:5 n-3	0.06 ± 0.02	0.10 ± 0.05	0.05 ± 0.02	0.07 ± 0.03	0.05 ± 0.05	0.06 ± 0.02	0.12 ± 0.06	0.04 ± 0.01	0.856	0.338	0.502
22:6 n-3 (DHA)	13.20 ± 0.20 *a*	11.20 ± 0.50 *b*	13.10 ± 0.70 *a*	13.50 ± 0.40 *a*	13.10 ± 0.30 *ab*	11.70 ± 0.50 *a*	13.70 ± 0.40 *b*	13.40 ± 0.50 *b*	0.479	**0.0002**	0.765
Total n-6 PUFA	14.20 ± 0.20 *a*	12.60 ± 0.40 *ab*	12.00 ± 0.50 *b*	11.10 ± 0.30 *b*	14.80 ± 0.90 *a*	13.20 ± 0.60 *ab*	12.70 ± 0.20 *b*	11.50 ± 0.40 *b*	0.093	**0.0001**	0.992
18:2 n-6	0.44 ± 0.05	0.43 ± 0.07	0.39 ± 0.03	0.43 ± 0.04	0.44 ± 0.09	0.39 ± 0.06	0.40 ± 0.04	0.43 ± 0.05	0.724	0.884	0.963
20:2 n-6	0.04 ± 0.01	0.08 ± 0.03	0.14 ± 0.05	0.10 ± 0.05	0.04 ± 0.02	0.07 ± 0.02	0.09 ± 0.03	0.09 ± 0.02	0.463	0.067	0.766
20:3 n-6	0.26 ± 0.02 *a*	0.21 ± 0.02 *a*	0.29 ± 0.02 *a*	0.42 ± 0.03 *b*	0.23 ± 0.01 *a*	0.23 ± 0.02 *a*	0.29 ± 0.02 *ab*	0.40 ± 0.05 *b*	0.562	**0.0001**	0.777

20:4 n-6 (AA)	9.37 ± 0.21 *a*	8.10 ± 0.39 *ab*	8.28 ± 0.39 *ab*	7.55 ± 0.28 *b*	8.95 ± 0.25	8.66 ± 0.50	8.70 ± 0.22	7.88 ± 0.26	0.354	**0.002**	0.508
22:4 n-6	3.31 ± 0.09 *ac*	3.23 ± 0.14 *a*	2.76 ± 0.09 *b*	2.57 ± 0.07 *b*	3.33 ± 0.06 *a*	3.07 ± 0.15 *ac*	2.89 ± 0.08 *bc*	2.64 ± 0.15 *b*	0.837	**0.0001**	0.610
22:5 n-6	0.77 ± 0.13 *a*	0.54 ± 0.09 *a*	0.18 ± 0.07 *b*	0.02 ± 0.005 *b*	1.07 ± 0.26 *a*	0.77 ± 0.10 *a*	0.38 ± 0.14 *b*	0.03 ± 0.017 *c*	**0.032**	**0.0001**	0.507
DHA:AA	1.40 ± 0.03 *a*	1.39 ± 0.05 *a*	1.58 ± 0.04 *b*	1.79 ± 0.02 *c*	1.48 ± 0.01 *ad*	1.36 ± 0.03 *a*	1.58 ± 0.03 *bd*	1.76 ± 0.03 *c*	0.998	**0.0001**	0.400
Total SFAs	38.70 ± 0.40	35.50 ± 0.90	36.70 ± 1.40	37.00 ± 1.40	36.60 ± 0.60	36.90 ± 1.20	37.70 ± 0.90	36.60 ± 0.80	0.961	0.409	0.216
14:0	0.22 ± 0.03 *a*	0.24 ± 0.04 *a*	0.13 ± 0.04 *ab*	0.09 ± 0.03 *b*	0.16 ± 0.04	0.18 ± 0.05	0.14 ± 0.02	0.10 ± 0.03	0.363	**0.007**	0.544
16:0	17.60 ± 0.50 *a*	14.70 ± 0.70 *b §*	16.40 ± 0.60 *ab*	16.40 ± 0.40 *ab*	16.20 ± 0.50	17.30 ± 0.90 §	17.20 ± 0.60	16.30 ± 0.70	0.305	0.501	**0.034**
18:0	20.10± 0.30	18.90 ± 0.50	19.30 ± 0.30	19.60 ± 0.30	19.30 ± 0.20	18.30 ± 0.60	19.60 ± 0.40	19.40 ± 0.30	0.298	0.104	0.673
20:0	0.31 ± 0.02	0.41 ± 0.03	0.29 ± 0.04	0.38 ± 0.02	0.28 ± 0.02	0.33 ± 0.03	0.27 ± 0.03	0.35 ± 0.04	0.068	**0.012**	0.771
22:0	0.22 ± 0.04	0.38 ± 0.05	0.23 ± 0.06	0.25 ± 0.09	0.26 ± 0.01	0.19 ± 0.06	0.19 ± 0.06	0.25 ± 0.09	0.299	0.335	0.302
24:0	0.31 ± 0.09 ab	0.78 ± 0.18 a	0.34 ± 0.10 ab	0.26 ± 0.13 b	0.38 ± 0.10	0.53 ± 0.14	0.27 ± 0.08	0.39 ± 0.11	0.669	**0.020**	0.438
Total MUFAs	25.40 ± 0.40 *a*	30.50 ± 0.80 *b*	26.50 ± 1.20 *ac*	30.00 ± 0.80 *bc*	27.30 ± 0.90 *ab*	27.90 ± 1.40 *ab*	26.50 ± 0.80 *a*	30.20 ± 0.90 *b*	0.615	**0.001**	0.054

16:1 n-9	0.22 ± 0.05	0.22 ± 0.06	0.27 ± 0.06	0.36 ± 0.05	0.20 ± 0.03	0.37 ± 0.06	0.18 ± 0.06	0.37 ± 0.06	0.748	**0.021**	0.206
16:1 n-7	0.43 ± 0.03	0.42 ± 0.01	0.44 ± 0.02	0.48 ± 0.04	0.39 ± 0.02 *a*	0.46 ± 0.04 *ab*	0.44 ± 0.03 *ab*	0.50 ± 0.02 *b*	0.972	**0.038**	0.588
18:1 n-9	18.20 ± 0.50	21.20 ± 0.80	18.50 ± 0.70	20.60 ± 0.40	19.30 ± 0.60	19.30 ± 1.20	19.00 ± 0.40	20.20 ± 0.40	0.698	0.026	0.166
18:1 n-7	3.62 ± 0.05	3.58 ± 0.16	3.45 ± 0.15	3.77 ± 0.12	3.38 ± 0.04	3.39 ± 0.13	3.46 ± 0.17	3.59 ± 0.11	0.258	0.096	0.769
20:1 n-9	2.03 ± 0.18 *a*	3.04 ± 0.19 *b*	2.18 ± 0.17 *ac*	2.73 ± 0.16 *abc*	2.28 ± 0.20	2.21 ± 0.21	2.11 ± 0.20	2.82 ± 0.31	0.364	**0.007**	0.071
22:1 n-9	0.17 ± 0.02	0.21 ± 0.03	0.18 ± 0.01	0.18 ± 0.04	0.21 ± 0.01	0.18 ± 0.02	0.13 ± 0.04	0.18 ± 0.04	0.718	0.400	0.509
24:1 n-9	1.37 ± 0.09	1.96 ± 0.18	1.49 ± 0.10	1.90 ± 0.16	1.56 ± 0.11 *ab*	1.14 ± 0.24 *a*	1.33 ± 0.13 *a*	2.03 ± 0.23 **b**	0.172	**0.008**	**0.02**
Total DMA	8.52 ± 0.27	9.82 ± 0.47	8.60 ± 0.32	8.23 ± 0.51	9.46 ± 0.24	8.61 ± 0.44	9.07 ± 0.52	8.48 ± 0.72	0.751	0.320	0.170

PUFA; Polyunsaturated fatty acid, 20:5 n-3 EPA; Eicosapentaenoic acid, 22:5 n-3; Docosapentaenoic acid, 22:6 n-3 DHA; Docosahexaenoic acid, 18:2 n-6; Linoleic acid, 20:2 n-6; Eicosadienoic acid, 20:3 n-6; Dihomo-gamma-linolenic acid, 20:4 n-6 AA; Arachidonic acid, 22:4 n-6; Adrenic Acid, 22:5 n-6 Docosapentaenoic acid, DHA:AA; Docosahexaenoic acid to Arachidonic acid ratio, SFA; Saturated fatty acid, 14:0; Myristic acid, 16:0; Palmitic acid, 18:0; Stearic acid, 20:0; Eicosanoic acid, 22:0; Docosanoic acid, 24:0; Tetracosanoic acid, MUFA; Monounsaturated fatty acid, 16:1 n-9; palmitoleic acid, 16:1 n-7; palmitoleic acid, 18:1 n-9; Oleic acid, 18:1 n-7; cis-Vaccenic acid, 20:1 n-9; 11-Eicosenoic acid, 22:1 n-9; Erucic acid 24:1 n-9; Nervonic acid. Brain fatty acid composition of experimental animals (n=5/6 per group) Data is % of total fatty acids and mean value ± SEM. 2-way ANOVA. Different superscripts denote significant difference between group means, whilst § indicates significant genotype difference (Tukey or Sidak post hoc).

Table S5 Full Erythrocyte fatty acid composition of all experimental animal groups (n=5/6 per group)

	APOE3				***APOE4***				***Genotype P value***	***Intervention P value***	***Interaction P value***
Fatty acid	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**	***HF Sham***	***HF VCD***	**HF VCD LFO**	**HF VCD HFO**			
Total n-3 PUFA	2.05 ± 0.35 *ab*	1.89 ± 0.45 *a*	2.08 ± 0.21 *ab*	4.60 ± 0.94 *b*	1.19 ± 0.19 *a*	1.65 ± 0.55 *ac*	2.49 ± 0.31 *cb*	3.94 ± 0.39 *b*	0.288	**0.0001**	0.367
22:6 n-3 (DHA)	1.95 ± 0.33 *ab*	1.85 ± 0.44 *a*	2.08 ± 0.21*ab*	3.98 ± 0.80 *b*	1.19 ± 0.19 *a*	1.65 ± 0.55 *a*	2.31 ± 0.30 *ab*	3.68 ± 0.38 *b*	0.333	**0.0001**	0.511
Total n-6 PUFA	32.30 ± 3.80 *a*	32.64 ± 4.15 *a*	22.27 ± 0.86 *b*	22.38 ± 0.68 *b*	24.28 ± 1.25	26.57 ± 2.64	20.91 ± 1.28	20.98 ± 0.46	**0.009**	**0.0003**	0.399
18:2 n-6	12.35 ± 1.40	12.71 ± 1.36	12.70 ± 0.43	15.14 ± 0.65	11.39 ± 0.55	11.06 ± 0.73	11.25 ± 0.96	12.63 ± 0.85	0.074	**0.0140**	0.856
20:3 n-6	0.63 ± 0.06	0.81 ± 0.11	0.47 ± 0.13	0.83 ± 0.10	0.70 ± 0.01	0.54 ± 0.19	0.58 ± 0.18	0.87 ± 0.05	0.875	0.149	0.479

20:4 n-6 (AA)	16.10 ± 2.61 *a*	16.47 ± 3.28 *a*	7.43 ± 0.49 *b*	6.94 ± 0.61 *b*	11.07 ± 0.83 *ab*	13.00 ± 1.66 *a*	7.58 ± 0.52 *b*	6.46 ± 0.38 *b*	0.0730	**0.0001**	0.379
22:4 n-6	1.01 ± 0.16 *ab*	1.24 ± 0.20 *a*	0.46 ± 0.15 *bc*	0.22 ± 0.14 *c*	0.56 ± 0.15	0.64 ± 0.29	0.65 ± 0.18	0.64 ± 0.18	0.409	**0.042**	**0.0254**
DHA:AA	0.12 ± 0.01 *a*	0.11 ± 0.01 *a*	0.29 ± 0.03 *b*	0.48 ± 0.01 *c*	0.11 ± 0.01 *a*	0.12 ± 0.03 *a*	0.38 ± 0.10 *b*	0.57 ± 0.05 *c*	0.530	**0.0001**	0.624
Total SFAs	47.81 ± 3.18	45.70 ± 3.95	51.71 ± 1.22	53.07 ± 1.22	52.00 ± 0.77	49.52 ± 3.20	50.64 ± 2.08	51.83 ± 0.68	0.373	0.199	0.453
14:0	0.21 ± 0.02	0.28 ± 0.04	0.23 ± 0.05	0.16 ± 0.04	0.25 ± 0.03	0.12 ± 0.06	0.21 ± 0.05	0.19 ± 0.05	0.404	0.626	0.146
16:0	30.43 ± 2.21	30.53 ± 3.0	33.19 ± 1.35	32.02 ± 1.22	34.37 ± 0.47	32.19 ± 2.14	31.15 ± 1.23	32.55 ± 0.93	0.387	0.930	0.325
18:0	15.81 ± 1.03	13.26 ± 0.86	16.73 ± 1.02	16.54 ± 1.32	15.48 ± 0.56	15.31 ± 1.37	17.45 ± 1.27	17.51 ± 0.63	0.276	0.046	0.785
20:0	0.20 ± 0.02	0.19 ± 0.02	0.31 ± 0.03	0.30 ± 0.01	0.22 ± 0.02	0.19 ± 0.03	0.27 ± 0.03	0.27 ± 0.03	0.614	**0.0006**	0.642
22:0	0.48 ± 0.10	0.64 ± 0.10	0.55 ± 0.10	0.79 ± 0.06	0.66 ± 0.04	0.57 ± 0.20	0.67 ± 0.08	0.63 ± 0.08	0.485	0.286	0.176
24:0	0.68 ± 0.05	0.78 ± 0.12 *§*	0.87 ± 0.05	0.96 ± 0.08	1.02 ± 0.10 *ab*	1.52 ± 0.41 *a §*	0.89 ± 0.08 *ab*	0.68 ± 0.15 *b*	**0.024**	0.420	**0.023**
Total MUFAs	17.84 ± 1.00 *a §*	19.78 ± 1.05 *ab*	23.94 ± 1.37 *b*	20.29 ± 1.48 *ab*	22.53 ± 0.68 *§*	22.59 ± 0.68	22.69 ± 1.25	23.25 ± 0.68	**0.006**	0.052	0.059

16:1 n-7	0.60 ± 0.06	0.88 ± 0.20	0.88 ± 0.13	0.79 ± 0.12	0.97 ± 0.08	1.00 ± 0.16	0.78 ± 0.09	0.79 ± 0.14	0.293	0.613	0.300
18:1 n-9	15.08 ± 0.77	16.51 ± 1.02	20.26 ± 1.11	17.49 ± 1.23	19.10 ± 0.85	19.38 ± 0.19	19.03 ± 1.03	19.64 ± 0.69	0.698	0.026	0.166
18:1 n-7	1.23 ± 0.15	1.48 ± 0.12	1.54 ± 0.15	1.40 ± 0.19	1.62 ± 0.05	1.75 ± 0.04	1.72 ± 0.21	1.33 ± 0.35	0.165	0.398	0.678
20:1 n-9	0.21 ± 0.01	0.20 ± 0.02	0.36 ± 0.11	0.27 ± 0.09	0.24 ± 0.02	0.36 ± 0.16	0.59 ± 0.16	0.71 ± 0.20	**0.038**	0.234	0.465
24:1 n-9	0.41 ± 0.05	0.44 ± 0.13	0.55 ± 0.15	0.34 ± 0.12 *§*	0.54 ± 0.06 *ab*	0.38 ± 0.16 *a*	0.71 ± 0.08 *ab*	0.93 ± 0.19 *b §*	**0.034**	0.241	0.103

PUFA; Polyunsaturated fatty acid, 22:6 n-3 DHA; Docosahexaenoic acid, 18:2 n-6; Linoleic acid, 20:3 n-6; Dihomo-gamma-linolenic acid, 20:4 n-6 AA; Arachidonic acid, 22:4 n-6; Adrenic Acid, DHA:AA; Docosahexaenoic acid to Arachidonic acid ratio, SFA; Saturated fatty acid, 14:0; Myristic acid, 16:0; Palmitic acid, 18:0; Stearic acid, 20:0; Eicosanoic acid, 22:0; Docosanoic acid, 24:0; Tetracosanoic acid, MUFA; Monounsaturated fatty acid, 16:1 n-7; palmitoleic acid, 18:1 n-9; Oleic acid, 18:1 n-7; cis-Vaccenic acid, 20:1 n-9; 11-Eicosenoic acid, 24:1 n-9; Nervonic acid. Brain fatty acid composition of experimental animals (n=5/6 per group) Data is % of total fatty acids and mean value ± SEM. 2-way ANOVA. Different superscripts denote significant difference between group means, whilst § indicates significant genotype difference (Tukey or Sidak post hoc)