The influence of dietary lipids on cognition, cerebral blood volume and amyloid pathology in the APP/PS1 mouse model of Alzheimer's Disease

Yael Reijmer[1]
Supervisors: Amanda Kiliaan[1] & Carlijn Hooijmans[1]

[1]*Department of Anatomy, Nijmegen, Radboud University Nijmegen Medical Centre*

High serum cholesterol and low docosahexaenoic acid (DHA) intake are risk factors for Alzheimer's disease (AD). However, how these parameters influence the pathology is still a topic of debate. The present study assessed the influence of a cholesterol (typical western diet: TWD) and a DHA containing diet on spatial memory, amyloid beta (Aβ) deposition and relative cerebral blood volume (rCBV) in a 15-month-old APP/PS1 mouse model of Alzheimer's disease and wildtype littermates. rCBV was determined by contrast enhanced MRI and Aβ deposition by using immunohistochemistry.
APP/PS1 mice showed impaired spatial learning and memory in the Morris Water Maze test. Furthermore, transgenic mice showed a decrease in cortical rCBV. The cholesterol enriched TWD diet decreased the rCBV in the cortex compared to a standard diet in both APP/PS1 and control mice without affecting Aβ deposition. APP/PS1 mice on a DHA diet showed a decrease in vascular Aβ deposition and improved memory performance. In conclusion, these results show disease relevant behavioural and cognitive changes in an APP/PS1 mouse model accompanied by a decrease in rCBV. This study further indicates an important role for dietary lipids in the development of Alzheimer's disease by influencing the rCBV and vascular Aβ.

Keywords: Alzheimer's disease, APP/PS1, amyloid beta, cerebral blood volume, hypoperfusion, DHA, cholesterol

Correspondence to: Amanda Kiliaan, Radboud University Nijmegen Medical Centre, Department of Anatomy, PO BOX 9101, 6500HB Nijmegen, The Netherlands

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia. Millions of people are affected world wide and this population is still increasing. To date, there is no effective treatment as the cause of AD remains to be elucidated. Therefore, it is of crucial importance to find out which factors play a causal role in the development of the disease and see if these are treatable.

The Alzheimer pathology is characterized by elevated levels of the amyloid beta peptide (Aβ) in the brain, which is generated by proteolytic cleavage of the amyloid precursor protein (APP) by the enzymes β– and γ-secretase (Kang et al., 1987). Extracellular deposition and accumulation of Aβ are known as senile plaques (Citron et al., 1992; Masters et al., 1985), while cerebral amyloid angiopathy (CAA) refers to Aβ deposition in the walls of the blood vessels. Familial AD, is caused by autosomal dominant inheritance of mutations in APP or presenilin genes (i.e. PS1, part of the γ-secretase complex) and contributes to 5% of all AD patients (Citron et al., 1992; Kwok et al., 1997; Van Broeckhoven et al., 1992). This connection has led to the hypothesis that Aβ plays a causal role in the degenerative cellular changes of the disease (Selkoe, 1991). However, the majority of AD cases (sporadic AD) are not due to genetic mutations. Nowadays, there is growing evidence that the cerebrovascular pathology is the primary trigger in the development of sporadic AD (de la Torre, 2000, 2004). Recent emphasis on co-morbidity of AD and cerebrovascular diseases indicate that neurovascular dysfunction could play a major role in the pathogenesis of AD (Prins et al., 2005; Ruitenberg et al., 2005).

Besides age and a family history of dementia, cardiovascular risk factors like high serum cholesterol levels (Kivipelto et al., 2002; Kivipelto et al., 2005), obesity (Kivipelto et al., 2005), hypertension (Heijer et al., 2003) diabetes (Biessels & Kappelle, 2005) and atherosclerosis (van Oijen et al., 2007) are risk factors for developing AD. Furthermore, a genetic risk factor of sporadic AD is the possession of the apolipoprotein E4 (APOEε4) allele, which codes for a cholesterol transporter (Poirier, 2003). These data suggest an association between high cholesterol intake and the development of the disease (Martins et al., 2006). However, the way cholesterol influences the AD pathology is still a topic of extensive debate.

1.1 Cerebral hypoperfusion hypothesis

According to the cerebral hypoperfusion hypotheses high serum cholesterol levels can lead to a compromised vasculature and decreased cerebral perfusion (de la Torre, 2006; Roher et al., 2006). Hypoperfusion will lead to structural microvascular changes, disruption of the blood brain barrier, lowered energy metabolism and finally to the formation of plaques and neuronal cell death (Farkas & Luiten, 2001; Velliquette, O'Connor, & Vassar, 2005; Zlokovic, 2005). Occlusion of the middle cerebral artery in rats resulted in aggregation of APP and Aβ in the thalamus, which supports the relation between hypoperfusion and plaque formation (van Groen, Puurunen, Maki, Sivenius, & Jolkkonen, 2005). The hypoperfusion theory is further strengthened by evidence from patient studies showing that cerebral perfusion is indeed decreased in patients with Alzheimer's disease (Farkas & Luiten, 2001; Ruitenberg et al., 2005; Schreiber, Doepp, Spruth, Kopp, & Valdueza, 2005).

Research has not only focused on the negative effects of high cholesterol intake on vascular health, but also on dietary lipids that show a protective effect on the vasculature. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid, derived from fish oil and appears to be effective in the prevention of hypertension-associated vascular pathology (de Wilde, Farkas, Gerrits, Kiliaan, & Luiten, 2002; Farkas, de Wilde, Kiliaan, & Luiten, 2002). Cross-sectional studies have linked low DHA levels in the brain and plasma with dementia (Conquer, Tierney, Zecevic, Bettger, & Fisher, 2000; Heude, Ducimetiere, & Berr, 2003; Tully et al., 2003), while cohort studies show that dietary supplementation of DHA reduces the incidence of AD (Barberger-Gateau et al., 2002; Freund-Levi et al., 2006). It could be hypothesised that DHA can protect against cognitive decline and amyloid beta accumulation, by improving the cerbrovascular conditions.

1.2 Membrane fluidity hypothesis

However, cerebral perfusion is not the only mechanism via which cholesterol and DHA may influence the AD pathology. Both lipids are important components of the phospholipid bilayer of the neuronal cell membrane and can effect the membrane function by modulating the activity of membrane bound enzymes (Criado, Eibl, & Barrantes, 1982; Mitchell, Straume, Miller,

& Litman, 1990; Sinha, Shattil, & Colman, 1977). According to the *membrane fluidity hypothesis*, high serum cholesterol levels will decrease the fluidity of the cell membrane and this will lead to altered protein metabolism and increased plaque formation (Gibson Wood, Eckert, Igbavboa, & Muller, 2003; Refolo et al., 2000; Wolozin, 2001, 2004). Contrarily, high levels of DHA in the neuronal membranes increase the membrane fluidity and down-regulate the production of Aβ and the formation of senile plaques (Hashimoto, Hossain, Shimada, & Shido, 2006; Wolozin, 2004). So, both cholesterol and DHA show two possible mechanisms via which they can effect the AD pathology, namely by influencing the cerebral perfusion or the fluidity of the neuronal cell membrane.

Insight into pathogenesis and treatments of AD may be significantly advanced by transgenic animal models for the disease. A well established mouse model for AD is the double transgenic APPswe/PS1ΔE9 mouse (Spires & Hyman, 2005). This mouse model has shown to exhibit AD neuropathology, such as a progressive increase in brain Aβ levels as well as disease related cognitive and behavioural changes, like impaired spatial memory (Arendash et al., 2001; Lalonde, Kim, Maxwell, & Fukuchi, 2005; Pugh, Richardson, Bate, Upton, & Sunter, 2007) .

A previous study from our lab, assessed the effect of DHA and cholesterol in 8- and 19-month-old APP/PS1 mice. Results show no differences in cerebral blood volume (CBV) or plaques load between the diet groups at the age of 8 months. At the age of 19 months however, APP/PS1 mice on a DHA enriched diet showed an increase in CBV and a lower amount of vascular Aβ, compared to mice fed a standard diet. Furthermore, a cholesterol enriched diet increased the plaque formation in the hippocampus accompanied by elevated plasma cholesterol levels, without effecting the cholesterol content in the brain of APP/PS1 mice (Hooijmans, 2007). These findings support the view that long-term intake of dietary lipids can impact brain circulation and the deposition of Aβ plaques in the brain. The question remains whether reduced brain perfusion precedes increased Aβ deposition and therefore is a primary trigger, rather than a secondary symptom in the neuropathological progression of dementia. To investigate this, we now study the effects of dietary lipids in APP/PS1 mice at a younger age of 15 months.

The present study was designed to determine whether dietary supplementation of cholesterol and DHA can influence relevant cognitive changes via the two described mechanisms in 15-month-old APPswe/PS1ΔE9 mice. We expect that supplementation of dietary lipids can effect spatial memory and learning by primary influencing CBV and secondary, the Aβ production in a transgenic mouse model for Alzheimer's disease.

2. Materials and Methods

2.1 Animals and Diets

The APPswe/PS1dE9 founder mice were obtained from John Hopkins University, Baltimore, USA and a colony was established at the Radboud University, Nijmegen, the Netherlands. Mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harbouring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transfected genes co-integrate and co-segregate as a single locus (Jankowsky et al., 2001). The breeder mice were backcrossed to C57BL6/J for 5-6 generations to obtain mice for the current study.

Male APP/PS1 mice (n=26) and male wild-type littermates (n=39) were assigned to three different diet groups. One group was fed a Typical Western diet containing 1 % of cholesterol, the second group received a DHA diet supplemented with 0,50% of n3 DHA, and the third group received a Standard control diet. For the exact sources and contents of fatty acids in the experimental diets, see Table 1. The groups were balanced for weight. The diets started at the age of 2 months and were maintained for 13 months. Mice were housed in individual cages in a controlled environment and maintained under a 12-h light:dark cycle with food and water available *ad libitum*.

Mice were behaviourally tested during the light phase at the age of 14 months (range: 13-15 months) in three cohorts of 21-23 mice, balanced by genotype and diet. The experiments were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority Radboud University Nijmegen, the Netherlands.

2.2 Morris Water Maze

To investigate spatial learning and memory

abilities, mice were placed in a black pool with a diameter of 120 cm, containing water (22°C) made opaque by the addition of milk powder. Each trial mice were placed at different starting positions (North, East, South, West) and were supposed to find a submerged escape platform (1 cm below the water surface in the North-East quadrant) by using visual cues present on the four sides (three walls and a curtain) around the pool at a distance of 0,5 m. Parameters measured were latency to find the platform and time spent in the training quadrant (NE). During all trials the experimenter was located in the same position, in the room.

Acquisition was measured over a period of four days (four trials a day, 30 sec on the platform, trial interval 60 min). After the last acquisition trial on the fourth day, a 'probe trial' was performed, in which the platform was removed from the pool. Mice had to swim for 120 sec, while the time spent in the training quadrant (where the platform had been located) was recorded.

The *reverse Morris water maze* was performed right after the Morris water maze at day five. In this test new learning and memory capacity were investigated. The platform was replaced to the South-East quadrant and acquisition was measured over a period of two days (four trials a day). The probe trial took place on the last day as described before.

2.3 MR measurements

Following behavioural studies, the relative cerebral blood volume was determined by susceptibility induced contrast magnetic resonance imaging, using Ultra Small Particles of Iron Oxide (USPIO) as superparamagnetic blood-pooled contrast agent (AMI-277, Sinerem®, Guerbet Laboratories, France). The USPIO contrast agent provides a valuable tool to characterize tissue vascularity since it remains intravascular for a prolonged period of time and highly enhances the transverse water proton MR relaxation rates. The tail vein was catheterized for administration of the contrast agent. During the MR experiment mice were anaesthetized with 1.9% isoflurane in a mixture of oxygen and N_2O (1:2) trough a nose cone. The body temperature was maintained at 37.7 ± 0.8 °C using a heated water pad and monitored using a rectal fluoroptic temperature probe. Respiratory rate and composition of the gas mixture was monitored continuously during the experiment using an optical respiratory gating apparatus (Sirecust 401, Siemens) and gas analyzer (Datascope, Multinex) respectively. MR measurements were performed on a 7 Tesla magnet (Magnex Scientific, Abingdon, England) interfaced to a S.M.I.S. spectrometer (Surrey Medical Imaging Systems, Surrey, England) operating at 300.20 MHz for ^1H. The magnet had a free bore size of 200 mm and was equipped with a 150mT/m shielded gradient set. After slice positioning, coronal multislice gradient-echo imaging was performed prior to and 1 minute after administration of a bolus injection with USPIO (170 μg Fe/mouse). Imaging parameters were: field of view (FOV) 25 x 25 mm, matrix size 256 x 256, slice thickness 1 mm, echo time (TE) 7 ms, repetition time (TR) 2000 ms and 2 averages per image.

Pixel-by-pixel ΔR2* maps were obtained from the formula: $\Delta R2^* = (1/TE)\log(S_o^{bef}/S_o^{aft})$, where TE is the echo time and S_o the signal amplitude before (S_o^{bef}) and after (S_o^{aft}) USPIO injection. The mean ΔR2* were calculated by drawing a Region of Interest (ROI) on the ΔR2* maps and averaging the values of all pixels within the ROI. The mean ΔR2* is proportional to the rCBV in these regions. Selected ROIs were the hippocampus, cerebral cortex (all cortical areas above the corpus callosum), prelimbic area, diencephalon and the entire brain in a single brain slice (based on the mouse brain atlas of Franklin and Paxinos, 1997).

2.4 Immunohistochemistry

Mice were transcardially perfused with 0.1 phosphate buffered saline (PBS) followed by Somogyi's fixative (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer). After removal, the brains were post fixed for 15 hours in the same fixative and cryoprotected by immersion in 30% sucrose in phosphate buffer at 4°C. Coronal sections of 40 μm thick were sliced using a cryostat (Microm HM 440, Walldorf, Germany).

The Aβ load was immunostained using WO-2 antibody (mouse anti-human $A\beta_{4\text{-}10}$, Beyreuter) and performed by standard free-floating labelling procedures.

First, the sections were pretreated with sodium citrate solution at 85°C. Incubation with a primary antibody 1:20.000, diluted in PBS was performed overnight at room temperature. Following incubation the sections were rinsed thoroughly with PBS and transferred to the solution containing the secondary antibody; donkey-anti-mouse biotin 1:1500 (Jackson Immuno research). After incubation the sections were rinsed and transferred to a solution containing Vector ABC-elite 1:800 (Vector laboratories, Burlingame). Visualization of Aβ plaques was

achieved by incubation with DAB-Ni solution.

Visualisation of the capillaries was done by labelling of the glucose transporter-1 expressed on endothelial cells using the rabbit anti-Glut-1 (AB1340, Chemicon) as primary antibody. The immunostaining procedure was the same as described above, except that the sections were pretreated with perhydrol solution against endogenous peroxidase. Donkey-anti-Rabbit 1:1500 was used as second anti-body (Jackson Immuno research). All stained sections were mounted on gelatine-coated slides and dehydrated in alcohol series, cleared with Xylol and mounted in Entellan.

Quantification of the immunohistochemical staining was done with a computer assisted analysis system (Stereo Investigator) using Cavaleiri's probe. The area covered by Aβ (parenchymal or vascular) and the area covered by Glut-1 stained capillaries (capillary density) was defined as the percentage of the covered area of interest. The area's of interest for both Aβ and GLUT-1 quantification were the prelimbic area, gyrus cinguli (GC) and the CA1, CA3 and dentate gyrus (DG) of the hippocampus. Capillary density was also assessed in the retrospinal cortex (RSP), sensory cortex (SS) and auditory cortex (AUD).

2.5 Statistical analyses

Statistical analyses were conducted using SPSS 14.0 for Windows. A repeated measures ANOVA was used to asses the difference between genotype (wild-type, APP/PS1) or diet (STD, DHA, TWD) at repeated time points. A main affect of genotype or diet was analysed using an ANOVA or independent T-test. *Post hoc* comparisons (Tukey's HSD) revealed the difference between the separate diet groups. Statistical significance was set at $p \leq 0.05$. Data are expressed as mean ± SEM.

3. Results

3.1 APP/PS1 mice show impaired spatial learning and memory in the Morris Water Maze

Acquisition: Spatial learning and memory was evaluated with the MWM test. Both wild type and APP/PS1 animals learned to find the platform during the acquisition phase, which was revealed by a decrease in latency across trials [F(7,385)=23,31; p<0.001]. However, APP/PS1 mice needed significant more time to find the platform than their wild type littermates [F(1,55)=6,2; p=0.016] (Fig. 1). The diet groups did not differ on learning performance. No diet x time or genotype x time interaction effects were found.

Probe: Memory performance was determined by the percentage of time animals spent in the target quadrant (were the platform was placed before). APP/PS1 mice spent significantly less time in the target quadrant compared to wild type animals [F(1,50)=22,45; p<0.001].This difference was present in all diet groups (Table 1). Impaired spatial memory was also reflected by the number of platformcrossings, which was lower for transgenic animals compared with wild type mice [F(1,50)=4,53 p=0.038].

Furthermore, all animals on a DHA diet spent more time in the target quadrant than animals on a standard diet (p=0.051). This dietary effect was most pronounced within the APP/PS1 group (p=0.034). No significant differences between the TWD and standard diet group were found.

Reversal MWM: A learning effect was also visible during the reversal MWM, revealed by a decrease in latency across trials for all groups [F(3,108)=7,53;

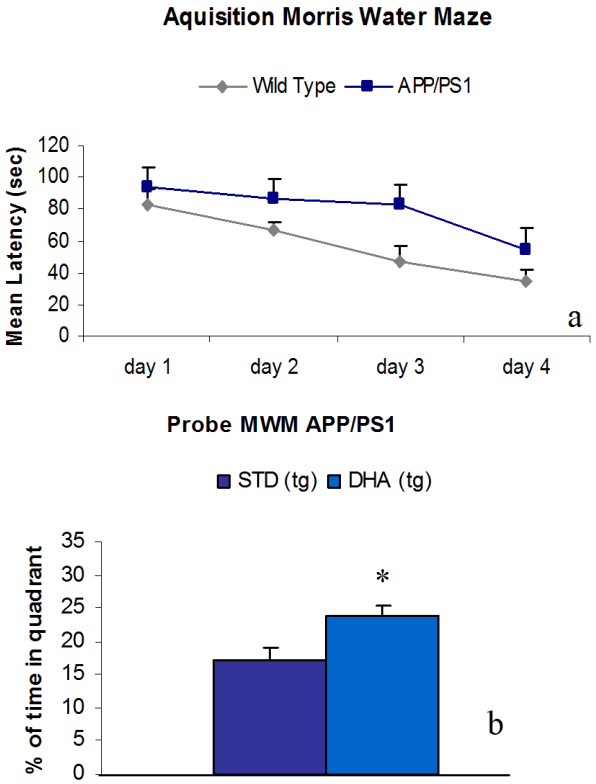

Figure 1. MWM a) Acquisition: Mean latency to find the platform across trials. Both transgenic and wild-type mice show a learning effect, although APP/PS1 mice need on average more time to find the platform. b) Probe test: APP/PS1 mice on a DHA diet spent more time in the target quadrant than transgenic mice on a STD diet.

p<0.001]. This *Reversal MWM:* A learning effect was also visible during the reversal MWM, revealed by a decrease in latency across trials for all groups [F(3,108)=7,53; p<0.001]. This means both APP/PS1 and wild type mice learned to find the 'new' platform. No differences between transgenic and wild types was observed in time needed to find the platform. The reversal test did not reveal any diet or interaction effects.

Tabel 1. Memory performance evaluated by the probe test of the MWM: The mean and SEM of the percentage of time spent in the target quadrant. # Significant mean difference between the DHA and STD diet (p=0.034). * Significant mean difference between wild type vs. APP/PS1 mice.

Diet	Wild Type	APP/PS1	p-value
DHA	35,24 % (2,1)	23,90 % (1,4)#	0.003*
STD	29,06 % (2,4)	17,25 % (1,6)	0.047*
TWD	34,06 % (4,3)	22,20 % (1,7)	0.030*
Total	32,66 % (1,8)	20,86 % (1,1)	<0.001*

3.2 Relative cerebral blood volume is decreased in APP/PS1 mice and in mice on a TWD diet

Differences in rCBV were observed between APP/PS1 and wild type animals on a standard diet (independent T-test). Transgenic animals showed a significant decrease in delta R2* (rCBV) in the cortex [T(1,11); p=0.017](Fig. 2a), but this effect of genotype was not present in the DHA or TWD diet group. In the hippocampus, the mean delta R2* was again lower in APP/PS1 mice compared with the wild type animals in all diet groups, but this difference was not significant [T(1,38)=1,31; p=0.198]. Diet x genotype interaction effects were not present in any of the regions.

Analysis of variance indicated a main effect of diet in the cortex as well [F(2,34)=2,73; p=0.013], were mice on a TWD diet showed a significant decrease in rCBV, compared to mice fed a standard diet [p=0.006](Fig. 2b). Within the transgenic group, cortical rCBV was enhanced for the animals on a DHA diet compared with those on a TWD diet [p=0.068](Fig. 2b). There was no effect of diet present in the hippocampus. Nor were there any differences in rCBV in the diencephalon or prelimbic area.

3.3 No dietary effects in plaques load, but mice on a DHA diet show a decrease in vascular Aβ

3.3.1 Parenchymalβ (plaques)

The amount of parenchymal Aβ was determined as the percentage of the area of interest covered by plaques. The plaque load was significantly higher in the DG compared to other hippocampal regions (CA1, CA3) and the prelimbic area (p<0.001), but not to the GC (Fig. 4). The percentage of Aβ in the prelimbic area was higher than in the CA3 region of the hippocampus (p=0.004). No difference in plaque load was found between the diet groups in any of the measured brain regions.

3.3.2 Vascular Aβ

The amount of vascular Aβ differed significant between the three measured regions (hippocampus vs. GC (p=0.04), hippocampus vs. prelimbic (p<0.001) and GC vs. prelimbic area (p=0.051)). The percentage was highest in the prelimbic area and lowest in the hippocampus (Fig. 5). No difference were found between the diet groups in the hippocampus and GC. However, in the prelimbic area the DHA diet group showed significant less vascular Aβ deposition than the TWD group (p=0.027) but not compared to the STD group (p=0,157).

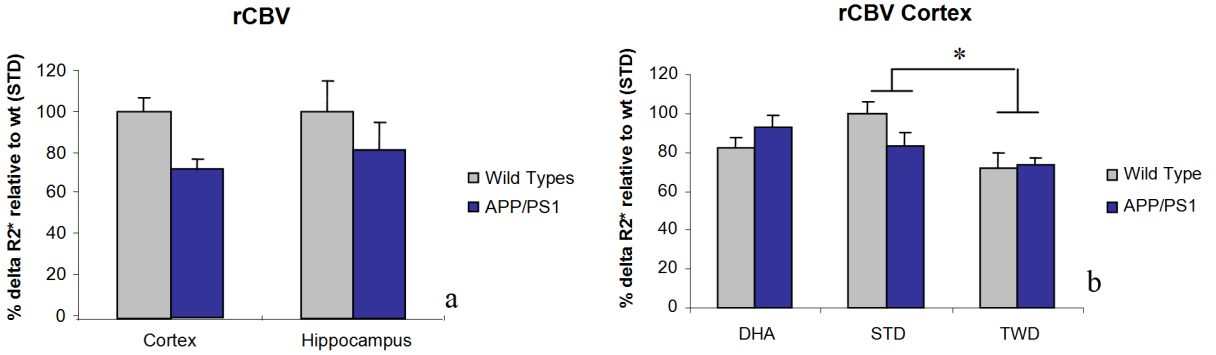

Figure 2. Percentage of delta R2* relative to wild type mice on a standard diet as a measure for rCBV a) between wild-type and APP/PS1 mice in the cortex and hippocampus and b) between different diet groups in the cortex.

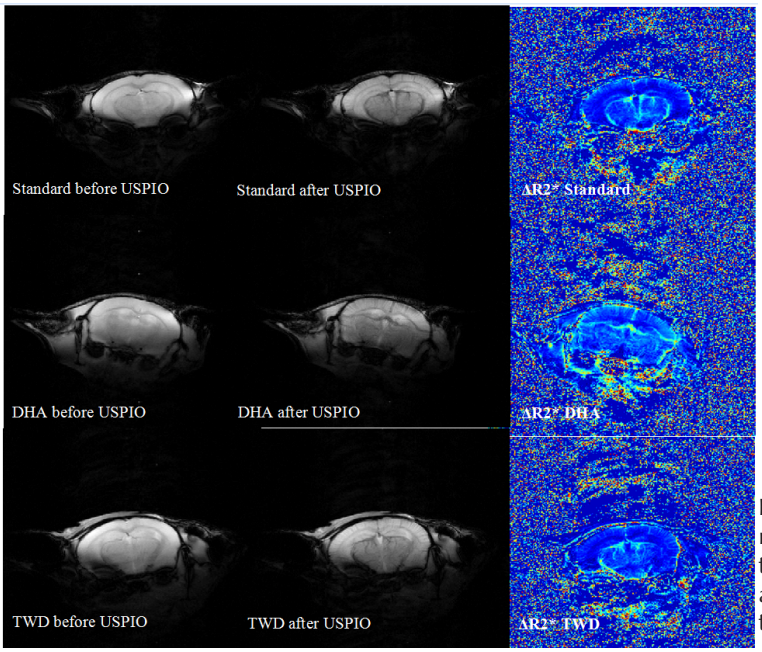

Figure 3. Gradient-echo MR images of the mouse brain, before and after injection with the paramagnetic contrast agent (USPIO) and the corresponding delta R2* maps for the different diet groups.

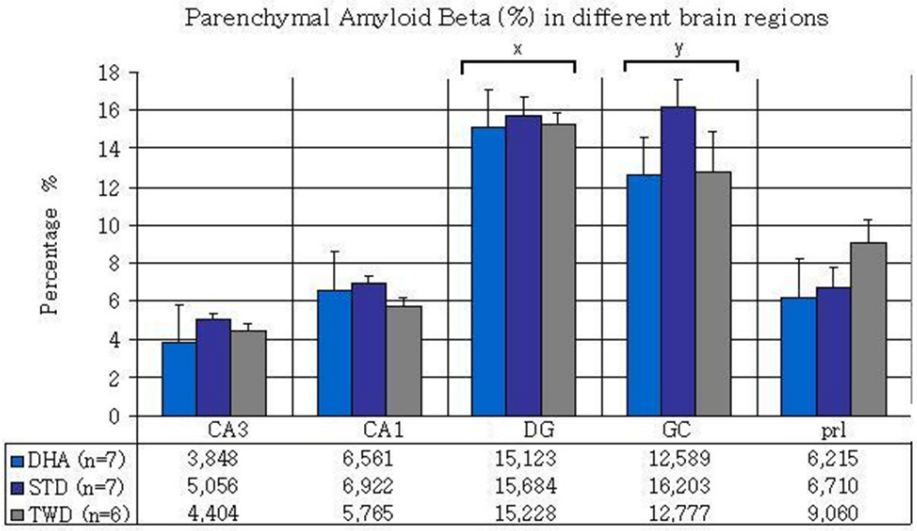

Figure 4. The percentage of parenchymal Aβ in the CA3, CA1 and dentate gyrus (DG) of the hippocampus, and in the gyrus cingulate (GC) and prelimbic area (Prl). Significant difference between the DG (x) and GC (y) compared to the CA3, CA1 and Prl area.

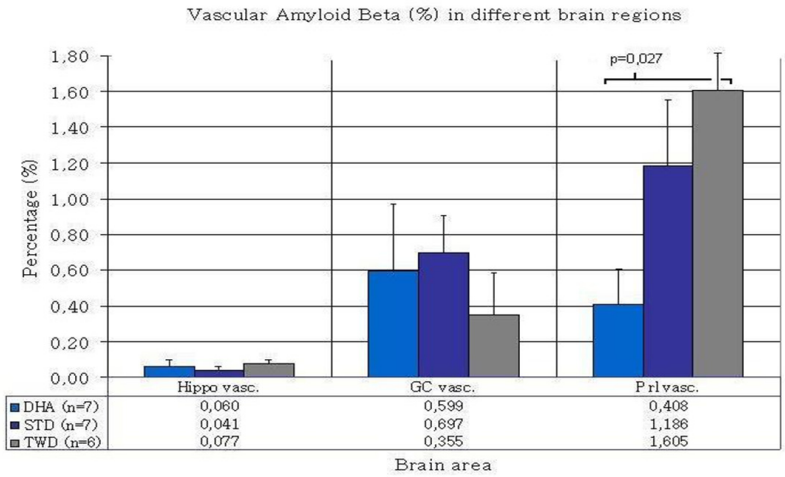

Figure 5. Percentage of vascular Aβ deposition in the hippocampus, gyrus cingulate (GC) and prelimbic area (Prl). Within the prelimbic area the amount of Aβ was significantly higher for the DHA group compared to the TWD diet group.

3.3.3 Minor differences in capillary density

Capillary density was measured as the percentage of the area of interest covered by capillaries. Within the investigated area's (prelimbic, GC, CA1, CA3, DG, RSP, SS and RSP), we found a significant, although not strong, difference in capillary density in the prelimbic area across all diet groups ($F(1,36)=4,03$; $p=0.05$). Except for the SS, APP/PS1 mice showed on average less capillaries per area than wild type mice (Fig. 6a). Because of an interaction effect between genotype and diet within the CA1 region of the hippocampus and the RSP region of the cortex, we examined the difference between transgenic and wild-type mice in those regions within the STD group. This difference became significant in the RSP ($F(1,9)=6,05$; $p=0.036$). We found no diet effects in any of the investigated area's.

3.3.4 Correlation between hippocampal rCBV and memory performance on the MWM

Because memory performance depends heavily on hippocampal function we investigated the relation between hippocampal rCBV and memory performance in the MWM. Indeed, a correlation exists within the STD group ($p=0.05$), but this correlation was not visible in the two other diet groups. The plaques load in the three hippocampal regions (CA1, DG, CA3) did not correlate with hippocampal rCBV.

No correlations were found between the rCBV in the cortex and the plaques load in the GC or memory performance on the MWM.

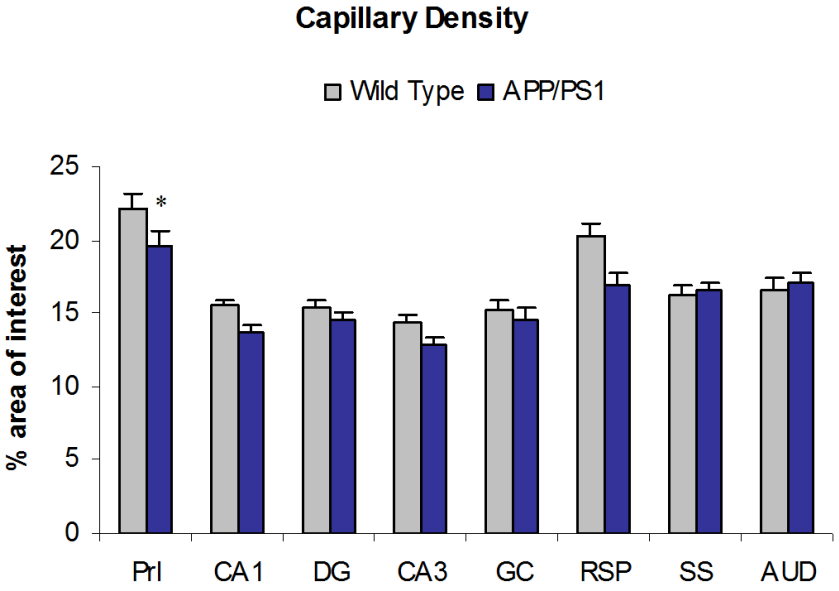

Figure 6. Capillary density as the percentage of the area of interest covered by capillaries. Prl = prelimbic; CA1, DG CA3 = hippocampal regions; GC = gyrus cingulate; RSP = retrospinal cortex; SS = somatosensory cortex; AUD = auditory cortex. Shown are the means over all diet groups, except for the CA1 and RSP which include only the mice on a STD diet.

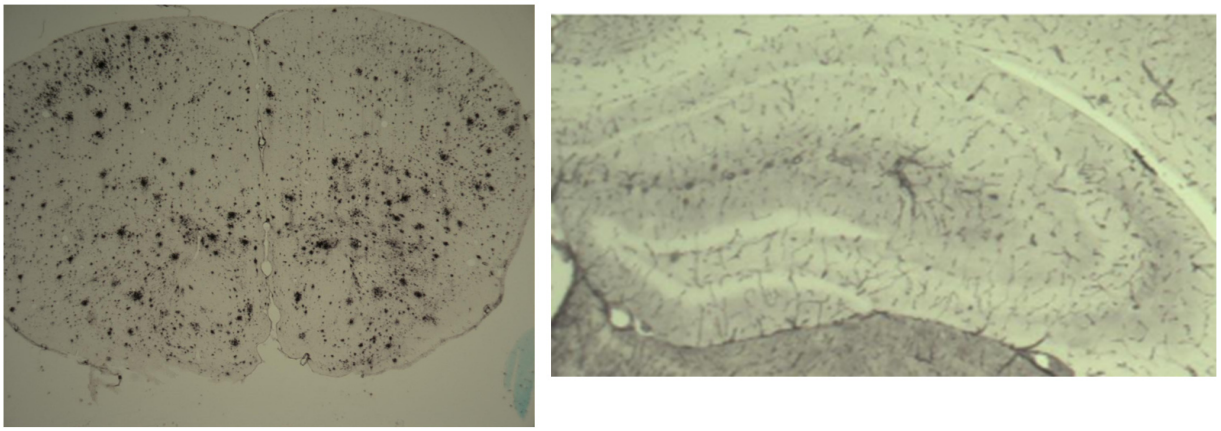

Figure 7. a) Plaque deposition in the prelimbic area of a transgenic APP/PS1 mouse. b) Capillary density in the hippocampus of a wild type mouse on a DHA diet.

4. Discussion

Mice co-expressing human APPswe and PS1dE9 genes have been generated as a model to investigate amyloid pathology associated with AD (Games et al., 1995; Jankowsky et al., 2004). In the present study, we used this mouse model at the age of 15 months to examine the long-term effects of dietary cholesterol and DHA supplementation on spatial learning and memory, haemodynamic parameters and Aβ deposition. Our results demonstrate three main findings. First, cognitive changes in the APP/PS1 mouse model are accompanied by a lowered relative cerebral blood volume (rCBV). Second, a TWD diet decreased rCBV without affecting plaque deposition. Third, transgenic mice on a DHA enriched diet showed a reduction in the amount of vascular Aβ, and improved spatial memory.

In accordance with previous research, APP/PS1 mice exhibit impaired spatial learning and memory compared to wild type littermates (Arendash et al., 2001; Lalonde et al., 2005; Pugh et al., 2007; Puolivali et al., 2002; Savonenko et al., 2005). Even though cognitive changes evident in the human AD population are more heterogeneous than demonstrated in the APP/PS1 mouse model, impaired spatial memory is the most important characteristic of AD (Mega, Cummings, Fiorello, & Gornbein, 1996). Besides Aβ pathology and memory impairment, APP/PS1 mice exhibit AD related changes in haemodynamic parameters. This makes the APP/PS1 a well suited mouse model to investigate Alzheimer pathology.

Several studies have reported haemodynamic alterations in AD patients (Ruitenberg et al., 2005; Schreiber et al., 2005). The present study is one of the first to show differences in cortical rCBV in 15-month-old APP/PS1 mice. In addition, rCBV in the hippocampus, a structure known to be involved in spatial memory, was correlated with memory performance. This is similar to data obtained from patient studies, were a correlation was found between cognitive status and the degree of cerebral hypoperfusion (Farkas et al., 2002).

It is difficult to determine the exact reason for the reduction in rCBV as we were not able to investigate cerebral blood flow. The small blood volume in the mouse brain and the quick recirculation of the blood, makes CBF measurements extremely difficult with contrast based imaging techniques. Hooijmans et al. (2007) recently estimated rCBF in 18-month-old APP/PS1 mice using MR spectroscopy and deuterium oxide (D2O) as freely diffusible tracer. They did not found any differences in rCBF nor rCBV in transgenic mice compared to wild type animals on a standard diet.

A reduction in rCBV in our APP/PS1 mouse model can be explained by enhanced vasoconstriction, possibly mediated by Aβ deposition in the vasculature. Vascular Aβ has previously been suggested to have a vasoactive role through the production of free radicals (Crawford, Suo, Fang, & Mullan, 1998). The effect of Aβ on vasoregulation was strengthened by the observation that addition of Aβ lead to consistent enhancement of contraction in the rat aortae (Thomas, Thomas, McLendon, Sutton, & Mullan, 1996). Enhanced vasoconstriction may impact vascular responses, leading to cerebrovascular dysfunction and encourage neuronal loss and degeneration (Crawford, Soto et al., 1998). Hence, these vasoactive mechanisms could be an important factor in the pathogenesis of AD.

Alternatively, the reduced cortical rCBV in 15-month-old transgenic mice could be a result of degeneration of the microvessels. Indeed, we found a reduction of capillary density in the prelimbic area and the retrospinal cortex of transgenic mice, although this difference was not observed in other cortical area's. Kouznetsova et al. (2006) demonstrated a reduction in capillary density in the cortical regions of APP mice compared to wild types present from the age of 15 months, with a significant difference at the age of 18 months. The question is whether the amyloid pathology contributes to degeneration of microvessels or cerebrovascular dysfunction. Some studies reported a congruency between regions with high plaque load and low density of microvessels (Kouznetsova et al., 2006; Paris et al., 2004). In contrast, we did not find any correlation between capillary density and plaque deposition in transgenic mice. Similarly, there was no relation observed between amyloid plaques and capillary degeneration in brain tissue obtained from AD patients (Kawai, Kalaria, Harik, & Perry, 1990). On the other hand, animal models have shown that chronically reduced CBF can trigger the degeneration of the capillaries in the brain (Farkas & Luiten, 2001), indicating a possible link between hypoperfusion and a lowered capillary density in AD. The previous discussed vascular mechanisms are closely related and interact, inferring that most likely changes in CBF, vasoconstriction and capillary density all contributed to the lowered rCBV observed in this experiment.

In the present study, impaired cognitive performance in APP/PS1 mice is accompanied

with reduced cortical and hippocampal rCBV. No correlation was observed between plaque load and cognitive performance, suggesting that other factors such as cerebrovascular factors play an important role in the development of AD. To clarify the relation between CBV, Aβ pathology and cognition, we examined the effects of long term dietary intake of cholesterol and DHA on these different parameters.

In line with human population studies suggesting a negative influence of cholesterol on our vasculature, dietary cholesterol supplementation resulted in a decreased rCBV in all mice. A compromised vasculature may account for the lowered rCBV, since the TWD diet did not effect capillary density. Long term cholesterol intake can lead to atherosclerosis, which is associated with hypoperfusion, lowered energy metabolism, dementia and Aβ pathology (Farkas & Luiten, 2001; Honig, Kukull, & Mayeux, 2005). Besides a profound affect on the vasculature, high intracellular brain cholesterol levels are thought to modulate the production of APP and Aβ by decreasing the fluidity of the cell membrane (Martins et al., 2006). However, it is unclear how cholesterol can effect the Aβ production by altering the membrane function as it is not able to cross the blood-brain barrier. The same holds for certain cholesterol lowering statins, which are known to reduce the risk for AD, but cannot cross the blood-brain barrier either (Gibson Wood et al., 2003). Studies in mice expressing APP, show a strong connection between plasma cholesterol levels and Aβ generation, but brain cholesterol levels seem unaffected (Gibson Wood et al., 2003; Refolo et al., 2000), suggesting that cholesterol may not directly effect Aβ production by elevating cellular brain cholesterol levels.

Our cholesterol enriched diet did not induce increased Aβ deposition at the age of 15-months. A parallel study from Oksman et al. (2006), using the same TWD diet, also remained to find any change in plaques load in 9-month-old APP/PS1 mice (Oksman et al., 2006). However, previous findings have shown that a TWD diet did lead to an increase in plaque load, together with reduced rCBV in APP/PS1 mice at the age of 19-months (Hooijmans, 2007). Taking these results together, a lowered rCBV is observed before changes in Aβ deposition become apparent. This supports the *cerebral hypoperfusion hypothesis* indicating that decreased rCBV induced by dietary cholesterol intake, is a cause rather than an effect of increased plaques formation.

We were not able to demonstrate the positive effects of DHA dietary intake on cerebral perfusion, compared to a standard diet in our 15-month-old mouse model. Nevertheless, transgenic mice on a DHA diet did show an elevated rCBV compared to the cholesterol enriched diet. This was again not related to a change in capillary density, however, it was accompanied by a decrease in vascular Aβ deposition which may indicate improved vascular conditions in APP/PS1 mice on a DHA enriched diet. Although these changes were moderate and not yet significant, our previous study looking at 19-month-old APP/PS1 mice, do show that the effect of dietary DHA intake on vascular Aβ levels become more pronounced with increasing age and even resulted in an increased rCBV compared with mice on a standard diet (Hooijmans, 2007). Excessive Aβ deposition in the cerebrovasculature, leading to cerebral amyloid angiopathy, can result in hypoperfusion and stroke in AD patients and may contribute to the development and severity of dementia (Rensink, de Waal, Kremer, & Verbeek, 2003). Dietary DHA could be protective against this mechanism by modulating amyloid production and potential downstream toxicity (Cole & Frautschy, 2006; Lim et al., 2005) leading to improved cerbrovascular conditions.

Interestingly, we did observe improved memory performance in transgenic animals on a DHA diet. Previous studies have demonstrated DHA-induced memory improvement in rats (Gamoh et al., 1999; Hashimoto et al., 2006) and patients (Fontani et al., 2005; Freund-Levi et al., 2006), but the mechanisms underlying this beneficial effect are still unknown. Supplementation of omega-3 fatty acids has shown to enhance nervous system activity in rodents by reducing oxidative stress (Cole & Frautschy, 2006; de Wilde et al., 2002; Hossain, Hashimoto, Gamoh, & Masumura, 1999) and improving the condition of cerebrocortical capillary walls (de Wilde et al., 2002; Farkas et al., 2002). At a synaptic level, a major player in memory, CaM kinase II alpha, was markedly reduced by DHA depletion in APPswe mouse (Calon et al., 2005; Cole & Frautschy, 2006). Moreover, DHA promotes neuronal survival by Akt signalling and dietary depletion increases neuronal susceptibility to apoptosis (Akbar, Calderon, Wen, & Kim, 2005; Calon et al., 2004).

These DHA mediated mechanisms leading to improved neuronal activity, neurovascular conditions and increased brain perfusion, may enhance the suppressive effect of DHA on vascular Aβ toxicity. Together this could explain why dietary DHA intake can protect against impaired memory performance in AD.

In summary, we observed spatial learning and memory impairment in the APP/PS1 mouse model accompanied by a decrease in rCBV. Furthermore, dietary cholesterol supplementation seemed to induce a decreased rCBV before affecting plaque deposition. These data provide direct evidence for the *hypoperfusion hypothesis*, suggesting that changes in haemodynamics are a primary trigger in the development of AD. In addition, a DHA enriched diet did not only improve the vascular condition in transgenic mice by slightly increasing the rCBV and reducing the amount of Aβ in the vessels, but improved spatial memory as well. A DHA containing diet may protect against neurovascular dysfunction and thereby the development of AD.

These findings have significant implications for public health as impaired vascular conditions can be improved by lifestyle changes. Adopting a healthy lifestyle with low cholesterol and high fish oil intake will likely improve the prognosis for people already suffering, and also reduce the risk of developing Alzheimer's disease.

References

Akbar, M., Calderon, F., Wen, Z., & Kim, H. Y. (2005). Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. *Proc Natl Acad Sci U S A, 102*(31), 10858-10863.

Arendash, G. W., King, D. L., Gordon, M. N., Morgan, D., Hatcher, J. M., Hope, C. E., et al. (2001). Progressive, age-related behavioral impairments in transgenic mice carrying both mutant amyloid precursor protein and presenilin-1 transgenes. *Brain Res, 891*(1-2), 42-53.

Barberger-Gateau, P., Letenneur, L., Deschamps, V., Peres, K., Dartigues, J. F., & Renaud, S. (2002). Fish, meat, and risk of dementia: cohort study. *Bmj, 325*(7370), 932-933.

Biessels, G. J., & Kappelle, L. J. (2005). Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? *Biochem Soc Trans, 33*(Pt 5), 1041-1044.

Calon, F., Lim, G. P., Morihara, T., Yang, F., Ubeda, O., Salem, N., Jr., et al. (2005). Dietary n-3 polyunsaturated fatty acid depletion activates caspases and decreases NMDA receptors in the brain of a transgenic mouse model of Alzheimer's disease. *Eur J Neurosci, 22*(3), 617-626.

Calon, F., Lim, G. P., Yang, F., Morihara, T., Teter, B., Ubeda, O., et al. (2004). Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. *Neuron, 43*(5), 633-645.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., et al. (1992). Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature, 360*(6405), 672-674.

Cole, G. M., & Frautschy, S. A. (2006). Docosahexaenoic acid protects from amyloid and dendritic pathology in an Alzheimer's disease mouse model. *Nutr Health, 18*(3), 249-259.

Conquer, J. A., Tierney, M. C., Zecevic, J., Bettger, W. J., & Fisher, R. H. (2000). Fatty acid analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment. *Lipids, 35*(12), 1305-1312.

Crawford, F., Soto, C., Suo, Z., Fang, C., Parker, T., Sawar, A., et al. (1998). Alzheimer's beta-amyloid vasoactivity: identification of a novel beta-amyloid conformational intermediate. *FEBS Lett, 436*(3), 445-448.

Crawford, F., Suo, Z., Fang, C., & Mullan, M. (1998). Characteristics of the in vitro vasoactivity of beta-amyloid peptides. *Exp Neurol, 150*(1), 159-168.

Criado, M., Eibl, H., & Barrantes, F. J. (1982). Effects of lipids on acetylcholine receptor. Essential need of cholesterol for maintenance of agonist-induced state transitions in lipid vesicles. *Biochemistry, 21*(15), 3622-3629.

de la Torre, J. C. (2000). Impaired cerebromicrovascular perfusion. Summary of evidence in support of its causality in Alzheimer's disease. *Ann N Y Acad Sci, 924*, 136-152.

de la Torre, J. C. (2004). Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. *Lancet Neurol, 3*(3), 184-190.

de la Torre, J. C. (2006). How do heart disease and stroke become risk factors for Alzheimer's disease? *Neurol Res, 28*(6), 637-644.

de Wilde, M. C., Farkas, E., Gerrits, M., Kiliaan, A. J., & Luiten, P. G. (2002). The effect of n-3 polyunsaturated fatty acid-rich diets on cognitive and cerebrovascular parameters in chronic cerebral hypoperfusion. *Brain Res, 947*(2), 166-173.

Farkas, E., de Wilde, M. C., Kiliaan, A. J., & Luiten, P. G. (2002). Systemic effects of dietary n-3 PUFA supplementation accompany changes of CNS parameters in cerebral hypoperfusion. *Ann N Y Acad Sci, 977*, 77-86.

Farkas, E., & Luiten, P. G. (2001). Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol, 64*(6), 575-611.

Fontani, G., Corradeschi, F., Felici, A., Alfatti, F., Migliorini, S., & Lodi, L. (2005). Cognitive and physiological effects of Omega-3 polyunsaturated fatty acid supplementation in healthy subjects. *Eur J Clin Invest, 35*(11), 691-699.

Freund-Levi, Y., Eriksdotter-Jonhagen, M., Cederholm, T., Basun, H., Faxen-Irving, G., Garlind, A., et al. (2006). Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. *Arch Neurol, 63*(10), 1402-1408.

Games, D., Adams, D., Alessandrini, R., Barbour, R.,

Berthelette, P., Blackwell, C., et al. (1995). Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature, 373*(6514), 523-527.

Gamoh, S., Hashimoto, M., Sugioka, K., Shahdat Hossain, M., Hata, N., Misawa, Y., et al. (1999). Chronic administration of docosahexaenoic acid improves reference memory-related learning ability in young rats. *Neuroscience, 93*(1), 237-241.

Gibson Wood, W., Eckert, G. P., Igbavboa, U., & Muller, W. E. (2003). Amyloid beta-protein interactions with membranes and cholesterol: causes or casualties of Alzheimer's disease. *Biochim Biophys Acta, 1610*(2), 281-290.

Hashimoto, M., Hossain, S., Shimada, T., & Shido, O. (2006). Docosahexaenoic acid-induced protective effect against impaired learning in amyloid beta-infused rats is associated with increased synaptosomal membrane fluidity. *Clin Exp Pharmacol Physiol, 33*(10), 934-939.

Heijer, T., Skoog, I., Oudkerk, M., de Leeuw, F. E., de Groot, J. C., Hofman, A., et al. (2003). Association between blood pressure levels over time and brain atrophy in the elderly. *Neurobiol Aging, 24*(2), 307-313.

Heude, B., Ducimetiere, P., & Berr, C. (2003). Cognitive decline and fatty acid composition of erythrocyte membranes--The EVA Study. *Am J Clin Nutr, 77*(4), 803-808.

Honig, L. S., Kukull, W., & Mayeux, R. (2005). Atherosclerosis and AD: analysis of data from the US National Alzheimer's Coordinating Center. *Neurology, 64*(3), 494-500.

Hooijmans, C. (2007). Changes in cerebral blood volume and amyloid pathology in aged Alzheimer APP/PS1 mice on a docosahexaenoic acid (DHA) diet or cholesterol enriched typical western diet. *Journal for Neurobiology of Disease*.

Hossain, M. S., Hashimoto, M., Gamoh, S., & Masumura, S. (1999). Antioxidative effects of docosahexaenoic acid in the cerebrum versus cerebellum and brainstem of aged hypercholesterolemic rats. *J Neurochem, 72*(3), 1133-1138.

Jankowsky, J. L., Slunt, H. H., Gonzales, V., Jenkins, N. A., Copeland, N. G., & Borchelt, D. R. (2004). APP processing and amyloid deposition in mice haplo-insufficient for presenilin 1. *Neurobiol Aging, 25*(7), 885-892.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., et al. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature, 325*(6106), 733-736.

Kawai, M., Kalaria, R. N., Harik, S. I., & Perry, G. (1990). The relationship of amyloid plaques to cerebral capillaries in Alzheimer's disease. *Am J Pathol, 137*(6), 1435-1446.

Kivipelto, M., Helkala, E. L., Laakso, M. P., Hanninen, T., Hallikainen, M., Alhainen, K., et al. (2002). Apolipoprotein E epsilon4 allele, elevated midlife total cholesterol level, and high midlife systolic blood pressure are independent risk factors for late-life Alzheimer disease. *Ann Intern Med, 137*(3), 149-155.

Kivipelto, M., Ngandu, T., Fratiglioni, L., Viitanen, M., Kareholt, I., Winblad, B., et al. (2005). Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. *Arch Neurol, 62*(10), 1556-1560.

Kouznetsova, E., Klingner, M., Sorger, D., Sabri, O., Grossmann, U., Steinbach, J., et al. (2006). Developmental and amyloid plaque-related changes in cerebral cortical capillaries in transgenic Tg2576 Alzheimer mice. *Int J Dev Neurosci, 24*(2-3), 187-193.

Kwok, J. B., Taddei, K., Hallupp, M., Fisher, C., Brooks, W. S., Broe, G. A., et al. (1997). Two novel (M233T and R278T) presenilin-1 mutations in early-onset Alzheimer's disease pedigrees and preliminary evidence for association of presenilin-1 mutations with a novel phenotype. *Neuroreport, 8*(6), 1537-1542.

Lalonde, R., Kim, H. D., Maxwell, J. A., & Fukuchi, K. (2005). Exploratory activity and spatial learning in 12-month-old APP(695)SWE/co+PS1/DeltaE9 mice with amyloid plaques. *Neurosci Lett, 390*(2), 87-92.

Lim, G. P., Calon, F., Morihara, T., Yang, F., Teter, B., Ubeda, O., et al. (2005). A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. *J Neurosci, 25*(12), 3032-3040.

Martins, I. J., Hone, E., Foster, J. K., Sunram-Lea, S. I., Gnjec, A., Fuller, S. J., et al. (2006). Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. *Mol Psychiatry, 11*(8), 721-736.

Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., & Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A, 82*(12), 4245-4249.

Mega, M. S., Cummings, J. L., Fiorello, T., & Gornbein, J. (1996). The spectrum of behavioral changes in Alzheimer's disease. *Neurology, 46*(1), 130-135.

Mitchell, D. C., Straume, M., Miller, J. L., & Litman, B. J. (1990). Modulation of metarhodopsin formation by cholesterol-induced ordering of bilayer lipids. *Biochemistry, 29*(39), 9143-9149.

Oksman, M., Iivonen, H., Hogyes, E., Amtul, Z., Penke, B., Leenders, I., et al. (2006). Impact of different saturated fatty acid, polyunsaturated fatty acid and cholesterol containing diets on beta-amyloid accumulation in APP/PS1 transgenic mice. *Neurobiol Dis, 23*(3), 563-572.

Paris, D., Townsend, K., Quadros, A., Humphrey, J., Sun, J., Brem, S., et al. (2004). Inhibition of angiogenesis by Abeta peptides. *Angiogenesis, 7*(1), 75-85.

Poirier, J. (2003). Apolipoprotein E and cholesterol metabolism in the pathogenesis and treatment of Alzheimer's disease. *Trends Mol Med, 9*(3), 94-101.

Prins, N. D., van Dijk, E. J., den Heijer, T., Vermeer, S. E.,

Jolles, J., Koudstaal, P. J., et al. (2005). Cerebral small-vessel disease and decline in information processing speed, executive function and memory. *Brain, 128*(Pt 9), 2034-2041.

Pugh, P. L., Richardson, J. C., Bate, S. T., Upton, N., & Sunter, D. (2007). Non-cognitive behaviours in an APP/PS1 transgenic model of Alzheimer's disease. *Behav Brain Res, 178*(1), 18-28.

Puolivali, J., Wang, J., Heikkinen, T., Heikkila, M., Tapiola, T., van Groen, T., et al. (2002). Hippocampal A beta 42 levels correlate with spatial memory deficit in APP and PS1 double transgenic mice. *Neurobiol Dis, 9*(3), 339-347.

Refolo, L. M., Malester, B., LaFrancois, J., Bryant-Thomas, T., Wang, R., Tint, G. S., et al. (2000). Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. *Neurobiol Dis, 7*(4), 321-331.

Rensink, A. A., de Waal, R. M., Kremer, B., & Verbeek, M. M. (2003). Pathogenesis of cerebral amyloid angiopathy. *Brain Res Brain Res Rev, 43*(2), 207-223.

Roher, A. E., Garami, Z., Alexandrov, A. V., Kokjohn, T. A., Esh, C. L., Kalback, W. M., et al. (2006). Interaction of cardiovascular disease and neurodegeneration: transcranial Doppler ultrasonography and Alzheimer's disease. *Neurol Res, 28*(6), 672-678.

Ruitenberg, A., den Heijer, T., Bakker, S. L., van Swieten, J. C., Koudstaal, P. J., Hofman, A., et al. (2005). Cerebral hypoperfusion and clinical onset of dementia: the Rotterdam Study. *Ann Neurol, 57*(6), 789-794.

Savonenko, A., Xu, G. M., Melnikova, T., Morton, J. L., Gonzales, V., Wong, M. P., et al. (2005). Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitter abnormalities. *Neurobiol Dis, 18*(3), 602-617.

Schreiber, S. J., Doepp, F., Spruth, E., Kopp, U. A., & Valdueza, J. M. (2005). Ultrasonographic measurement of cerebral blood flow, cerebral circulation time and cerebral blood volume in vascular and Alzheimer's dementia. *J Neurol, 252*(10), 1171-1177.

Selkoe, D. J. (1991). The molecular pathology of Alzheimer's disease. *Neuron, 6*(4), 487-498.

Sinha, A. K., Shattil, S. J., & Colman, R. W. (1977). Cyclic AMP metabolism in cholesterol-rich platelets. *J Biol Chem, 252*(10), 3310-3314.

Spires, T. L., & Hyman, B. T. (2005). Transgenic models of Alzheimer's disease: learning from animals. *NeuroRx, 2*(3), 423-437.

Thomas, T., Thomas, G., McLendon, C., Sutton, T., & Mullan, M. (1996). beta-Amyloid-mediated vasoactivity and vascular endothelial damage. *Nature, 380*(6570), 168-171.

Tully, A. M., Roche, H. M., Doyle, R., Fallon, C., Bruce, I., Lawlor, B., et al. (2003). Low serum cholesteryl ester-docosahexaenoic acid levels in Alzheimer's disease: a case-control study. *Br J Nutr, 89*(4), 483-489.

Van Broeckhoven, C., Backhovens, H., Cruts, M., De Winter, G., Bruyland, M., Cras, P., et al. (1992). Mapping of a gene predisposing to early-onset Alzheimer's disease to chromosome 14q24.3. *Nat Genet, 2*(4), 335-339.

van Groen, T., Puurunen, K., Maki, H. M., Sivenius, J., & Jolkkonen, J. (2005). Transformation of diffuse beta-amyloid precursor protein and beta-amyloid deposits to plaques in the thalamus after transient occlusion of the middle cerebral artery in rats. *Stroke, 36*(7), 1551-1556.

van Oijen, M., Jan de Jong, F., Witteman, J. C., Hofman, A., Koudstaal, P. J., & Breteler, M. M. (2007). Atherosclerosis and risk for dementia. *Ann Neurol.*

Velliquette, R. A., O'Connor, T., & Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *J Neurosci, 25*(47), 10874-10883.

Wolozin, B. (2001). A fluid connection: cholesterol and Abeta. *Proc Natl Acad Sci U S A, 98*(10), 5371-5373.

Wolozin, B. (2004). Cholesterol and the biology of Alzheimer's disease. *Neuron, 41*(1), 7-10.

Zlokovic, B. V. (2005). Neurovascular mechanisms of Alzheimer's neurodegeneration. *Trends Neurosci, 28*(4), 202-208.