Impact of nutrition on brain structure and function

A magnetic resonance imaging approach in

Alzheimer mouse models

Valerio Zerbi
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**General introduction**

Alzheimer’s disease (AD) is named after the German psychiatrist and neuropathologist Alois Alzheimer, who described his first case, a 55 years old female patient (i.e. Auguste Deter), in 1906. The pathology of Auguste was characterized by deteriorated memory, speaking, physical, and social abilities. After her decease, the autopsy revealed brain atrophy, atherosclerosis in larger cerebral vessels, neuronal loss and numerous small foci distributed over the entire cortex, which were perceivable even without staining. It took over 70 years to reveal that those foci consist of aggregates of extracellular loads of small peptides called amyloid-β (Aβ), that are considered today one of the hallmarks of the disease (figure 1) (Masters, et al., 1985). Over more than a century, AD transformed from what was thought to be a rare, sporadic, and non-inherited disease into one of the major socioeconomic problems of our time, and the most common form of dementia in elderly (Wimo, et al., 2006). According to the World Alzheimer Report, in 2010 approximately 35.6 million people worldwide suffered from AD. Mainly due to the increased aging of the population, this number is expected to increase to over 100 million people by 2050, corresponding to 1 in 85 persons (Clegg, et al., 2001, Zheng and Koo, 2006). To date, no treatments are available to prevent the progression of AD. Several different pharmacological agents only ameliorate or provide temporary alleviation of the symptoms (Selkoe and Schenk, 2003).

**Clinical aspects and etiology of the Alzheimer’s disease**

AD affects a person’s ability to carry out daily activities and is characterized by gradual memory loss, anomia (i.e. difficulties with word finding), apraxia (i.e. difficulties with complex movements), confusion, and general withdrawal. As the disease progresses, severe cognitive impairment, impaired executive functions and personality changes worsen the lifestyle condition. Patients in more advanced stages become very susceptible to infections, pneumonia, and decubitus ulcers. Life expectancy after a diagnosis of AD is usually between 7 and 10 years (Helzner, et al., 2008, Xie, et al., 2008). AD can be divided into two major forms termed early onset or presenile AD, and late onset or sporadic AD. Patients are classified as early onset AD when the first symptoms of AD occur at an age earlier than 65 (see: [http://www.alz.org/downloads/facts_figures_2012.pdf](http://www.alz.org/downloads/facts_figures_2012.pdf)). Early onset AD accounts for 5% of all AD cases worldwide and is caused by
Figure 1. The Aβ is generated by sequential cleavage of the transmembrane amyloid precursor protein (APP), via groups of enzymes named α-, β- and γ-secretases (Allinson, et al., 2003, Van Dam and De Deyn, 2006). When the APP protein is cleaved by the α-secretase, a soluble amino (N)-terminal ectodomain (sAPPα) and a C-terminal fragment (CTF) are released. Thereby the formation of Aβ is prohibited, because the cleaving occurs inside the Aβ region (Wisniewski T, 2010). This is known as the “non-amyloidogenic” pathway. The “amyloidogenic” pathway generates Aβ when the precursor protein is cleaved by a β-secretase (BACE: β-site APP cleaving enzyme) at the N-terminal domain, releasing a soluble N-terminal fragment (sAPPβ) and the remaining C-terminal part (b-CTF). The CTF and b-CTF are then cleaved in the transmembrane domain by the γ-secretase to release either extracellular p2 or Aβ respectively. Cleavage by γ-secretase takes place at position 40 or 42 of the protein, which generates amyloid-β 40 (Aβ40) or amyloid-β 42 (Aβ42), respectively. Approximately 90% of the residues consist of Aβ40. However, the Aβ42 aggregates more readily. For this reason, Aβ42 is the most abundant isoform in amyloid plaques.

genetic mutations, as the persons affected generally have a positive family history (Selkoe and Schenk, 2003). Several mutations in the transmembrane amyloid precursor protein (APP) on chromosome 21, in presenilin 1 (PS1) on chromosome 12 and presenilin 2 (PS2) on chromosome 1 have shown to be autosomal-dominant inheritable (Rademakers, et al., 2003). Mutations in PS1
and PS2 lead to an increased production of the strong self-aggregating Aβ_{42} peptide by elevating the γ-secretase activity. This causes the most aggressive form of AD with an early onset that can occur even earlier than age of 40. However, mutations in APP, PS1 and PS2 have been found to cause less than 30% of the early onset AD. So far little is known about the other genetic disorders that may be responsible for the remaining cases of early onset (Tanzi and Bertram, 2001).

Patients with late onset AD account for the remaining 95% of AD cases. The exact causes of late onset AD are still elusive. Despite that aging is known as the most important risk factor for AD (Mayeux, 2003), a large number of risk factors for the development of AD has been identified; these include smoking, dietary fat intake, stroke, hypertension, heart disease, hypercholesterolemia, and diabetes mellitus (table 1) (Kivipelto, et al., 2001, Van Duijn, et al., 1994). Interestingly, most of these factors are linked to pathologies of the vascular system. The only gene that has currently been identified as a risk factor for the late onset AD is the ε4 allele of the cholesterol transport protein, apolipoprotein E (apoE) (Corder, et al., 1993, Strittmatter, et al., 1993). ApoE is a 34-kDa cholesterol transport glycoprotein that exists in three isoforms: apoE-ε2, -ε3 and -ε4, which differ between each other by one or two amino acid residues 112 and 158 (Zannis, et al., 1982). This difference strongly affects the conformation and the structure of apoE and influences its ability to bind lipids, receptors and amyloid-β (Aβ) (Frieden and Garai, 2012).

<table>
<thead>
<tr>
<th>Alzheimer’s disease risk factors</th>
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<td>Ageing</td>
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<td>APOE ε4 genotype</td>
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<td>Alcoholism</td>
<td>Overweight</td>
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<td>White matter lesions</td>
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*Table 1. Risk factors for Alzheimer’s disease*
The highest expression of apoE is found in the liver, followed by the brain where it is mainly expressed by astrocytes and to a certain extent by microglia (Grehan, et al., 2001, Pitas RE, 1987). Neurons are also capable of producing apoE under certain conditions, but much less than astrocytes (Xu, et al., 1999, Xu, et al., 2006). Extensive epidemiological studies demonstrated that the inheritance of one apoE-ε4 allele is associated with an increased risk of developing AD by 2-5 fold and the inheritance of both ε4 alleles elevates this risk by 4-10 fold compared to expression of the most common isoform ε3 (Mahley and Rall, 2000). The ε4 allele is also associated with earlier onset and with an increased conversion from mild cognitive impairment (MCI) to AD (Blacker, et al., 1997, Tierney, et al., 1996). The inheritance of the ε2 allele, on the contrary, appears to protect against AD pathogenesis (Corder, et al., 1994). Furthermore, apoE-ε4 carriers show significantly higher levels of Aβ, even in the absence of neurological symptoms. The apoE-ε4 isoform is also strongly associated with the presence of Aβ in the vascular walls and the occurrence of cerebral amyloid angiopathy (CAA) (Kinnekom, et al., 2007). The mechanisms by which the apoE-ε4 promotes the disease have been recently reviewed and classified as both gain of toxic functions and loss of neuronal protection (Liu, et al., 2013).

**Amyloid cascade hypothesis**
To date, many theories about the causes of AD have been postulated; among them, the “amyloid cascade hypothesis” and the “vascular hypothesis” are the most widely accepted. As described above, genetic risk factors for AD include mutations in genes expressing APP, PS1, PS2, and ApoE4. Most of these mutations result in an elevated Aβ peptide production or failure of Aβ clearance mechanisms, leading to an abnormal accumulation of Aβ in the brain. Furthermore, patients with Down syndrome develop AD early in life and show overproduction of Aβ and plaque deposition in the brain before other AD lesions (Lemere, et al., 1996). Based on these observations, the accumulation of Aβ in the brain was more than 20 years ago described by John Hardy and David Allsop as the key event in the pathogenesis of AD (Hardy and Allsop, 1991). The so called “amyloid cascade hypothesis” suggested that the accumulation and aggregation of Aβ, specifically Aβ42, would trigger further pathological events that causes AD. However, later studies revealed that the amount of fibrillar Aβ deposits in AD brain poorly correlated with the severity of cognitive impairment (Dickson, et al., 1995, Terry, et al., 1991).
Figure 2. Revision of the amyloid hypothesis. Amyloid-β (Aβ) monomers are formed by β- and γ-secretase cleavage of the amyloid precursor protein (APP). These monomers accumulate in the extracellular space and trigger several processes associated with AD pathophysiology. Aβ monomers can initiate the formation of fibrillar Aβ aggregates, possibly by an initial aggregation in the form of Aβ dimers, or non-fibrillar Aβ aggregates, by an initial aggregation in the form of Aβ trimers. Accumulation of Aβ also results in an activation of microglial cell, which increase the production of cytokines that can damage neuronal health when expressed in high concentrations. Furthermore, Aβ can aggregate in the cerebral blood vessels causing cerebral amyloid angiopathy (CAA), impairing neurovascular function and decreasing oxygen and nutrients supply to brain cells.

Instead, non-fibrillar oligomeric Aβ species were found to be highly neurotoxic and their levels much better correlated with severity of disease and synaptic loss (Lue, et al., 1999, McLean, et al., 1999). These findings formed the basis for a revision of the amyloid hypothesis by Hardy and Selkoe, pointing towards the oligomeric Aβ species as the initiator culprits of AD rather than Aβ plaques (Hardy and Selkoe, 2002) (see figure 2). Reports confirmed that soluble Aβ oligomers inhibit neuronal function (Walsh and Selkoe, 2007), induce dendritic spine loss (tShankar, et al., 2007), and alter neuronal plasticity (Li, et al., 2009). A recent study from Selkoe and colleagues reported that Aβ dimers could trigger tau hyperphosphorylation and tau-dependent cytoskeletal microtubule abnormalities in primary neurons (Jin, et al., 2011). To date, the revisited amyloid cascade hypothesis is still holding up fairly well. Upcoming methodologies for the
isolation and identification of proprieties for specific Aβ oligomers (dimers, trimers and high-molecular weight oligomers) will possibly bring the amyloid cascade hypothesis at another level of complexity in the near future (Larson and Lesne, 2012).

Vascular hypothesis
Although the amyloid hypothesis provides a good explanation of how and why amyloid species are formed in the brain, several flaws remain to explain the development of AD. Most importantly, there is still no solid prove supporting the neurotoxic properties of insoluble Aβ in vivo. Secondly, there is not a general agreement that Aβ plaques are the cause of neural impairment, rather than the result of sick neurons (de la Torre, 2004). In addition, amyloid deposition does not correlate well with neuron loss (Schmitz, et al., 2004) and presence of Aβ plaques is also seen in non-demented subjects (Arriagada, et al., 1992,Davis, et al., 1999). Finally, different treatment approaches that target amyloid clearance or plaque formation have not yet provided any significant therapeutic improvement (Kurz and Perneczky, 2011).

The first description of a possible vascular, instead of an amyloid cause of AD, was reported in 1993 (de la Torre and Mussivand, 1993). Since then, increasing evidence from epidemiological, pharmacological and neuroimaging studies, has suggested that vascular disorders with chronic cerebral hypoperfusion might trigger the cascade of AD-related metabolic, anatomical and cognitive impairments (de la Torre, 2002,de la Torre, 2004,Grammas, 2000,Iadecola, 2004,Kalaria, 2002). In 2000, de la Torre hypothesized that aging in combination with decreased cerebral perfusion could lead to oxidative stress and decreased energy metabolism, followed by increased glutamate production (de la Torre, 2000b). These events may directly contribute to progressive synaptic loss, increased Aβ and NFTs production, increased brain inflammation, brain tissue atrophy, neurodegeneration and cognitive decline (de la Torre, 2000a,de la Torre, 2000c,de la Torre and Stefano, 2000). Recent studies further suggest that impaired brain vasculature plays a causative role in AD pathology progression (Altman and Rutledge, 2010,Zlokovic, 2011).

Diagnosis of AD: the use of biomarkers
In 2011, the National Institute on Aging (NIA) and the Alzheimer’s Association recommended new diagnostic criteria and guidelines for AD (Jack, et al., 2011).
According to these criteria and guidelines, three stages of the disease are identified: preclinical AD, MCI due to AD and dementia due to AD. Importantly, it is proposed that new technologies have the potential to identify brain changes related to Alzheimer that occur before clinical symptoms are evident.

To establish a diagnosis of dementia, a physician must determine the cause of the dementia-like symptoms, based on the criteria given in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (Cooper, 1995). The diagnosis of AD is often associated with a low score in cognitive and memory tests, such as the mini-mental state examination (Folstein, et al., 1975), the clock-drawing test (Berger, et al., 2008), and other sensory-related tests (e.g. the odor identification test (Wilson, et al., 2006)). Once the cognitive deficits have been objectively demonstrated, biomarker evaluation that accurately indicates the presence or absence of disease, or the risk of later developing a disease is demanded. The new criteria and guidelines identify two biomarker categories: (1) biomarkers showing the level of beta-amyloid accumulation in the brain and (2) biomarkers showing that brain is injured or actually degenerating. Routinely, these AD biomarkers are identified based on magnetic resonance imaging (MRI) and positron emission tomography (PET).

The presence of Aβ plaques in the brain can be readily revealed in-vivo with the use of a PET-imaging agent that binds amyloidosis deposits, known as Pittsburgh Compound-B (PiB) (Klunk, et al., 2004). PiB has been used to follow the evolution of amyloidosis during the progression of the disease, and to study its relationship with cognitive decline and possible treatments (Pike, et al., 2007; Rinne, et al., 2010). Other emerging imaging techniques, like MR T2* susceptibility changes, showed good co-localization with Aβ plaques in-vivo, yet improvements in shortening the acquisition time are required (Baltes, et al., 2011). Despite the advantages of direct plaque imaging, these methods present several limitations; for example, they may display Aβ also in the brain of non-demented subjects (Mintun, et al., 2006) and they don’t readily detect soluble oligomeric forms of Aβ, which are considered highly pathogenic (Selkoe, 2008). For those reasons, complementary methods for the detection of other biomarkers, which better describes structural and functional changes related to AD, are often required by physicians.

Compared to other methodologies, MRI is very versatile and offers several tools to detect AD biomarkers, with the advantages of being non-invasive, highly informative on the disease status and easily accessible by most of the patients. In
MRI, protons in water, which are abundantly present in the body, generate a signal in the presence of a magnetic field. This signal is used to reconstruct images. Because it is sensitive to various local magnetic and other conditions, a large range of different anatomical and functional contrasts can be recorded for these images. The details of MRI have been described in several handbooks (Bernstein, et al., 2004, Haacke, et al., 1999). The most promising MR imaging tools and their applications in the field of AD research and clinical routine are discussed more in detail in the next section.

**MR biomarkers for detection of AD progression**

A recent study from the US Alzheimer’s Association predicted that by delaying the onset of AD by just 5 years, it would bring a reduction of the related costs for the society of approximately 50% (http://www.alz.org/documents_custom/trajectory.pdf). Such an ambitious plan requires further research to assess novel therapeutic strategies combined with an early diagnosis of the disease. As previously mentioned, MRI offers a range of advanced neuroimaging tools that might not only improve diagnostic accuracy but also accelerate treatment discovery. These novel neuroimaging techniques and scanning methods could eventually be used to detect neurodegeneration before clinical symptoms are obvious, so that physicians can identify treatment candidates for future preventive therapies. Traditionally, MR neuroimaging techniques have been categorised as either structural or functional, according to the primary information they provide.

**Brain atrophy**

The most obvious age-related brain change is atrophy, which occurs during normal aging, but can drastically accelerate in specific areas in case of dementia, including AD, mainly due to synaptic and neuronal loss (Josephs, et al., 2008). In patients with AD, atrophy is particularly evident in the medial temporal area, including the hippocampus and the entorhinal cortex, together with an enlargement of the ventricles (Neary, et al., 2005). Standard structural MRI approaches, commonly by recording high-resolution T1-weighted or T2-weighted images, can reveal these changes by comparing brain scans taken over the years from the same patient. Several studies reported a high correlation between rate of atrophy in the entorhinal cortex and hippocampus and an increased risk of
developing AD (Apostolova, et al., 2006; Stoub, et al., 2005). However, these changes might not be specific to AD and occur also in other dementia disorders.

**Water diffusion imaging**

A more advanced imaging approach to identify microstructural differences between normal subjects and AD patients relies on the measure of directionality (and anisotropy) of water diffusion. Diffusion tensor MR imaging (DT-MRI) models the degree of water diffusion measured in multiple orientations into a diffusion ellipsoid. Because in the brain water diffusion is restricted by cell membranes, axonal bundles and other organelles, the size and the shape of the diffusion ellipsoid reflect local fibre orientation, neuronal organization and tissue architecture integrity. When the ellipsoid is reconstructed in every voxel of interest, DT-MRI provides unique information of neuronal connectivity and microstructural integrity in white matter (WM) and, in some extent, also in grey matter (GM) (Basser, et al., 1994).

With advances in acquisition and analysis methods, DT-MRI is gaining acceptance as preferred technique for assessing structural integrity in aging and dementia. In AD patients, changes in DT-MRI parameters are particularly consistent, showing reduced degree of diffusion anisotropy (or fractional anisotropy, FA) in white matter tracts and increased mean diffusivity (MD) in grey matter areas, specifically in the hippocampus (Clerx, et al., 2012). Most of these studies suggested that such differences in AD brains reflect myelin degradation and axonal degeneration in WM, and neuronal loss in GM. Interestingly, increased MD in GM predicted the conversion from MCI to AD better than hippocampal volumetric changes in two independent studies (Fellgiebel, et al., 2006). DT-MRI has also been reported to differentiate between AD patients from MCI patients (Zhang, et al., 2007) and from those with dementia with Lewis bodies (Firbank, et al., 2007). Finally, diffusion-related changes were also shown in pre-symptomatic carriers of AD mutation (Ringman, et al., 2007). Taken together, these findings point out the advantage of DT-MRI in providing complementary information to the macrostructural changes by conventional MRI, for early diagnosis and disease characterization. However, more studies are needed to specify the biological underpinnings of diffusion changes, in relationship with the development of pathology in AD brains.
**Perfusion imaging**

As explained by the “vascular hypothesis”, the presence of cerebrovascular pathologies, such as brain hypoperfusion, might contribute to the initial expression of AD (Altman and Rutledge, 2010; Zlokovic, 2011). The early detection of cerebrovascular pathology in patients with suspected AD is therefore of primary importance for the initiation of preventive strategies. Deficits in the cerebrovascular system can be readily assessed *in vivo* with MRI. Two important parameters measurable with MRI are cerebral blood volume (CBV), which describes the cerebrovascular integrity and blood reserves, and cerebral blood flow (CBF), to measure brain perfusion; both these parameters are correlated with neural tissue health and function. In most of the cases, CBV and CBF are measured either with dynamic susceptibility contrast perfusion MRI (DSC-MRI), which require the intravenous injection of a contrast agent (eg. gadolinium compounds), or by using arterial spin labeling (ASL) techniques, whereby arterial blood is magnetically tagged before it enters the tissue of interest (Calamante, et al., 1999). These techniques have been widely used to study hemodynamic alterations and cerebral hypoperfusion in AD patients (Alsop, et al., 2000; Johnson, et al., 2005; Ruitenberg, et al., 2005; Schreiber, et al., 2005) and have served as diagnostic tool to distinguish AD from other forms of dementia (Du, et al., 2006). Improving the knowledge and characterizing the vascular changes in relation with other AD biomarkers is an essential challenge for future research to clarify the role of vascular impairments in the development of the disease.

**Functional MRI**

Neural activity is associated with a local transient increase in blood flow (Roy and Sherrington, 1890). The resulting changes in deoxyhemoglobin-hemoglobin ratio modulate the magnetic proprieties of the blood, which can be detected with spin-echo or gradient-echo MRI sequences. By measuring the fluctuations in blood oxygen content (often called the blood oxygenation level dependent, or BOLD signal), either during a specific designed task that the patient needs to perform in the MR scanner, or at rest, maps of neural activity can be assessed (Kwong, et al., 1992; Ogawa, et al., 1992). The temporal correlation of the BOLD fluctuations between different brain regions is often defined as functional connectivity.
The applications of functional MRI (fMRI) are enormous, from basic research on the functions of the human brain, to clinical assessment of brain and behaviour pathologies (Minati, et al., 2007). In particular, the examination of functional connectivity has generated a big deal of interest among neuroscientists, as it provides new insights into the reorganization of neural processes during aging and diseases (Damoiseaux, et al., 2008, van den Heuvel, et al., 2009). A large number of fMRI studies have revealed that the patterns of activation or deactivation are changed in AD patients during the performance of tasks (Buckner, et al., 2000, Gould, et al., 2005). Interestingly, many deactivated brain regions appear to be largely task independent (Raichle and Mintun, 2006). Consequently, resting-state fMRI, in which no stimulation and responses are required, has attracted considerable attention of researchers in recent years. With this method, the spontaneous neuronal activity and resting functional connectivity can be measured (Fox and Raichle, 2007). Resting-state fMRI studies in AD patients have revealed a decreased activity in the posterior cingulate cortex and hippocampus, suggesting a disrupted connectivity between these two regions (Greicius, et al., 2004). Similar evidence of disrupted functional connectivity between hippocampus and several other regions, such as the medial prefrontal cortex and the ventral anterior cingulate cortex, were also shown in AD patients (Allen, et al., 2007). Furthermore, significant differences in resting brain activity have been reported comparing cognitively normal carriers of the APOE-ε4 and APOE-ε3 alleles (Bookheimer, et al., 2000). Despite the numerous and consisting evidence that relate AD (or AD risk factors) to region-specific decreased neural activity and impaired functions, the exact meaning of resting-state fMRI changes is still a point of discussion. An issue needing further exploration is the degree to which hemodynamic response alter fMRI fluctuations, in particular when pathologies that affect the neurovascular coupling are examined (Buckner, et al., 2000).

**Magnetic resonance spectroscopy (MRS)**

MR spectroscopy (MRS) is another technique that has become well established as a tool to study biological systems *in vivo* and *in vitro*. The physical basis of MRS is the chemical shift effect, which arises because nuclei located in different molecular environments (e.g. different molecules or different locations within a molecule) sense slightly different magnetic fields, causing them to precess at different rates. *In vivo* MRS provides plots (or spectra) of signal intensity, which is
proportional to concentration, versus precession rate shift with respect to a reference, expressed in parts per million (ppm). Molecular groups generate specific resonance patterns in the spectra, either as single peaks, doublets or more complex shapes. A given molecule therefore may have multiple corresponding peaks, only some of which may be observable. Even though any nucleus with nonzero spin could in principle be studied, clinical MRS is most commonly using the hydrogen nucleus (Govindaraju, et al., 2000)(de Graaf, 2013). The set of molecules visible by neuro-MRS in vivo is limited to 15 - 20 and usually comprises creatine, choline, N-acetyl-aspartate (NAA), myoinositol, glutamate/glutamine and lipids. Quantitation of these NMR-observable metabolites can provide considerable biochemical information, and can help clinical investigators in understanding the role of metabolites in normal and pathological conditions. In patients with AD and MCI, the central spectroscopy findings are an age-related reduction in the concentration of NAA, indicating reduced neural volume and/or viability, accompanied by a significant increase in the level of myoinositol, likely caused by gliosis and metabolic dysfunction (Catani, et al., 2001, Chantal, et al., 2004, Chantal, et al., 2002). These findings motivate further studies, aimed at evaluating the potential role of MRS as a diagnostic tool to detect the neurometabolic correlates of decline in cognitive function.

In conclusion, we foresee that advanced MR neuroimaging methods will be increasingly used as a tool to explore the structural and functional abnormalities of AD patients, due to the relatively easy applicability and the unique information provided. It is now believed that future treatments to slow or stop the progression of AD will be most effective when administered during the preclinical and MCI stages of the disease. In this scenario, biomarker tests will be essential to identify which individuals are in these early stages and should receive disease-modifying treatment when it becomes available. They also will be critical for monitoring the effects of such treatments.

**Dietary intervention as a strategy to treat AD**

An extensive search to find a treatment for AD has been pursued for several decades. However, no treatment is yet available nor seems near. Preventive approaches have therefore consistently emerged as key policy priorities in recently formulated national dementia strategies. These approaches include
modifying health-compromising behaviours that can lead to AD, such as lifestyle and dietary intake. For example, high consumption of fruit, vegetables and legumes, moderate consumption of fish and the use of olive oil as the main source of fats, collectively known as the Mediterranean diet, have been associated with a reduced risk for AD (Scarmeas, et al., 2006, Sofi, et al., 2008) and with a lower mortality in AD (Scarmeas, et al., 2007). The mechanisms by which dietary intake influence the pathology onset and progression are still a matter of investigation. One possible explanation is given by the beneficial effect that some nutrients, such as omega 3 (n3) long-chain polyunsaturated fatty acids (LCPUFA), express on the vascular system (Lee, et al., 2008). The AD prevalence would then decrease by reducing the exposure to AD vascular risk factor, like atherosclerosis (Thies, et al., 2003), high blood pressure (Geleijnse, et al., 2002) and cardiovascular diseases (Daviglus, et al., 1997, Hu, et al., 2002, Thies, et al., 2003, von Schacky and Harris, 2007). Other nutrients may instead directly act in restoring or reducing synaptic loss; for instance, the formation of phosphatidylcholine, the most common phosphatide in the brain and a major component of the synaptic membrane, is enhanced when its precursors are supplemented in the diet (Weiss and Kennedy, 1956, Wurtman, et al., 2010) (figure 3). Several preclinical studies confirmed these findings, showing that animals supplemented with all these membrane precursors had increased levels of brain phospholipids, dendritic spines and neurite outgrowth, with beneficial effects on cognition (Cansev and Wurtman, 2007, Sakamoto, et al., 2007, Wurtman, et al., 2006). Based on these evidences, a novel combination diet that also includes other precursors and cofactors in membrane synthesis (such as phosphatidylcholine, B-vitamins and antioxidants) has been proposed for the dietary management of AD (Kamphuis and Scheltens, 2010). To date, two randomized controlled clinical trials have shown improvements in the delayed verbal recall task and better cognitive performance in mild AD patients supplemented with this multi-nutrient component diet (Cummings, 2012, Scheltens, et al., 2010, Scheltens, et al., 2012). The processes by which these dietary nutrients influence the pathophysiology of AD yet need to be elucidated, and there is the need for more studies to confirm their efficacy.
Figure 3. The Kennedy cycle. Essential precursors for phosphatidylcholine (PC) formation are uridine (mostly as uridine monophosphate (UMP)), choline, docosahexaenoic acid (DHA) and other omega-3-fatty acids (e.g. eicosapentaenoic acid (EPA)). The formation of PC consists of 3 sequential enzymatic reactions. In short, ATP and choline are utilized to form phosphocholine, followed by combination of cytidine triphosphate (CTP; mostly derived from uridine (Wurtman, et al., 2000)) and the newly formed phosphocholine to form a CDP-choline. Lastly CDP-choline is bound to diacylglycerol (DAG; which is formed from DHA and indirectly from other omega-3-fatty acids) which will yield a PC (Weiss and Kennedy, 1956, Wurtman, et al., 2010).
Thesis overview

The two main aims of this thesis are to describe work done:

- to provide the tools to study brain structure and function with magnetic resonance imaging in mouse models for AD and vascular risk factors
- to investigate the mechanism by which specific multi-nutrient diets can avert AD pathology onset and progression

To this end, the work presented in this thesis is divided in two parts. The first part, which includes chapter 2, chapter 3 and chapter 4, is dedicated to the development of novel MR methods to assess AD biomarkers for preclinical studies. In the second part, covered by chapter 5 and chapter 6, we studied the effect of specific nutritional treatment approaches in mouse models for genetic AD and for vascular risk factors.

One of the main open questions in AD research is how deficits in brain vasculature affect the development of the disease. In particular, it is still not clear in which way the different forms of amyloid beta - aggregates and oligomers - can trigger micro and macrovasculature defects. In order to address this question, in chapter 2 we developed an MR imaging method to follow longitudinally the progression of cerebral blood volume (CBV) in a mouse model of AD. The CBV results were compared in the same study with the amount of capillaries measured with immunohistochemistry, with the amount of Aβ deposition in parenchyma and in blood vessel walls and with Aβ oligomers.

In chapter 3 we aimed to assess brain diffusion changes in an AD mouse model, and to specify the biological underpinning of such changes. The differences in diffusion-related parameters found in white matter tracts well-matched previous findings of axonal disconnection and myelin degradation in AD patients. Furthermore, we noted an increased mean diffusivity in the granulate molecular layer of the dentate gyrus, which we relate to neuronal loss as visualized with immunohistochemical staining for neurons and myelin.

The study presented in chapter 4 is directed to explore the functional connectivity (FC) changes measured with resting-state fMRI in aging apoE-ε4 and
apoE-ko mouse models. In the same animals, cerebral blood flow (CBF), DT-MRI and post-synaptic density were also measured, with the scope of understanding their possible role in FC signal changes.

The application of these methods to study the efficacy of specific multi-nutrient diets against AD progression is presented in chapter 5 and chapter 6. In chapter 5 we combined CBV and MR spectroscopy measures with behavioural tests and neuropathology to evaluate the effect of long-term intake of a multi-component diet in apoE-ε4 and apoE-ko mice. This diet is found to increase CBV, to reduce anxiety and to reduce the blood cholesterol levels in these mice.

In chapter 6, two specific multinutrient diets have been supplied in AD transgenic mice. CBF and DT-MRI analyses revealed a possible beneficial effect of the diets by improving contemporarily brain perfusion and protecting against neurodegeneration.

Chapter 7 and 8 are dedicated to a summary and a general discussion of these findings and suggestions for future studies.
Microvascular cerebral blood volume changes in aging $\text{APP}_{\text{swe/PS1}_{\text{dE9}}}$ AD mouse model: a voxel-wise approach

Published as
Abstract
Vascular disorders can either be cause or consequence in the pathophysiology of Alzheimer’s disease (AD). To comprehensively characterize the occurrence of vascular impairment in a double transgenic mouse model for AD (APP_{swe}/PS1_{dE9}) during aging, we developed a new method to obtain microvascular relative cerebral blood volume (rCBV_{micro}) maps from gradient echo MR imaging by histogram evaluation and we applied a voxel-wise approach to detect rCBV_{micro} changes. With this methodology the development of cerebral microvascular impairments can be described in vivo with 0.16 millimeter isotropic resolution for the whole mouse brain. At 8 months of age, impaired rCBV_{micro} appeared in some cortical regions and in the thalamus, which spreads over several sub-cortical areas and the hippocampus at 13 months of age. With a ROI-based approach, we further showed that hippocampal rCBV_{micro} in 13-month-old wild type and APP_{swe}/PS1_{dE9} mice correlates well with capillary density measured with immunohistochemical staining. However, no differences in capillary density were detected between genotypes. The rCBV_{micro} values showed no significant correlation with amyloid-β (Aβ) plaque deposition, Aβ at bloodvessel walls and biochemically measured levels of Aβ_{1-40}, Aβ_{1-42} oligomers and fibrillar forms. These results suggest that rCBV_{micro} reduction is caused by an impaired vasoactivity of capillaries and arterioles, which is not directly correlated with the amount of Aβ deposition in parenchyma nor bloodvessel walls.
Introduction
Well-known neuropathological hallmarks of Alzheimer’s disease (AD) are the presence of β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) in cerebral tissue and a massive loss of neuronal cells and of the white matter. Besides many reports on the causative role for Aβ in AD (Walsh and Selkoe, 2007), large epidemiological studies demonstrated that vascular disorders are important risk factors, with chronic brain hypoperfusion and cerebrovascular pathology among the earliest markers of AD (Breteler, 2000, de la Torre, 2002, de la Torre, 2004, Grammas, 2000, Iadecola, 2004, Kalaria, 2002, Meyer, et al., 2000, Skoog and Gustafson, 2002). In 2000, de la Torre hypothesized that aging in combination with decreased cerebral perfusion could lead to oxidative stress and decreased energy metabolism, followed by increased glutamate production (de la Torre, 2000b). These events contribute to progressive synaptic loss, increased Aβ and NFTs production, increased brain inflammation, brain tissue atrophy, neurodegeneration and cognitive decline (de la Torre, 2000a, de la Torre, 2000c, de la Torre and Stefano, 2000). Recent studies further showed evidence for impaired vasculature being also a causative factor in AD pathology progression (Altman and Rutledge, 2010, Zlokovic, 2011).
Transgenic mouse models with targeted expression of mutant amyloid precursor protein (APP) reflect many of the behavioral and neuropathological traits of AD. Besides the parenchymal Aβ accumulation, these mice also manifest early impairment in cerebrovascular autoregulation and cerebral blood flow and abnormalities in the cortical microvasculature similar to AD brains (El Tayara Nel, et al., 2010, Meyer, et al., 2008, Niwa, et al., 2002a, Niwa, et al., 2002b). These deficits in the cerebrovascular system can be readily assessed in vivo with magnetic resonance imaging (MRI). An important parameter measurable with MRI is cerebral blood volume (CBV), which describes the cerebrovascular integrity and, indirectly, the neural tissue physiological status and function. A reduction of relative CBV (rCBV) in a 4 months old transgenic APP mouse model (V717F, K670N/M671L) was first described by Wu and colleagues with intravascular superparamagnetic susceptibility contrast enhanced (CE) MRI (Wu, et al., 2004). Other studies in different mice strains showed similar findings (Weidensteiner, et al., 2009). In these studies the methodology applied for the analysis of vascular defects used a region of interest (ROI) based approach with no further spatial characterization. Thus, a ROI approach generally doesn’t
consider the whole brain area and may not reveal subtle or local CBV variation that occurs in a fraction of the selected regions.

In this paper, we aimed for a more comprehensive and spatially determined analysis of the rCBV changes occurring during ageing in the double transgenic AD mice (APP<sub>swe</sub>/PS1<sub>dE9</sub>) (Jankowsky, et al., 2004, Jankowsky, et al., 2001). These mice exhibit progressive parenchymal and vascular Aβ accumulation, leading to cerebral amyloid angiopathy (CAA) (Garcia-Alloza, et al., 2006). In our previous experiments we found a decreased rCBV in the hippocampus and in cortical regions at 15 months of age (Hooijmans, et al., 2009).

For this study we employed APP<sub>swe</sub>/PS1<sub>dE9</sub> mice of the same breeding at 8 and 13 months of age and we spatially localize vascular changes with an explorative whole-brain voxel-wise approach. As second aim, we wanted to investigate the advantages of a vascular compartment separation for the analysis of rCBV changes. For this purpose, we developed a post-processing method based on histogram analysis of high-resolution rCBV maps in order to distinguish between blood volume changes of large cerebral vessels and capillaries. We validated this approach with an immunohistochemical staining for capillaries in order to determine capillary density. This non-invasive methodology allows to monitor local cerebrovascular changes over time which can be used in translational research on AD treatment in the earlier stages of the disease with vascular impairment.

**Materials and methods**

**Animals**

For this study, we used the APPswe mice, line 85, which expresses a Mo/Hu APPswe construct in conjunction with the exon-9-deleted variant of human presenilin 1 (PS1-dE9) (Jankowsky, et al., 2004, Jankowsky, et al., 2001) (Jankowsky, et al., 2004, Jankowsky, et al., 2001) (Jankowsky, et al., 2004, Jankowsky, et al., 2001). This line was originally maintained in a hybrid background by backcrossing to C3HeJ × C57BL6/J F1 mice for 9 generations. The APP<sub>swe</sub>/PS1<sub>dE9</sub> founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) and a colony was established at the Central Animal Facility (CDL) of the Radboud University Nijmegen Medical Centre, the Netherlands. Throughout the experiment the
Microvascular cerebral blood volume changes in aging APP<sub>swe</sub>/PS1<sub>dE9</sub> AD mouse model

Animals were housed in groups of 2-3 mice per cage in a controlled environment with room temperature at 21°, an artificial 12:12 light:dark cycle and they were fed ad libitum. The experiment was performed according to the Dutch federal regulations for animal protection and was approved by the Veterinary Authority of the Radboud University Nijmegen Medical Centre, the Netherlands.

**MR imaging**

7 APP<sub>swe</sub>/PS1<sub>dE9</sub> and 9 non-transgenic littermates mice underwent rCBV measurements at 8 and 13 months of age. The MRI measurements were performed on a shielded 7T/300mm horizontal-bore MR magnet interfaced to a clinical console (ClinScan, Bruker BioSpin, Ettlingen, Germany). A circular polarized ¹H transmit volume coil (200mm/154mm outer/inner diameter) was used for excitation and was combined with a circular polarized ¹H receive surface coil with a shape adapted to the mouse head for signal reception. Before the MR measurements, all mice were weighed and received an intravenous (i.v.) tail vein catheter. During the experiment mice were anesthetised using 2% isoflurane (Abott, Cham, Switzerland) in a 2:1 oxygen and N₂O mixture. Isoflurane was chosen as it induces low cerebral hemodynamic changes (Hansen, et al., 1989).

Mice were placed in a stereotactic holder to prevent unwanted movement during the scanning. Body temperature was maintained at physiological temperature with heated airflow and monitored with a rectal optical temperature probe. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). Initially, multislice Turbo Spin Echo images in the coronal, transversal and longitudinal orientation were acquired to visualize the anatomy and the morphology of the mouse brain structures. Imaging parameters were FOV = 25×25 mm, matrix size = 256×256, slice thickness = 0.5 mm, TE = 46 ms, TR = 3500 ms.

**rCBV measurement**

To assess changes in rCBV, we used a susceptibility contrast enhanced MRI technique using ultra small particles of iron oxide (USPIO – AMI-277, Sinerem®, Guerbet Laboratories, France) as previously described (Dennie, et al., 1998, Le Duc, et al., 1999). By allowing the contrast agent (CA) to distribute in the vasculature after intravenous injection, the rCBV can be estimated as the transverse relaxation rate increase, which can be calculated from T₂ or T₂* images acquired before and after the CA injection. Both empirical data (Belliveau, et al.,
1990) and Monte Carlo modeling (Boxerman, et al., 1995) indicate an approximately linear relationship between the increase of relaxation rate calculated with spin echo (SE) and gradient echo (GE) sequences ($\Delta R_2$ and $\Delta R_2^*$ respectively) and rCBV fraction over the physiologically relevant range. In this work, a 3D multi echo GE FLASH sequence was performed prior to and 1 min after administration of an i.v. injection with USPIO at the dose of 140μg Fe/mouse. Imaging parameters were: FOV = 30x30x14 mm, matrix size = 192x192x88, total resolution = 0.16x0.16x0.16 mm, TEs = 5.13 – 7.28 – 9.40 ms, TR = 50 ms, resulting in a total scan time of 35 min per mouse.

**Data pre-processing and cerebral blood volume calculation**

For the 8- and 13-months-old mice groups, individual image sets were spatially normalized into a standardised coordinate space defined by a study-specific template of the mouse brain. The creation of the template was performed using Advanced Normalization Tools (ANTS. V1.9, http://picsl.upenn.edu/ANTS/). GE datasets from wild type and transgenic animals were co-registered to this template. Briefly, spatial normalization was achieved by applying a 12-parameter affine transformation and then followed by non-linear deformations using a diffeomorphic model (Avants, et al., 2008). A mask that included the entire brain was manually defined in the normalized coordinate space and applied to all the images. Spatially normalized and masked datasets were then imported in MatLab and, for each mouse, a pixel-by-pixel delta relaxation rate $\Delta R_2^*$ map was obtained using the formula:

$$\Delta R_2^* = \frac{1}{TE} \log \left( \frac{S_{bef}}{S_{aft}} \right)$$

where $S$ is the signal amplitude before-CA ($S_{bef}$) and after-CA ($S_{aft}$). Image sets from the three TEs were used for the calculation of three $\Delta R_2^*$ values and thereafter these values were averaged for $\Delta R_2^*$ maps. The TE values were chosen as a compromise between signal-to-noise ratio and the $T_1$-weighting of CA effects that occurs at short TR (Tropres, et al., 2001).

**Analysis of the histogram**

While the $\Delta R_2$, measured with spin echo sequences, has an intrinsic higher sensitivity for small caliber vessels, the $\Delta R_2^*$, obtained with GE-based sequences,
is equally sensitive to all vessel radii for a sufficient dose of contrast agent (Boxerman, et al., 1995, Dennie, et al., 1998, Tropres, et al., 2001). Nevertheless, the measurement of blood volume in fast-flowing arteries and veins acquired with GE sequences is more susceptible to noise and quantification errors, and this may result in inaccurate values. To circumvent these problems and to focus on the microvascular compartment (e.g., capillaries, arterioles and venules with diameters ranging approximately from 3 to 20 μm), we developed an histogram analysis technique on the ΔR₂* to mask pial and other large perforating arteries and veins. First, an averaged ΔR₂* map was defined in the normalized space and for each slice the corresponding histograms were computed with number of bins equal to the maximum ΔR₂* intensity value (MV). The histograms were then fitted with a polynomial function. Subsequently, peak height position (PHP) and the Full Width Half Maximum (FWHM) of the curve were calculated; these values were used as thresholds to create masks of rCBV in the microvascular (rCBV\text{micro}) and macrovascular (rCBV\text{Macro}) compartment with the following criteria:

1) \( \text{PHP} - \left( \frac{\text{FWHM}}{2} \right) < \text{rCBV}_{\text{micro}} < \text{PHP} + \left( \frac{\text{FWHM}}{2} \right) \)

2) \( \text{PHP} + \text{FWHM} < \text{rCBV}_{\text{Macro}} < \text{MV} \)

These thresholds were then applied to each individual spatially normalized ΔR₂* maps. Only the voxels included in the rCBV\text{micro} mask, which were non-zeros in all mice, were kept for further calculations. To validate this approach, we correlated the individual non-normalized rCBV\text{micro} data with the capillary density measured with Glucose transporter-1 staining determined by immunohistochemistry in the hippocampus region. Then, to ensure that all large blood vessels were excluded, visual identification of the rCBV\text{Macro} maps was performed with Image J (3D volume viewer tool, ver. 1.41, National Institute of Health, USA) and compared with the vasculature from an anatomical atlas (Dorr, et al., 2007). Because rCBV was mapped as ΔR₂*, several experimental variation such as USPIO dosage and mouse body weight composition could influence its values. To avoid these problems, normalization to the individual averaged rCBV\text{micro} of the entire brain was performed.
Chapter 2

Voxel-wise group comparison

Regional differences in spatially normalized rCBV_{micro} maps between APP_{swe}/PS1_{dE9} mice and wild type littermates were assessed voxel-wise using MATLAB R2008a (Mathworks, Natick, MA, USA) and statistical parametric mapping 5 (SPM5, Wellcome Department of Clinical Neurology, London) with SPMMouse toolbox (Sawiak, et al., 2009). Spatially normalized rCBV_{micro} datasets were first smoothed with a 500μm isotropic Gaussian kernel to correct for imperfect registration and subsequently a two-group t test was performed to identify genotype-wise changes in the framework of the general linear model (GLM). Statistical significance for an individual voxel was established at p<0.01, uncorrected for multiple comparisons (Ashburner and Friston, 2000). The locations of significant voxels were compared to the anatomical atlas of (Franklin K, 1997,Franklin and Paxinos, 1997).

ROIs based approach

Normalized ∆R_{2}^{*} maps and rCBV_{micro} maps were additionally measured for each animals in their original coordinate system with a region of interests (ROIs) group comparison. For the ROI-based approach, we evaluated significant effects of genotype and aging. ROIs that included hippocampus, cerebral cortex (all cortical areas above the corpus callosum), and thalamus were drawn based on the atlas of (Franklin K, 1997,Franklin and Paxinos, 1997).

Immunohistochemistry

For immunohistochemical staining we used a separate group from the same breeding of 8-month-old mice (7 APP_{swe}/PS1_{dE9} and 7 wild type littermates) and the 13-month-old mice (7 APP_{swe}/PS1_{dE9} and 9 wild type littermates), which were sacrificed directly after MR measurements. The 8 months old mice were transcardially perfused starting with 0.1M phosphate buffered saline (PBS) followed by Somogyi’s fixative (4% paraformaldehyde, 0.05% gluteraldehyde and 0.2% picric acid in 0.1M phosphate buffer, pH 7.2). The group of 13 months old mice was sacrificed after the MR experiment by cervical dislocation and the brains cut mid sagittally for immunohistochemistry (immersion fixation of left hemisphere) and biochemistry. Six series of 40μm thick coronal sections were cut using a sliding microtome (Microm HM 440, Walldorf, Germany).
Amyloid-β load

Amyloid-β deposits were visualized using WO-2 antibody (mouse anti-human Aβ4–10 from K. Beyreuther, Centre for Molecular Biology, University of Heidelberg, Germany). In brief, sections were pretreated with sodium citrate solution 0.05M at 85 °C for 30 min and incubated for 18h at room temperature with primary antibody mouse anti-human Aβ4–10 (1:20.000 in TBS-T). The next day sections were rinsed in TBS-T and incubated for 90 min with the secondary antibody donkey-anti mouse biotin (1:1500, Jackson ImmunoResearch). Thereafter, sections were transferred to a solution containing Vector ABC-Elite 1:800 (Vector laboratories, Burlingame, CA, USA). The sections were incubated in DAB-Ni solution with perhydrol for 10 min and mounted on gelatin-coated slides. After dehydration in alcohol series, slices were cleared with xylol and mounted in Entellan.

GLUT-1

The glucose transporter type 1 (GLUT-1) is a membrane protein responsible for the active glucose transport across the blood brain barrier and is primarily localized in vascular endothelial cells (Choeiri, et al., 2002); In this study, GLUT-1 staining was performed to quantify capillary bloodvessels density as previously described (Hooijmans, et al., 2007b). The amount of GLUT-1 was visualized using rabbit anti GLUT-1 transporter antibody (AB 1340, Chemicon International, Inc., Temecula, CA, USA). Briefly, the sections were pre-treated against endogenous peroxidase for 30 min with 0.3% H2O2 in 0.1 M PBS. After sequentially rinsing in PBS and pre-incubation in 0.1 M PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS–BT) the section were incubated for 18h with anti-GLUT-1 (1:10.000 in 0,1M PBS-BT). Following incubation, the sections were rinsed thoroughly with PBS and transferred to donkey-anti-rabbit biotin 1:1500 (Jackson ImmunoResearch) for 90 min. The sections were rinsed three times and transferred to a solution containing Vector ABC-elite 1:800 (Vector laboratories) for another 90 min. Visualization of the GLUT-1 amount was achieved by incubation with DAB–Ni solution. All stained sections were mounted on gelatin-coated slides and dehydrated in alcohol series, cleared with xylol and mounted in Entellan.
Quantification

The amount of β-amyloid and GLUT-1 was determined in appropriate sections digitized using a Carl Zeiss Axioskop microscope, equipped with hardware and software of Microbrightfield (Williston, USA). Serial images with 20× and 40× magnifications were used for the quantification by two double blind investigators.

Extracellular Aβ plaque load was quantified with a computer-assisted analysis system (Stereo Investigator, Microbrightfield, Williston, USA) using Cavalieri’s probe. Contours along the hippocampal regions (cornu Ammonis area 1 and 3 (CA1, CA3) and dentate gyrus (DG)) and in the frontal prelimbic area (PLA) and anterior cingulate gyrus (ACg) were drawn on the selected slices. Measurement of Aβ load was defined as the percentage of area covered by Aβ plaques. In addition, we determined the amount of Aβ in and around the vascular walls in the entire hippocampus in 13 months old APP_swe/PS1<sup>dE9</sup> mice. Measurements were defined as the percentage of area covered by Aβ in the bloodvessels, including the vessels in the hippocampal fissure.

GLUT-1 density was quantified with a computer-assisted analysis system (Stereo Investigator) using Cavalieri’s probe. Contours were drawn along the CA1, CA3 and DG on the selected slices. Measurement of GLUT-1 density was defined as the percentage of the area covered by GLUT-1 immunoreactivity compared to the total area of the region measured.

Soluble Amyloid-β oligomers biochemical analysis

For biochemical analysis we used half brain of 13-month-old mice (7 APP<sub>swe/PS1<sub>dE9</sub> and 8 wild type). The brains were homogenized as previously described (Maier, et al., 2008). Briefly, frozen hemispheres were homogenized in a carbonate buffer (CB: 100mM Na<sub>2</sub>CO<sub>3</sub>, 50mM NaCl, pH 11.5) containing protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and centrifuged at 16.000g for 30min at 4°C. Then, the pellet was resuspended in guanidine chloride buffer and extracted for 4 hours.

We separated the brain homogenates into three different fractions: the TBS-fraction contains more soluble monomeric Aβ, the TBS-T (TBS plus 1% Triton) is related to membrane-associated Aβ and enriched with oligomeric Aβ and the guanidine-HCl fraction, which contains mainly highly aggregated Aβ. Since we were mainly interested in Aβ oligomers and aggregated forms, presence of Aβ<sub>40</sub>
and Aβ42 were measured in the TBS-T and guanidine-HCL fractions using the Aβ kits from Invitrogen (KHB3442 and KHB3482, Karlsruhe, Germany). Result was normalized to the protein concentration of the sample (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munich, Germany).

Statistical analysis.
All statistical analyses were performed using SPSS v16.0 (SPSS Inc. Chicago, IL, USA). Resulting data are expressed as mean ± SEM. Differences between genotypes and/or aging within multiple ROIs for rCBV measurements and vessel density measurements were analysed with multivariate ANOVA’s with Bonferroni correction. The one-way ANOVA test was used to evaluate regional differences in Aβ- load and in vessel density. If overall analysis revealed a significant difference, the separate groups were analyzed post hoc by using Tukey HSD. Other data were analyzed with an independent t-test. Correlation analyses were done with bivariate Pearson’s correlation method. Statistical significance was established at p<0.05.

Results

Cerebral blood volume
To measure changes in rCBV, susceptibility-induced contrast MR imaging was used. Relaxation rate maps ($\Delta R_2^*$) were obtained from T$_2^*$-weighted images before and after i.v injection of USPIO (Figure 1a-c). Histograms of the $\Delta R_2^*$ maps were calculated as described in the Material and Methods section. Overall, the whole-brain $\Delta R_2^*$ histograms showed a right-skewed distribution that consisted of two peaks that we associated with contributions of macro- and microvascular compartments to the rCBV (Figure 1d and Figure 2). For the voxel-wise comparison, we used rCBV$_{\text{micro}}$ maps. For the ROI-based analysis, $\Delta R_2^*$ maps without masking were also considered as measure of rCBV of the total vasculature. The 3D representation of rCBV$_{\text{Macro}}$ maps showed good agreement with the anatomy of major mouse brain vessels (see video, http://link.springer.com/content/esm/art:10.1007/s00429-012-0448-8/file/MediaObjects/429_2012_448_MOESM1_ESM.mpeg) (Dorr, et al., 2007)
ROI-based analyses

The analysis focused on the hippocampus, cerebral cortex and thalamus (see Table 1). In 8 months old mice no significant differences were found in the rCBV indexes between APP\textsubscript{swe}/PS1\textsubscript{dE9} and wild type mice. In 13 months old mice the total rCBV, expressed by $\Delta R_2^*$, was lower in the hippocampus of the APP\textsubscript{swe}/PS1\textsubscript{dE9} compared with non-transgenic littermates, but not reaching a statistical significant difference ($p=0.081$). However, the rCBV\textsubscript{micro} was significantly lower in the hippocampus of the APP\textsubscript{swe}/PS1\textsubscript{dE9} mice ($p=0.024$). In the cortex and thalamus no significant differences in rCBV between genotypes were found. These results suggest that in the hippocampal area of APP\textsubscript{swe}/PS1\textsubscript{dE9} mice a rCBV reduction in microvasculature is present that becomes apparent when the contribution of large vessels is excluded.

Aging effects

No significant differences were found between 8 and 13 months old mice, except a reduction in hippocampal rCBV\textsubscript{micro} in APP\textsubscript{swe}/PS1\textsubscript{dE9} mice ($p=0.010$, not shown). This suggests that the decrease in the hippocampal microvascular rCBV is a pathological aging process in the APP\textsubscript{swe}/PS1\textsubscript{dE9} mice.

Voxel-wise differences using SPM

To determine the location of rCBV\textsubscript{micro} impairment at higher spatial resolution, we applied a voxel-wise statistical approach with the co-registered brains as described in the material and methods section. The analysis of spatially normalized rCBV\textsubscript{micro} at 8 and 13 months of age exhibits the evolution of the vascular impairment in APP\textsubscript{swe}/PS1\textsubscript{dE9} mice. Compared to wild-type littermates, some areas with a significant decreased rCBV\textsubscript{micro} were found already at 8 months of age (figure 3). Voxels of significantly decreased rCBV\textsubscript{micro} values were found in the cortical infralimbic area (ILA), in the anterior group of the dorsal thalamic region and, with smaller amount, also in the hypothalamic lateral zone (LZ), medulla, pons and cerebellum (figure 3).
Figure 1. a) Representative MRI gradient echo images of 13 months old wild type mouse brain before USPIO injection in four coronal slices. The images were acquired using a 3D multi GE FLASH sequence. ROIs of the entire brain, cerebral cortex, hippocampus and thalamus were selected approximately at 0.38 up to -2.46 posterior to bregma. b) Corresponding slices obtained after 1min of i/v USPIO injection c) Relaxation rate images ($\Delta R_2^*$) show sensitivity for macro- and microvasculature rCBV. Data are expressed in arbitrary units d) Microvasculature rCBV maps ($rCBV_{micro}$) are obtained after selective thresholding based on the $\Delta R_2^*$ histogram analysis and expressed in arbitrary units. Pixels that have anatomical correspondence with major brain arteries and veins are discarded in the $rCBV_{micro}$ maps.
<table>
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<th></th>
<th>Wild-type</th>
<th>APP&lt;sub&gt;swe/PS1&lt;sub&gt;dE9&lt;/sub&gt;</th>
<th>Difference (%)</th>
<th>p value</th>
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**Table 1.** Comparison of normalized blood volume changes between control C57BL6/J mice and APP<sub>swe/PS1<sub>dE9</sub> mice at 8- and 13 months of age. The ΔR<sub>2</sub> is considered a measure of the total blood volume, while rCBV<sub>micro</sub> a measure of the microvascular blood volume. Data show hippocampal rCBV<sub>micro</sub> decrease at 13 months of age in the APP<sub>swe/PS1<sub>dE9</sub> mice. Values represent mean ± STD.
Figure 2. Representation of the histogram of the $\Delta R_2^*$ map of the entire brain in 13 months old wild type mouse. a) The distribution of $\Delta R_2^*$ intensities consisted of two peaks that we associated with contributions of macro- and microvascular compartments to the rCBV. From the first peak of the histogram, point high peak (PHP), full width half maximum (FWHM) and maximum value (MV) are determined and used to define thresholds for microvasculature rCBV ($rCBV_{\text{micro}}$ = gray area) and macrovasculature rCBV ($rCBV_{\text{macro}}$ = dark gray area). b) Representation of the $\Delta R_2^*$ distribution in the hippocampus of one 13-month-old APP$_{swf}$/PS1$_{de9}$ mouse compared to a wild type littermate. The difference between APP$_{swf}$/PS1$_{de9}$ and wild type is more pronounced in the range of low $\Delta R_2^*$ intensities, which is assigned to the microvascular compartment.
Figure 3. Normalized $rCBV_{\text{micro}}$ t-value maps superimposed on anatomical images in axial and sagittal view revealed the occurrence of early microvascular blood volume reduction in the APP$_{\text{swe/PS1\text{de9}}}$ mice at 8 months of age in the infralimbic cortical area (ILA) and in the dorsal thalamic regions. Hypothalamic lateral zone, medulla, pons and cerebellum seem also partly affected. Voxels showing decreased $rCBV_{\text{micro}}$ corresponding to p values <0.01 for a two-tailed test, uncorrected, are shown in the t-maps.

At 13 months of age, a more severe decrease in $rCBV_{\text{micro}}$ was detected in APP$_{\text{swe/PS1\text{de9}}}$ mice as illustrated in figure 4. At this age, several areas of the thalamus, hypothalamus, ventral striatum, medulla, pons, cerebellum and hippocampus showed decreased normalized $rCBV_{\text{micro}}$. In the cortex, clusters of significant $rCBV_{\text{micro}}$ decrease were found mostly in the primary somatosensory areas (SSp) and in the secondary somatomotor area (MOs). Some clusters were also found in the olfactory bulb, superior colliculus and cerebral cortex. Overall, the inferior part of the brain seems to be more affected in APP$_{\text{swe/PS1\text{de9}}}$ mice.
Figure 4. In 13 month old transgenic APP<sub>swe</sub>/PS1<sub>dE9</sub> mice, normalized rCBV<sub>micro</sub> t-value maps revealed the evolution of blood volume decrease. A more severe and widespread pathology seems to occur in several areas of thalamus, hypothalamus, ventral striatum, medulla, pons, cerebellum and hippocampus. The somatosensory areas and the secondary somatomotor area appear the most affected cortical regions. Some voxels of the olfactory bulb, superior colliculus and cerebral cortex also showed significantly decreased rCBV<sub>micro</sub>. Voxel showing decreased rCBV<sub>micro</sub> in APP<sub>swe</sub>/PS1<sub>dE9</sub> mice and corresponding to p values <0.01 for a two-tailed test, uncorrected, are shown in the t-maps.

GLUT-1 density per area
Immunohistochemical staining with rabbit anti GLUT-1 transporter was used to visualize the capillaries and quantify the capillary density per area in percentage (see Figure 5a and 5b). Both at 8 and 13 months of age no difference could be found in the capillary density between genotypes and ROIs (see Figure 5c-d). In order to verify our method, we specifically looked at the relation between capillary density measured with GLUT-1 staining and non-normalized hippocampal rCBV<sub>micro</sub> measured with MRI. A strong positive correlation was
found between capillary density and both rCBV\textsubscript{micro} and PHP of the hippocampus measured in the 13-months-old wild type mice ($p=0.003$ and $p=0.004$ respectively, see Figure 5e).

In the APP\textsubscript{swe}/PS1\textsubscript{de9} mice a similar but weaker correlation was also found, possibly due to the lower number of animals or small variation in the amount of CA injected (for $p=0.037$ and $p=0.036$, not shown). As we were interested in determining the effects of the compartment separation, we further evaluated the correlation between capillary density and non-masked rCBV values, and we found no significant correlations. These results reflect the dependence between rCBV\textsubscript{micro} as measured with our technique and capillary density as visualized with immunohistochemistry. Moreover, they confirm the use of proper vascular compartment masking as a tool to increase the sensitivity of the measure towards the capillaries in GE derived images.

**Amyloid-β load**

**8-month-old mice**

Aβ deposition was visualized with immunohistochemical staining with *mouse anti*-human Aβ\textsubscript{4-10} antibody in CA1, CA3 and DG hippocampal regions and in PL and ACg cortex regions (see figure 6a-c). Wild type mice did not shown any immunoreactivity with this antibody. As illustrated in figure 6d in the APP\textsubscript{swe}/PS1\textsubscript{de9} mice, most Aβ plaques were present in the DG (4.54±2.0% of total area, $p<0.001$ compared to other areas) and only rare deposits were seen in the CA1 and in the CA3 (CA1: 0.69±0.9% and CA3: 0.35±0.6% of total area). In cortical areas much less deposits were detected (PL: 1.2±0.5% and ACg: 2.1±0.7% of total area).

**13-months-old mice**

Transgenic mice showed increased Aβ deposits in the CA1 and CA3 hippocampal regions compared to 8 months (CA1: 4.2±1.8%, $p=0.001$ and CA3: 2.9±3.8% of total area $p=0.005$) and in cortical regions (PL: 5±3.9% and ACg: 7.5±4.1% of total area, $p=0.002$); in the DG the percentage of parenchymal Aβ plaques increased, although not significantly (5.6±2.4% of total area, $p=0.463$). No significant regional variation in Aβ plaque burden could be found at this age and no correlations were found between Aβ burden in the hippocampus and GLUT-1 density or rCBV\textsubscript{micro}. 
Microvascular cerebral blood volume changes in aging APP<sub>swe</sub>/PS1<sub>DE9</sub> AD mouse model

**Figure 5.** Representative images of the blood vessels stained with glucose transporter-1 (GLUT-1) in brain regions of 13-month-old wild type mice. A: hippocampal region, magnification 5×, Scale Bar=250μm; B: magnification 20×, Scale Bar=50μm; C, D: the area covered by GLUT-1 reflect the capillary density in the dentate gyrus (DG), CA1 and CA3. No significance genotype difference was found in 8- and 13-month old mice. Values represent mean ± SEM. E: Correlation between capillary density and microvasculature rCBV measured with the non-normalized peak height position (PHP) of the hippocampal ΔR<sub>2</sub>* in 13 months old wild type mice. The line depicts the linear regression (Bivariate Pearson’s = 0.004).

Apart from parenchymal Aβ plaque load, we also determined the Aβ deposition in the vascular walls of the hippocampus, including vessels in the hippocampal fissure. The amount of Aβ was significantly lower than the plaque load in the total hippocampus (2.2±1.1% and 4.2±2.2% respectively, p=0.005). No significant correlation between Aβ in/around blood vessels and rCBV<sub>micro</sub> could be found.

**Soluble amyloid-β concentration**

Presence of oligomeric Aβ and high-molecular weight Aβ aggregates were measured in 13 months old APP<sub>swe</sub>/PS1<sub>DE9</sub> mice. Both in TBS-T and guanidine-HCL fractions, the concentration of Aβ<sub>42</sub> found was significantly higher than the concentration of Aβ<sub>40</sub> (in TBS-T, Aβ<sub>42</sub>: 85.7±6 ng/mg brain and Aβ<sub>40</sub>: 54.9±4 ng/mg
brain, \( p<0.01 \). In guanidine-HCL \( \text{A\beta}_{42} \): 0.10±0.05 ng/mg brain and \( \text{A\beta}_{40} \): 0.05±0.02 ng/mg brain, \( p<0.01 \); see figure 6e).

The ratio of \( \text{A\beta}_{40}/42 \) observed was similar in the two fractions (0.66±0.2 and 0.64±0.01 respectively), as expected from the \( \text{APP}_{\text{swe/PS1}_{\text{dE9}}} \) strain (Garcia-Alloza, et al., 2006). Strong positive correlation was found between \( \text{A\beta}_{40} \) and \( \text{A\beta}_{42} \) (Bivariate Pearson’s <0.01). No other correlations could be found.

Discussion

Vascular disorders have been associated with clinical symptoms of AD (Breteler, 2000, Helzner, et al., 2009, Iadecola, 2010, Larson and Lesne, 2012). The early detection of cerebrovascular pathology in patients with suspected AD is therefore of primary importance for the initiation of preventive strategies. Because changes in functional physiological parameters, like microvascular deficits, can be subtle and region-specific, a methodology that offers sensitive quantification together with a high spatial resolution is required for the detection of these deficits.

In this study, a voxel-wise analysis (VBA) approach was applied to the microvascular rCBV maps (rCBV_{micro}) to spatially localize the cerebrovascular changes in the \( \text{APP}_{\text{swe/PS1}_{\text{dE9}}} \) mouse model, not limited to predetermined neuroanatomical locations. Because of its non-invasiveness, we were able to follow rCBV_{micro} changes longitudinally. We showed some clusters of voxels indicating early rCBV_{micro} impairment in 8 months old \( \text{APP}_{\text{swe/PS1}_{\text{dE9}}} \) mice in cortical areas such as the infralimbic area (ILA), in the anterior group of the dorsal thalamus and in the hypothalamic lateral zone (LZ). At 13 months of age we described a widespread decreased rCBV_{micro} in several cortical and subcortical brain regions of old \( \text{APP}_{\text{swe/PS1}_{\text{dE9}}} \) mice. These findings were based on the analyses of gradient echo (GE) derived \( \Delta R_{2}^{*} \) maps combined with a histogram analysis developed in this study for the selection of the microvascular compartment. For this purpose, a 3D GE sequence was chosen. Because of the short repetition time needed, GE sequences offer the possibility to acquire a whole-brain volume with a very high isotropic resolution in a reasonable acquisition time. This is of particular importance when a voxel-wise approach is applied, in order to have a better co-registration of the individual datasets.
Figure 6. Parenchymal and soluble β-amyloid pathology in the brains of APP<sub>swe</sub>/PS1<sub>dE9</sub> mice. Photomicrographs at 5× magnifications show the β-amyloid load in different brain regions of 13 months old APP<sub>swe</sub>/PS1<sub>dE9</sub> mice based on the mouse brain atlas of Franklin and Paxinos, 1997. Scale bar=250μm. A, B, C: hippocampus was quantified from -2.18 to -2.46 posterior to bregma and the CA1, CA3 and DG regions were defined. The ACg and PLA were quantified at level +1.10 to +0.86 and at level +1.98 up to +1.78 anterior to bregma respectively. D: Area covered by β-amyloid increases differently in different brain regions due to aging. DG dentate gyrus, PLA prelimbic area, ACg anterior cingulated gyrus. E: presence of Aβ<sub>40</sub> and Aβ<sub>42</sub> in TBS-T and guanidine-HCL fractions in 13 months old APP<sub>swe</sub>/PS1<sub>dE9</sub> mice. Result are normalized to the protein concentration of the sample. Values represent mean ± SEM. *p<0.05.

Moreover, we employed a 3D excitation to minimize the inflow effect and yields a more reliable signal (Wu, et al., 2003). Previous studies demonstrated an equal susceptibility weightings for all vessels’ sizes for the ΔR<sup>2*</sup> index (Boxerman, et al., 1995, Dennie, et al., 1998, Tropres, et al., 2001). This feature of ΔR<sup>2*</sup> limited its use in rCBV evaluation, as the evaluation of capillary rCBV is often preferred in neurological studies. For this study, we developed a method to shift the sensitivity of ΔR<sup>2*</sup> images towards the microvascular compartment based on analysis the distribution of ΔR<sup>2*</sup> values in the whole brain. Because the capillary density in rodents does not differ significantly in distinct brain regions (Weiss, et
al., 1982), it is likely that the $\Delta R_2^*$ will mostly dependent on the percentage of macro- and microvessels included in the region of interest selected. For example, in a region that contains only capillaries, we observed a normal distribution around a mean capillary blood volume fraction (data not shown). In contrast, if a voxel partially or totally includes a major artery or vein, the blood volume fraction is substantially higher, and the resulting $\Delta R_2^*$ value can be easily separated with a threshold-based approach from the capillary distribution.

Indeed our analysis for the whole brain showed a distribution of $\Delta R_2^*$ that consisted of two peaks that we associated with contributions of macro- and microvascular compartments. In support of our methodology, we found a strong positive correlation between the capillary density, as reflected by GLUT-1 staining, and the rCBV indexes of the microvascular compartment. The major surrounding and penetrating brain vessels obtained from the rCBV$_{Macro}$ maps were in good correspondence with an anatomical atlas of mouse brain vasculature (Dorr, et al., 2007). Hence, the analysis of the $\Delta R_2^*$ distribution provides a useful division of tissue vasculature, that can be employed as an attractive tool for noninvasive brain analysis.

The results obtained in the ROI based approach demonstrated the importance of this two compartments separation, as we were able to detect statistical significant differences in the hippocampus of 13 months old APP$_{swe}$/PS1$_{DE9}$ mice only after removing the contribution of the macrovasculature. Cerebral macrovascular abnormalities, such as narrowing, vessel voids, constrictions and partial occlusions, were also described in AD patients and animal models (Beckmann, et al., 2003, El Tayara Nel, et al., 2010, Krucker, et al., 2004). However, in this study we decided not to consider the $\Delta R_2^*$ from the large cerebral vessels because of the difficulties with correct quantification and determination of rCBV$_{Macro}$ due to partial volume effects, and susceptibility artifacts due to the higher concentration of CA. As the brain vascular network includes a large variety of vessels such as capillaries, arterioles, venules, arteries and veins, each with its own size, the two-component model is an approximation of the vessels included in a voxel.

The reduced microvascular rCBV$_{micro}$ found in our APP$_{swe}$/PS1$_{DE9}$ mice matches well with other studies in AD mouse models (Wu, et al., 2004; Hooijmans et al 2009). The technique developed in our study enabled us to determine maps of reduced blood volume with the highest possible spatial resolution – equal to the voxel size. From these maps, we could identify several areas of reduced CBV that
correspond to AD pathogenesis and relate to areas of spatial memory and learning (e.g. hippocampus, primary somatosensory area (SSp) and secondary somatomotor area (MOs). Notably, in 2008 Liu and colleagues found robust losses (~50%) of monoaminergic (MAergic) neuronal axonal losses in these same areas in 12 months old APP\textsubscript{swt}/PS1\textsubscript{de9} mice (Liu \textit{et al} 2008). The occurrence of axonal degeneration followed the load of Aβ deposits and preceded atrophy of cell bodies and loss of MAergic neurons. These results imply an Aβ-dependent effect on neuronal degeneration although in absence of precise co-localization.

To confirm whether the same hypothesis could be applied for the CBV results in our study and to analyze the plausible relation between neuronal degeneration and decreased CBV, further multi-modal longitudinal analyses will be necessary. In this scenario, the use of a voxel-based approach, as presented here, would be required to spatially relate multiple parameters and biomarker changes over time.

A significantly reduced rCBV\textsubscript{micro} is also apparent in the inferior part of the brain, adjacent to the circle of Willis, and following the internal carotid artery (IC), anterior cerebral artery (ACA) and the basilar artery (BA). Although these major arteries were excluded from the VBA, it is possible that blood flow disturbances or structural flaw in these macrovessels could have caused a reduction in blood volume in the perfused areas. This hypothesis is in agreement with macroscopic vascular alterations in these large arteries that are found in APP×PS1 mice as the Aβ pathology progresses (El Tayara Nel \textit{et al} 2010)

In general, a decrease in CBV may be caused by vasoconstriction or capillary loss or disruption; the last hypothesis, however, is less likely since we did not find any difference in the hippocampal capillary density between genotypes after quantification of the amount of bloodvessels visualized immunohistochemically with GLUT-1 staining in both 8 and 13-month-old groups. Loss of GLUT-1 transporters in capillary endothelium is mediated by the presence of Aβ and visible in APP\textsubscript{swt}/PS1\textsubscript{de9} mice starting from 18 months of age (Hooijmans, et al., 2007a); the relatively younger age of the mice used for this study ensure that no significant GLUT-1 changes had occurred yet, that could compromise the vessel density quantification. Thus, diminished rCBV\textsubscript{micro} in our APP\textsubscript{swt}/PS1\textsubscript{de9} mice is more likely caused by vasoactivity impairment of the capillaries and small arteries and reduced cerebral blood flow.

The equilibrium between Aβ production, transport and clearance is dependent on lipid and lipoprotein content (Altman and Rutledge, 2010), on the
functionality of the blood brain barrier (BBB) (Zlokovic, 2008), and of the neurovascular unit, including pericytes, astrocytes and microglia (Iadecola, 2004, Peppiatt, et al., 2006). Overproduction and neurovascular accumulation of Aβ, as occur in APP<sub>swe/PS1<sub>dE9</sub></sub> mice, are capable to disturb this balance by several mechanisms and consequently induce hypoperfusion. These include the interaction with receptors for advanced glycation end products (RAGE) for the transport of Aβ across the BBB and the increased expression of proinflammatory cytokines and endothelin-1 (ET-1), that enhances the production of superoxide radicals and mediates Aβ-induced vasoconstriction (Zlokovic, 2008). A reduced vasoactivity in response to endothelium-dependent vasodilators, such as acetylcholine and bradykinin has also been shown in mice overexpressing Aβ (Iadecola, et al., 1999), suggesting a role of Aβ in inducing capillaries endothelial dysfunction. Altered endothelial function and BBB disruption therefore amplify the Aβ-induced stress by restricting clearance of Aβ. These events may then cause decreased blood flow and hypoxia of the neurovascular unit, resulting in accumulation of metabolic waste products and changes in brain microenvironment that leads to neurodegeneration. Furthermore, deposition of Aβ in bloodvessels walls results in the development of cerebral amyloid angiopathy (CAA), which may be the cause of structural damage of brain vessels and is reported to be lifelong increasing in APP<sub>swe/PS1<sub>dE9</sub></sub> mice (Ghiso and Frangione, 2002). Here, we quantified Aβ in the vessel walls in the hippocampus and found no relationship with the decreased rCBV<sub>micro</sub>. Levels of Aβ in the bloodvessels were significantly lower than plaque burden, with high inter-variability between mice. These results are comparable with other studies in this mouse strain (Hooijmans et al 2007a; Hooijmans et al 2009). Therefore it is unlikely that CAA alone is sufficient to cause the reduction in CBV reserves as found in the transgenic mice at this age. Because Aβ accumulation in bloodvessel walls is a lifelong process, we believe that CAA could induce severe vascular damage in older animals in an advanced stage of the pathology. Transgenic mice for β-amyloidosis show also increased levels of soluble forms of Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub>. Most studies have demonstrated that the soluble non-fibrillar species of Aβ are deleterious to blood vessels and neurons, rather than aggregated fibrillar forms (Haass and Selkoe, 2007). In particular, Aβ<sub>1-42</sub> oligomers are currently suspected to be the cause of synaptic dysfunction and neurodegeneration in AD, and were correlated with the severity of cognitive impairment in humans (McLean, et al., 1999) and associated with a reduction of
the number of synapses (Kamenetz, et al., 2003; Lue, et al., 1999). Instead, Aβ_{1-40} have a more pronounced effects on brain vascular activity. A dose-response reduced resting CBF has been described in mice due to superfusion of Aβ_{1-40} on the neocortex (Niwa, et al., 2000). Levels of Aβ_{1-40} and Aβ_{1-42} in APP_{swen}/PS1_{de9} mice at 13 months of age were comparable with data from other studies (Hao, et al., 2011). However, the expected correlation between blood volume changes and Aβ_{1-40} levels was not found, which might be because of Aβ_{1-40} was analyzed using tissue of the half-whole brain and not in co-localized brain structures that showed rCBV_{micro} reduction.

To summarize, in this paper we described a novel method to characterize vascular impairment with a voxel-wise approach, that is based on the analysis of ΔR_{2*} data acquired with a 3D GE sequence into microvasculature CBV maps by histogram evaluation. This methodology offers a valid non-invasive tool for the identification of early CBV deficits in mouse models for AD, and allows the evaluation of future therapeutic strategies targeting microvascular integrity as risk factor for neurodegeneration and Alzheimer’s disease with the highest possible spatial resolution.
Gray and white matter degeneration revealed by diffusion in an Alzheimer mouse model

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Abstract

In patients with Alzheimer’s disease (AD) the severity of white matter degeneration correlates with the clinical symptoms of the disease. In this study, we performed diffusion-tensor MRI (DT-MRI) at ultra-high field in a mouse model for AD (APP<sub>swe</sub>/PS1<sub>e9</sub>) in combination with a voxel-based approach and tractography to detect changes in water diffusivity in both white and gray matter, as these reflect structural alterations in neural tissue. We found substantial changes in water diffusion parallel and perpendicular to axonal tracts in several white matter regions like corpus callosum and fimbria of the hippocampus, that match with previous findings of axonal disconnection and myelin degradation in AD patients. Moreover, we found a significant increase in diffusivity in specific hippocampal sub-regions, which is supported by neuronal loss as visualised with the Klüver-Barrera staining.

This work demonstrates the potential of ultra-high field DT-MRI as a non-invasive modality to describe white and gray matter structural changes in mouse models for neurodegenerative disorders, and provides valuable knowledge to assess future AD prevention strategies in translational research.
**Introduction**

Alzheimer’s disease (AD) is the most common type of dementia and is characterized by a progressive loss of neuronal function, leading to gradual memory impairment, confusion and general withdrawal. Pathological hallmarks are the accumulation of extracellular amyloid plaques, caused by amyloid-β protein (Aβ) aggregation, and the presence of intracellular neurofibrillary tangles (NFTs), formed by aggregates of the hyperphosphorylated tau protein. These pathological changes originate in the medial temporal lobe, especially the entorhinal cortex and hippocampus, spreading further across the limbic cortex and neocortex (Arnold, et al., 1991; Braak and Braak, 1995). Along with Aβ- and NFTs gray matter pathology, histological studies identified several changes in white matter structures. Over 50% of confirmed cases of AD show white matter disease (WMD) in neuropathological examinations, with a widespread distribution in patients with moderate- to late-stage dementia (Englund and Brun, 1990). Several studies reported a correlation of the incidence of white matter lesions with severity of the underlying AD pathology (Bozzali, et al., 2002) (Bronge, et al., 2002, de Groot, et al., 2000). The etiology of AD-related white matter pathology remains to be fully elucidated, although some underlying processes have been proposed, including (1) interhemispheric disconnection through Wallerian degeneration (Tomimoto, et al., 2004), 2) axonal damage and gliosis following vascular disease (Englund, 1998) and (3) primary myelin degradation resulting in axonal disconnection (Medina, et al., 2006, Xie, et al., 2006).

Magnetic resonance imaging (MRI) offers tools to measure white and gray matter architecture in vivo. Diffusion weighted MRI measures the incoherent motion of water molecules for every imaged voxel and provides complementary information to conventional MRI on tissue microstructure (Le Bihan, et al., 1986). Since its first description (Basser, et al., 1994), diffusion tensor MRI (DT-MRI) has been widely used to investigate white matter because of the relatively coherent organization of axons in fiber bundles that results in a marked diffusion anisotropy, with greater diffusivity occurring along the axonal direction. By measurement of the diffusivity in multiple directions, DT-MRI can reconstruct an ellipsoid to model the diffusion in every voxel. The diffusion tensor is characterized by the magnitude of the diffusivity over its three axes.
(eigenvectors). The mean diffusivity (MD) is the average of these diffusivities and captures the size of the tensor. Other informative measures include the axial diffusivity ($\lambda_1$) aligned to the primary diffusion direction and the radial diffusivity (RD) that represent the diffusivity perpendicular to this main direction. The shape of the diffusion tensor is often quantified by the fractional anisotropy (FA), which is an index between 0 and 1 that indicates to what degree diffusivity is different over the three axes of the tensor.

DT-MRI is particular well suited for studies of neurological disorders, like AD, because structural changes in neural tissue, like neuronal cell death and white matter microstructural pathology, are reflected in shape and size of the diffusion tensor (Kantarci, et al., 2005). In AD patients changes in DT-MRI parameters are particularly consistent, showing increased diffusivity with loss of directionality - decreased FA - in white matter and increased MD in gray matter regions (Hanyu, et al., 1998, Kantarci, 2011, S.-K. Song, et al., 2004). Histological examinations in brains of AD mice models suggested that myelin loss, decrease in axonal density and axonal disconnection contribute to the diffusion changes in white matter (Chen, et al., 2011, S.K. Song, et al., 2004). In gray matter, the increased diffusivity has been attributed to neuronal loss, as the diffusivity of water molecules increases when less cell membrane restrict their random motion (Sykova, et al., 2005). Interestingly, changes in gray matter diffusion - e.g. elevated hippocampal MD - predicted the conversion from mild cognitive impairment (MCI) to AD similarly or even better than hippocampal volumetric changes in two independent studies (Fellgiebel, et al., 2006, Kantarci, et al., 2005). These results highlight the importance of characterizing the diffusion properties not only in white, but also in gray matter structures, as these contain unique and complementary information about the progression of the disease.

For translational research in AD it’s important to perform similar DT-MRI studies in animal models of the disease, but only recently the availability of new dedicated hardware and methods for acquisition and data analysis enabled the study of brain water diffusion in small animals. The results reported in mouse models of cerebral amyloidosis showed white matter diffusion changes similar to those in human studies, but no further investigation on diffusion in gray matter sub-structures has yet been performed (S.K. Song, et al., 2004, Sun, et al., 2005).

In this study, we aimed to assess water diffusion changes both in white and in gray matter in a double transgenic mouse model for AD (APP$_{swe}$/PS1$_{de9}$). We used
state-of-the-art methodology for in vivo DT-MRI data acquisition (Harsan et al 2010b) at ultra-high field (11.7T) with respiratory and cardiac motion correction and robust tensor estimation (L.A. Harsan, et al., 2010, Zwiers, 2010). Thereafter, we employed both ROI-based and whole-brain voxel-based approaches in combination with tractography algorithm to describe genotype differences with the highest possible spatial resolution. This enables an innovative and accurate description of diffusion changes, for a comprehensive spatial characterization of the underlying pathology in our AD mouse model.

**Material and methods**

**Animals**
The \(\text{APP}_{\text{sw}e}/\text{PS1}_{\text{dE9}}\) founders were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Department of Pathology). A colony was bred and established at the Central Animal Facility at the Radboud University Nijmegen Medical Centre, The Netherlands. The transgenic mice were created by co-transfection with chimeric mouse/human amyloid precursor protein \(\text{APP}_{\text{sw}e}\) (mouse APP695 harboring a human A\(\beta\) domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human presenilin 1 with deletion of exon 9 (PS1\(_{\text{dE9}}\)) vectors controlled by independent mouse prion protein promoter element (Jankowsky, et al., 2004, Jankowsky, et al., 2001). These two genes co-integrate and co-segregate as a single locus. Breeder mice were backcrossed to C57BL6/J for 12 generations to obtain the animals for this study. Throughout the duration of the experiment the mice were housed in groups. Room temperature was kept at 21° C with an artificial 12h light:dark cycle. Food and water were available \textit{ad libitum}. The experiments were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority of the Radboud University Nijmegen Medical Centre.

**MR imaging**
Twelve months old male \(\text{APP}_{\text{sw}e}/\text{PS1}_{\text{dE9}}\) transgenic mice (n=9) and age-matched wild type (WT) littermates (C57BL6/J n=15) were used for DT-MRI. Isoflurane (3.5% for induction and ~2% for maintenance) was used for anesthesia. The
anesthetic concentration was adjusted during the experiment in order to maintain the breathing frequency at 65-85 per minute. The mice were placed in a stereotactastic device in order to immobilize the head. Body temperature was measured using a rectal thermometer and maintained at 37° C using a heated air flow device.

MR measurements were performed on a 11.7 T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m. We used a circular polarized volume resonator for signal transmission and an actively decoupled mouse brain quadrature surface coil for signal reception (Bruker BioSpin).

Gradient echo (GE) images in the axial, sagittal and coronal orientation were acquired to visualize the anatomy and the morphology of the mouse brain structures. Imaging parameters were: echo time (TE) = 5 ms, repetition time (TR) = 630 ms, flip angle = 12 deg, field of view (FOV) = 40×40 mm, matrix size = 512×512, slice thickness = 0.345 mm.

Diffusion MRI was performed following a modified protocol of Harsan et al (L.A. Harsan, et al., 2010). In short, 31 axial slices covering the whole brain were acquired with a spin-echo planar imaging protocol (SE-EPI). B0 shift compensation, navigator echoes and automatic ghost correction algorithm were implemented to limit the occurrence of ghosts and artifacts. Encoding b-factors of 0 s/mm² (b0 images; 5×) and 1000 s/mm² were used and diffusion-sensitizing gradients were applied along 30 non-collinear directions of three-dimensional space. Other imaging parameters were: TE = 21.4 ms, TR = 7750 ms, time between the application of diffusion gradient pulses Δ = 10 ms, diffusion gradient duration δ = 4ms, number of segments = 4, total resolution 156×156×500 μm. This results in a total scan time of 18 minutes for each mouse.

Data preprocessing
For spatial normalization, a study-specific template was created of all WT and transgenic animals using Advanced Normalization Tools (ANTs. V1.9.x, http://picrosl.upenn.edu/ANTS/). A group-wise normalization procedure (SyGN, as implemented in the buildtemplateparallel.sh script V0.0.13) was employed on the realigned mean diffusion image of each mouse (Avants et al 2008). The default implementation was followed with four iterations using mutual information as the initial affine similarity metric and cross-correlation as ‘greedy
SyN’ diffeomorphic transformation metric. A mask that included the entire brain was manually defined in the normalized coordinate space and transformed to each animal’s native space for use in subsequent processing.

The diffusion tensor was estimated for every voxel using the PATCH algorithm (Zwiers, 2010). This method incorporates realignment and is robust against both regional (e.g. cardiac motion) and slice-wise (e.g. bulk motion) artifacts by providing a weight for every voxel that reflects the probability of being an outlier in the tensor fitting. From the eigenvalues of the diffusion tensor, the rotationally invariant indices fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity ($\lambda_1$) were calculated. The resulting volumes were spatially normalized to the template space using the affine parameters and deformation field obtained at template creation.

**Voxel-based group comparison**

Regional differences in spatially normalized FA, MD, RD and $\lambda_1$ maps between APP$_{swe}$/PS1$_{dE9}$ mice and WT littermates were assessed voxel-wise using MATLAB R2008a (Mathworks, Natick, MA, USA) and Statistical Parametric Mapping 5 (SPM5, Wellcome Department of Clinical Neurology, London, UK) with the SPMMouse toolbox (Sawik, et al., 2009). Two t-tests were performed to identify increase (APP$_{swe}$/PS1$_{dE9}$ > WT) or decrease (APP$_{swe}$/PS1$_{dE9}$ < WT) genotype-wise differences in the framework of the general linear model (GLM). Statistical significance for an individual voxel was established at $p<0.01$, uncorrected for multiple comparisons. The locations of significant voxels exceeding a minimum cluster size of 4 (to achieve cluster size ≈ 0.05 mm$^3$ as in (Dubois, et al., 2008)) were determined with an anatomical atlas (Franklin K, 1997, Franklin and Paxinos, 1997). The contrasts were then color coded and overlaid onto images derived from the template image.

**ROI-based group comparison**

To compare our results with other studies, the DTI parameter maps FA, MD, RD and $\lambda_1$ were evaluated for significant genotype differences with a region of interest (ROI) approach. For the ROI-based approach, ten white matter tracts were selected bilaterally across the mouse brain based on the atlas of (Franklin K, 1997,Franklin and Paxinos, 1997). These regions include the anterior commissure, anterior commissure posterior, and genu – body – splenium of the
corpus callosum, cerebral peduncle, external capsule, fimbria of the hippocampus, fornix and optic tract. The hippocampus and cerebral cortex (all cortical areas above the corpus callosum) were also defined approximately at -1.22 up to -2.54 posterior to bregma.

**Image analysis and tractography**

To determine the fiber systems involved in areas of significant difference between APP\textsubscript{swe}/PS1\textsubscript{dE9} and WT, we investigated those regions with tractography. We used MRtrix' (v.0.2.9) constrained spherical deconvolution (CSD) with spherical harmonic order 4, for obtaining fiber orientation distributions (FODs) (Tournier, et al., 2007). Deterministic CSD tractography was initiated from the VBA clusters of significant FA differences separately for APP\textsubscript{swe}/PS1\textsubscript{dE9} > WT and APP\textsubscript{swe}/PS1\textsubscript{dE9} < WT. Fibers were seeded from these regions until 10000 fibers with a minimum of 1 mm, maximum length of 20 mm and stepsize 0.02 mm. Termination criteria were set at: FA threshold < 0.1 and curvature radius > 0.05.

**Histology**

In order to compare the axonal organization in hippocampal and cortical gray matter structures with our DT-MRI images and to demonstrate the correlation between changes in water diffusion as found with DT-MRI and the degeneration of the brain tissue, we performed two staining methods: one for myelin and nerve cell bodies (Klüver-Barrera staining) and one for myelinated and unmyelinated nerve fibers (Bodian silver staining). These stainings were performed on brain tissue of two APP\textsubscript{swe}/PS1\textsubscript{dE9} and two wild type littermates of the scanned group of mice. After perfusion with saline the brains were removed from the skull and immersion fixed overnight in 4% phosphate buffered paraformaldehyde. After fixation, the brain tissue was dehydrated in alcohol series, cleared in xylene and embedded in paraffin. Sections of 5 µm were cut on a rotary microtome, mounted on albumen-coated glass slides and dried overnight at 37 °C. After removing the paraffin by xylene the sections were rehydrated in alcohol series and rinsed in distilled water. The brain sections (Bregma -2.92 mm, based on the atlas of Franklin and Paxinos, 1997) were stained according to Bodian (1936, 1937), or according to Klüver and Barrera (1953). After dehydration in alcohol series, sections were cleared with xylol and mounted in Entellan.
Statistical analysis

Statistical analyses for the ROI-based approach were performed using SPSS v16.0 (SPSS Inc. Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). Differences between genotypes in multiple ROIs were analysed with multivariate ANOVA’s with Bonferroni correction. Statistical significance was established at $p<0.05$.

Results

For each mouse, fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity ($\lambda_1$) maps were generated from the DT-MRI data (Figure 1-a). The high resolution of the images enabled us to recognize the organization of many structures in the mouse brain. This is illustrated in Figure 1-b for ten slices of color-coded fractional anisotropy (FA) maps of a wild type mouse, overlaid with the primary diffusion direction $\lambda_1$. These composite images incorporate in each voxel information from the anisotropy degree and the main diffusion orientation, and help to identify several white matter substructures that are difficult to distinguish by other image modalities (Jiang and Johnson, 2010). For example, neighboring white matter structures like the cerebral peduncle (cp) and the optic tract (ot) are readily discriminated from these images (Figure 1-b). Other white and gray matter structures are also easily identifiable, like the anterior commissure anterior (aca), anterior commissure posterior (acp), genu (gcc) – body (bcc) – splenium (scc) of the corpus callosum, external capsule (ec), fimbria of the hippocampus (fi) and fornix (f).

Voxel-based analysis and tractography

An anatomical template was overlaid with $p$-value maps ($p$-value with threshold at 0.01, minimum voxel cluster size set at 0.05 mm$^3$) in order to visualize the distribution of water diffusivity differences between the APP$_{swe}$/PS1$_{dE9}$ transgenic and wild type mouse brains. Voxels that differ significantly are indicated by the colored overlay (Figure 2). Radial diffusivity (RD), mean diffusivity (MD), axial diffusivity ($\lambda_1$) and FA were investigated using this method. The VBA indicates several areas of significant differences between the two genotypes. In several clusters in the corpus callosum (cc) and external capsule (ec), a decrease in all
Figure 1: Coronal images of diffusion parameter maps from a wild-type mouse. Fractional anisotropy (FA), mean diffusivity (MD), radial diffusivity (RD) and axial diffusivity ($\lambda_1$) maps are shown (a). Regions of interest were selected on a representative series of FA maps, color-coded by the diffusion orientation, overlaid with the primary diffusion direction $\lambda_1$ (b). Anterior commissure anterior (aca), anterior commissure posterior (acp), cerebral peduncle (cp), optic tract (ot), genu (gcc) – body (bcc) – splenium of the corpus callosum (scc), external capsule (ec), fimbria of the hippocampus (fi), fornix (f), hippocampus (hc) and cerebral cortex (ct) were defined approximately from bregma 1.10 mm to -3.16 mm, based on the atlas of Paxinos 1997.
Figure 2: Voxel based analysis indicate significant differences in radial diffusivity (RD), mean diffusivity (MD), axial diffusivity ($\lambda_1$) and fractional anisotropy (FA) in transgenic APP<sub>swe</sub>/PS1<sub>dE9</sub> mouse brains compared to wild type. Five rostral to caudal axial diffusion weighted maps are overlaid with voxels that showed a significant difference ($p < 0.01$, voxel cluster size $0.05 \text{ mm}^3$). The voxel color indicates a negative or positive change in the transgenic mice compared to the wild type mice. VBA results were overlaid on top of a template image derived from the dataset. A widespread increase in RD is found in the hippocampus and in the fimbria (fi), while decreases are present in the corpus callosum (cc), cerebral peduncle (cp) and in the lateral posterior thalamus nuclei (LP). The MD decrease is found amongst others in the internal capsule (ic) and in the cc. An increased MD in the hippocampus and fi is also present. An increase in $\lambda_1$ is found along the boundaries of the hippocampus, while a decrease is present in the splenium of cc (scc). FA reduction was observed in areas such as the scc, fi and cortex. Increases in FA are found in the cp, ic LP.
DT-MRI parameters (FA, MD, RD & \( \lambda_1 \)) was visible. The decrease in FA resulted from the stronger decrease in \( \lambda_1 \) than in RD. In the fimbria of the hippocampus, bilateral clusters of voxels showed a large increase in MD, RD and \( \lambda_1 \), with decreased FA. In the area corresponding to the cerebral peduncles, a decrease in RD is notable, together with an increase in FA and a slight decrease in \( \lambda_1 \).

Similar changes were detected in other areas, like the internal capsule (ic) and the lateral posterior thalamus nuclei (LP). The fiber tracts of a wild type mouse shown in Figure 3 were obtained from seeding fibers in the clusters of significant differences in FA from the VBA (overlaid in the pictures). The seeded voxels were differentiated in areas where \( \text{APP}_{\text{swe/PS1}}/\text{PS1}_{\text{de9}} \) mice showed lower FA values (Figure 3a, in blue) and higher FA values (Figure 3b, in red) compared to wild type. By tracking fibers from voxels of significantly lower FA in the \( \text{APP}_{\text{swe/PS1}}/\text{PS1}_{\text{de9}} \) mice, we identified several white matter fiber bundles involved in degeneration processes in these mice. Among them, the whole corpus callosum with its extensions into the cortex and the fimbria of the hippocampus were the areas most affected (Figure 3a). Conversely, the tracts resulting from seed voxels of higher FA in the transgenic mice did not form single coherent fiber bundles, but these voxels showed a marked co-localization with crossing fibers, originating from the thalamus, midbrain and hypothalamus (Figure 3b).

Interestingly, the VBA indicated clusters of significant genotype differences located in gray-matter structures. In both left and right hippocampus, the MD, RD and \( \lambda_1 \) increased in transgenic mice compared to the control (Figure 2). The FA did not change significantly. A closer look at these results shows that most of the differences are present in the LMol and in the Mol layers of the hippocampus (Figure 2). In some cortical areas, mainly in the visual cortex, a few clusters of decreased FA and \( \lambda_1 \) were also found.

**Regions of interest (ROI) based analysis**

For a quantitative assessment of genotype differences, we analyzed the DT-MRI indices in ROI drawn on several white and (two) gray matter structures. The results are shown in Table 1. A significant decrease in diffusion along the main fiber orientation (\( \lambda_1 \)) was seen in the anterior commissure posterior (\( p=0.030 \)), cerebral peduncle (\( p=0.020 \)), body of corpus callosum (\( p=0.010 \)) and fornix (\( p=0.025 \)) of transgenic mice. In the latter three regions, this was also associated with a decreased MD (\( p=0.004, p=0.044 \) and \( p=0.023 \) respectively). In the fimbria
of the hippocampus, we found a strong increase in RD \( (p<0.001) \), linked with a decreased FA \( (p=0.013) \) and increased MD \( (p=0.037) \). In the total hippocampal area, we detected a strong significant increase in MD, RD and \( \lambda_1 \) \( (p=0.001, p=0.003 \) and \( p=0.002 \) respectively). In the cerebral cortex, a decrease in FA was seen \( (p=0.035) \). Overall, diffusion values resemble the results from other similar studies (L.-A. Harsan, et al., 2010, Ruest, et al., 2011).

**Gray matter diffusion and genotype comparison with histology**

To illustrate the advantage of high resolution MR imaging to assess directional diffusivity in grey matter structures, magnifications of the DT-MRI images of hippocampal area (Figure 4) and cortical area (Figure 5) were compared with stainings for myelin and nerve cells (Kluver-Barrera) and staining for nerve fibers (Bodian). The anatomical layers which constitute the hippocampus are discernible by the different orientation of their diffusion tensors (Figure 4 a-b): two layers of marked anisotropy and medial-lateral orientation, correspond to the stratum radiatum (Rad) and the molecular layer of dentate gyrus (Mol), and they alternate with two thin layers of more isotropic diffusion: i.e. the lacunosum molecular layer (LMol) and the granular layer of the dentate gyrus (GrDG) / polymorph layer of the dentate gyrus (PoDG). The actual orientation of the axons is visualized in the hippocampal layers by the Klüver-Barrera staining (Figure 4 c-d) and by the Bodian staining (Figure 4e). In the latter, the alignment of the fibers in the direction radial to the cortical surface is discernible in the Rad and in the Mol layers. An incoherent alignment of the nerve fibers is found instead in the LMol, GrDG and PoDG. Overall, a good anatomical agreement is found between DT-MRI data and nerve fibers directionality and alignment. Sections of the same Bregma (-2.92 mm) stained according to Klüver-Barrera, are shown in order to demonstrate neurodegeneration in the APPswe/PS1dE9 (Figure 4d) compared with wild-type mice (Figure 4c). In the APPswe/PS1dE9, the GrDG layer is significantly thinner compared to WT mice due to a marked reduction of neurons as shown with the Klüver-Barrera staining (Figure 4d, arrows). In the magnification insert of the APPswe/PS1dE9 mouse (Figure 4g) a decreased amount of stained healthy neuronal cell bodies is visible in this layer and many more pycnotic dark stained cells are shown compared to the WT (Figure 4f), suggesting degenerated neurons. Spherical-shaped areas with no staining are present in the APPswe/PS1dE9 tissue, most likely related to the presence of Aβ
Figure 3: Example of fiber tracts of a wild type mouse seeded from the clusters of significant differences in FA. (a) shows tracts from the voxels where APPswe/PS1dE9 mice showed significantly lower FA values (overlaid as blue voxels). The tracts defined in (a) suggest the areas that appear to be more affected in transgenic mice, particularly corpus callosum, fimbria of hippocampus and fornix. (b) shows tracts from clusters where APP mice showed significantly higher FA values (overlaid as red voxels). The tracts in (b) spread out in multiple directions, suggesting the presence of crossing fibers.

plaque deposits (Figure 4d, asterisks). No major differences in amount of nerve fibres between APPswe/PS1dE9 and WT mice were detectable with the Bodian staining (not shown). In the cerebral cortex the primary diffusion direction radial to the cortical surface can be clearly identified (Figure 5).
Figure 4: Diffusion orientations in a representative wild type (WT) mouse hippocampus at 12 months of age (a, b). The primary diffusion tensor orientation is overlaid on FA maps, color-coded by the diffusion orientation (a). The diffusion is shown with fiber orientation distribution (FODs) overlaid on the FA (b). In the hippocampus, two layers of marked anisotropy and medial-lateral orientation alternate with two layers of more isotropic diffusion. The anatomical orientation of the axons is visualized in the hippocampal layers.
by the Klüver-Barrera staining in a WT and an APP<sub>swe</sub>/PS1<sub>dE9</sub> mouse (c, d respectively, 10× magnification) and in a WT mouse by the Bodian staining (e, 5× magnification). In the stratum radiatum (Rad) and in the molecular layer of the dentate gyrus (Mol) the fibers are oriented in the direction radial to the cortical surface, as visible in the Bodian staining (e). A less coherent alignment of the nerve fibers is found instead in the lacunoso-molecular layer (LMol), in the granulate layer of the dentate gyrus (GrDG) and in the polymorph layer of the dentate gyrus (PoDG). Overall, a good anatomical agreement is found between diffusion data and nerve fibers directionality and alignment. The Klüver-Barrera staining of a WT (c) and of a APP<sub>swe</sub>/PS1<sub>dE9</sub> (d) and their correspondent magnifications (f, g, 40× magnification) illustrate neurodegeneration in the transgenic mice, with thinner GrDG layer (d, arrows), loss of healthy neurons and many pycnnotic dark stained cells and spherical-shaped areas with no staining, possibly due to the presence of Aβ plaques (d, asterisks). DT-MRI images and staining sections were selected at Bregma -2.92 mm, based on the atlas of Franklin and Paxinos 1997. C, d, e: scale bar = 250 μm. F, g: scale bar = 50 μm.

This corresponds well with the axonal orientation, as shown with Klüver-Barrera and Bodian staining of the same areas (Figure 5c-d and Figure 5e respectively). Comparing APPswe/PS1dE9 (Figure 5d) and WT mice (Figure 5e), the only noticeable difference in the cortical tissue was the loss of both neurons and fibers in regions that are likely related to presence of the Aβ plaques (Figure 5d, asterisks).

**Discussion**

DT-MRI has become accepted in clinical practice as preferred tool for quantitative assessment of the structural integrity of white matter and, to some extent, of gray matter (Basser, et al., 1994, Beaulieu, 2002). Nowadays, it is possible to apply water diffusion MRI also in translational research and acquire high-resolution DTI images of the mouse brain in vivo (L.A. Harsan, et al., 2010). A recent study in rat brain validated the correlation between DTI parameters and microstructural tissue properties (Budde and Frank, 2012). This is of particular interest in experimental AD research to monitor white and gray matter degeneration in AD models for the investigation of new preventive strategies. A reduction of the apparent diffusion coefficient was found in neocortical areas of 25 months old APP23 transgenic mice, which was associated with fibrillar amyloid deposits and glial proliferation (Mueggler, et al., 2004).
Gray and white matter degeneration revealed by diffusion in an Alzheimer mouse model

## Table 1. Diffusion tensor parameters of the regions of interest. The mean (MD), radial (RD) and axial ($\lambda_1$) diffusivities are expressed in units of $10^{-3}$ mm$^2$/s. n.s.: non-significant

<table>
<thead>
<tr>
<th>Region</th>
<th>APPsw/PS1dE9</th>
<th>Wild type</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anterior commissure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0.51 ± 0.03</td>
<td>0.50 ± 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>MD</td>
<td>0.69 ± 0.03</td>
<td>0.70 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>$\lambda_1$</td>
<td>1.06 ± 0.08</td>
<td>1.08 ± 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>FA</td>
<td>0.45 ± 0.05</td>
<td>0.46 ± 0.06</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Anterior commissure posterior</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0.62 ± 0.08</td>
<td>0.65 ± 0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>MD</td>
<td>0.74 ± 0.06</td>
<td>0.77 ± 0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>$\lambda_1$</td>
<td>0.99 ± 0.04</td>
<td>1.02 ± 0.03</td>
<td>0.030</td>
</tr>
<tr>
<td>FA</td>
<td>0.31 ± 0.11</td>
<td>0.28 ± 0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Cerebral peduncle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0.42 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>MD</td>
<td>0.70 ± 0.02</td>
<td>0.74 ± 0.02</td>
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<tr>
<td>$\lambda_1$</td>
<td>1.27 ± 0.08</td>
<td>1.33 ± 0.04</td>
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<tr>
<td>FA</td>
<td>0.61 ± 0.04</td>
<td>0.60 ± 0.02</td>
<td>n.s.</td>
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<tr>
<td><strong>Corpus callosum - Body</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0.61 ± 0.05</td>
<td>0.64 ± 0.03</td>
<td>n.s.</td>
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<tr>
<td>MD</td>
<td>0.74 ± 0.05</td>
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<td>$\lambda_1$</td>
<td>1.18 ± 0.06</td>
<td>1.22 ± 0.05</td>
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<tr>
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<td>0.37 ± 0.04</td>
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<td>n.s.</td>
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<td><strong>Corpus callosum - Genu</strong></td>
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<tr>
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<td>0.59 ± 0.06</td>
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</tr>
<tr>
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<td>n.s.</td>
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<tr>
<td>FA</td>
<td>0.39 ± 0.06</td>
<td>0.44 ± 0.05</td>
<td>0.058</td>
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<td>0.79 ± 0.11</td>
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<td>1.03 ± 0.11</td>
<td>n.s.</td>
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<tr>
<td>$\lambda_1$</td>
<td>1.47 ± 0.09</td>
<td>1.52 ± 0.12</td>
<td>n.s.</td>
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<tr>
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<td>0.44 ± 0.08</td>
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<td>1.06 ± 0.02</td>
<td>n.s.</td>
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<tr>
<td>FA</td>
<td>0.29 ± 0.03</td>
<td>0.30 ± 0.02</td>
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<td><strong>Fimbria of the hippocampus</strong></td>
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<tr>
<td>RD</td>
<td>0.78 ± 0.08</td>
<td>0.65 ± 0.04</td>
<td>0.000</td>
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<tr>
<td>MD</td>
<td>1.05 ± 0.09</td>
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<tr>
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<td>0.59 ± 0.08</td>
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<td><strong>Optic tract</strong></td>
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<td>$\lambda_1$</td>
<td>1.20 ± 0.10</td>
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<td>0.48 ± 0.06</td>
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<td><strong>Hippocampus</strong></td>
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<td><strong>Cerebral cortex</strong></td>
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<td>n.s.</td>
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<td>0.91 ± 0.02</td>
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<td>FA</td>
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<td>0.18 ± 0.02</td>
<td>0.035</td>
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</tbody>
</table>
Figure 5. Diffusion orientations in a representative mouse brain cortex at 12 months of age (a,b). The primary diffusion direction is overlaid on color-coded FA maps (a). The diffusion is shown with FODs overlaid on the FA (b). The diffusion direction well resembles the actual axonal orientation radial to the cortical surface, as shown with the Klüver-Barrera staining of a WT and an APP<sub>swe</sub>/PS1<sub>δE9</sub> mouse (c-d, respectively) and with the Bodian staining of a WT (e) in the same areas (boxes in a and b). The Klüver-Barrera staining of a APP<sub>swe</sub>/PS1<sub>δE9</sub> mouse (d) revealed the presence of spherical-shaped areas characterized by omission of staining, were no fibers and cell bodies are present likely due to Aβ plaque deposits in the transgenic animals (d, asterisks). c, d, e: magnification = 10x. Scale bar = 250 μm.

In DT-MRI studies, changes in fractional anisotropy (FA) and longitudinal diffusivity (λ<sub>1</sub>) were found in 15 months old PDAPP mice and 12 months old Tg2576 mice in some white matter areas, including corpus callosum and external capsule, followed by an increase in radial diffusivity (RD) as the pathology progressed (S.K. Song, et al., 2004, Sun, et al., 2005). Remarkably, the same effects were not seen in an ex vivo study with APP<sub>swe</sub> mice brains, likely due to fixation-related alterations in post-mortem analysis (Harms, et al., 2006).
In this study, we aimed for a comprehensive analysis of the DT-MRI changes occurring not only in white but also in gray matter in 12 months old APP\textsubscript{swe}/PS1\textsubscript{dE9} mice \textit{in vivo}. At 12 months of age these transgenic mice already exhibit widespread parenchymal deposits of A\textbeta in several cortical and subcortical regions, together with progressive A\textbeta accumulation in the vascular walls, leading to cerebral amyloid angiopathy (CAA) (Garcia-Alloza, et al., 2006; Jankowsky, et al., 2004). Furthermore, deficits in learning and memory tasks were described at this age in the Morris water maze test, indicating an initial decline of neuronal function (Malm, et al., 2011).

White matter

A histological characterization of white matter degeneration of 24 months old APP/PS1 mice (with the Swedish KM670/671NL, and London mutation V717I introduced in human sequence APP751 × HMG PS1 M146L) has been recently reported (Chen, et al., 2011). This study demonstrated fibre tract volume reduction, loss of axonal neurofilaments and myelin breakdown in axonal bundles of the corpus callosum (cc) and the anterior commissure. Interestingly, atrophy of the corpus callosum is also classically observed in AD patients (Teipel, et al., 2002). Our DT-MRI results describe \textit{in vivo} changes in white matter diffusion that resemble the fibre tract anomalies reported by Chen et al, including changes in the cc. Compared to wild type littermates, the APP\textsubscript{swe}/PS1\textsubscript{dE9} mice display a smaller and more isotropic diffusion ellipsoid in some areas of the cc, particularly in the body of cc, with the largest reduction occurring in the direction of the fibre bundles ($\lambda_1$). This situation has been associated with a Wallerian-like degeneration in the presence of axonal disconnection and volume reduction of fiber tracts, as the water molecules have more restricted diffusion mostly along the tracts (Sun, et al., 2008). Wallerian-like degeneration and processes of retrogenesis are also described in the corpus callosum of mild AD patients (Di Paola, et al., 2010). We did not find an increase in RD in the cc, that has been linked to myelin degradation of the axonal bundles (Song, et al., 2005), but contrarily we found a minor decrease. This same trend has been shown already by Sun et al in 12 months old Tg2576 mice. In their experiment, mice were measured at multiple time points, and the increase in RD in the cc was detected only after 16 months of age (Sun, et al., 2005). Together these findings indicate that myelin degradation in the cc occurs in an advanced stage of the pathology.
This corresponds with histological findings, in which volume reduction of the corpus callosum in APP/PS1 mice – at 24 months of age – appeared to be largely caused by atrophy of fiber bundles, loss of axonal neurofilaments and only partly by myelin breakdown (Chen, et al., 2011). In our staining on myelin and nerve fibers, we could not detect well-defined differences in cc between APP\textsubscript{swe}/PS1\textsubscript{dE9} and WT mice. However, the lack of staining in some areas of the cc and the external capsule in the transgenic mice are likely related to deposits of Aβ, which could interfere with the shape and the integrity of the axonal bundles (Sun, et al., 2005). The presence of Aβ in white matter regions of APP\textsubscript{swe}/PS1\textsubscript{dE9} mice already has been reported at this age by ex vivo histopathological studies (Zerbi \textit{et al}, 2012). Contrary to what we expected, the VBA showed also regions of co-localized increased FA and decreased RD in the APP\textsubscript{swe}/PS1\textsubscript{dE9}, e.g. in the cerebral peduncles and thalamic areas below the hippocampus. This would suggest that in these regions wild type mice and not APP\textsubscript{swe}/PS1\textsubscript{dE9} mice show signs of white matter pathology and myelin degradation. However, as highlighted by our tractography images, in these regions the white matter is not coherently aligned as in the cc, ec or ac and crossing fibers are frequent. The FA in such regions is naturally lower than would be the case in any one of these bundles separately. In the depicted thalamic regions, the higher FA and lower RD that we found in APP\textsubscript{swe}/PS1\textsubscript{dE9} mice could therefore be caused by a degeneration of one fiber tract, in voxels that normally include more fibers with different orientations. This event would cause one fiber group to dominate in the diffusion quantification, resulting in a voxel with higher FA and lower RD. This concept has been discussed recently for AD patients, where the increase of FA in crossing-fiber areas was associated with early white matter alterations (Douaud, et al., 2011). A recent study on stained rat brain slices further demonstrated that these regions are likely to include multiple fibre populations (Budde and Frank 2012).

**Gray matter**

The interpretation of the data from gray matter diffusion imaging requires distinct considerations. Whereas the white matter consists of relatively coherent bundles of myelinated axons, in gray matter regions like cortex or hippocampus, the constituents are far more heterogeneous in size, shape and orientation. The larger volume fraction of cell bodies of neurons and glial cells compared to white matter fibre bundles yield an increased isotropic diffusion. As the dendrites are
only partially coherently organized and the axons are not homogeneously distributed and may be myelinated or unmyelinated, their contribution to the diffusion signal is uncertain and may vary in different substrate regions. Hence, if the analysis of FA do not account for these contributions, it may lose its descriptive meaning. Instead, other parameters like the averaged diffusivity along all the diffusion directions (MD) are considered more informative. There is growing evidence that the increased MD in the hippocampus is associated with loss of neurons and can be used as a good predictor of AD progression (Carlesimo, et al., 2010; Fellgiebel, et al., 2006); In the present study, a marked increase in MD was seen in the hippocampus of APP<sub>swe</sub>/PS1<sub>dE9</sub> mice, suggesting neurodegeneration. With the increased spatial sensitivity of the VBA, we were able to further circumscribe these changes to the lacunosum moleculare layer (LMol) and the molecular layer of the dentate gyrus (Mol). To the best of our knowledge, this is the first study that reports diffusion changes in AD models in specific sub-regions of the hippocampus, which is a key-area in AD pathogenesis, related to spatial memory and learning and among the earliest and most severely affected regions in AD pathology in these mice (Braak and Braak, 1991; Garcia-Alloza, et al., 2006).

The increase of MD in gray matter has been described in several human AD studies, and is often explained to be caused by neuronal degeneration and cell loss, resulting in less hindered water diffusion (Fellgiebel, et al., 2004). Although reports about neuronal loss in mice expressing APP mutations are still controversial, this explanation for our results is supported by histological analysis. In the Klüver-Barrera staining for myelin and nerve cells, we showed clear evidence of neuronal loss with thinning of the whole GrDG layer of the hippocampus in APP<sub>swe</sub>/PS1<sub>dE9</sub> mice compared to the WT. Interestingly, the neurodegeneration seems to occur adjacently to the Mol layer of the DG, where we demonstrated higher MD in APP<sub>swe</sub>/PS1<sub>dE9</sub> mice with DT-MRI.

Similar findings have been found in other studies in APP/PS1 double transgenic mice. Two studies reported a substantial age-related neuronal loss in the hippocampal pyramidal cell layer of APP/PS1 mice that could relate to our results (Ramos, et al., 2006; Schmitz, et al., 2004). Lower levels of glucose uptake were measured with fluoro-D-glucose PET (FDG-PET) in APP/PS1 mice compared to PS1 mice in the LMol and in the stratum radiatum of the hippocampus (Dubois, et al., 2010). Moreover, in 12 months old APP<sub>swe</sub>/PS1<sub>dE9</sub> mice, robust axonal loss (~50%)
of monoaminergic neurons in hippocampal and cortical regions was observed, which was correlated with progressive atrophy of cell bodies and loss of monoaminergic neurons (Liu, et al., 2008). Vascular alterations are closely associated with amyloid angiopathy, and could contribute to a progressive neuronal loss due to chronic hypoperfusion and/or ischemia (de la Torre, 2002). Many reports, including some from our group, described a reduction in cerebral blood volume in the hippocampus of APP/PS1 mice (Hooijmans, et al., 2009; Wu, et al., 2004; Zerbi, et al., 2012). Furthermore, several changes in behavior and memory impairment are known in these mice, and suggest hippocampal neuronal malfunction or loss (Hooijmans, et al., 2009). The histological staining methods did not reveal neuronal loss in the cerebral cortex of APP_{swe}/PS1_{dE9} mice. However in this area, the absence of staining of neurons and nerve fibres in the spherically shaped areas is likely due to a severe cortical Aβ plaque load. The presence of Aβ plaques might result in a more isotropic diffusion on the voxel level, because the structure of the Aβ is unlikely to contribute to anisotropy. This is in agreement with the reduction in cortical FA in our DT-MRI results for the APP_{swe}/PS1_{dE9} mice compared to WT.

**Methodological considerations**

For achieving fast and high-resolution EPI images with a good signal-to-noise ratio (SNR), the use of ultra-high field magnet strength coupled with powerful gradients and highly sensitive radiofrequency coils are prerequisites. Especially at ultra-high field (> 7T), susceptibility inhomogeneities that can cause image distortion must be properly adjusted by careful shimming. Furthermore, respiration and head movement must be avoided during the acquisition (e.g. by using a stereotactic holder) and should be further corrected with post-processing methods (Zwiers, 2010). All these criteria were fulfilled in this study. The advantages in SNR allowed us to acquire high spatial resolution data in a short acquisition time. The use of high resolution is necessary to describe diffusion proprieties in small brain structures, and is particularly important to reduce the partial volume effect in structures neighboring cerebrospinal fluid (CSF) or at the interface between GM and WM. In these areas, the presence of different structures in one voxel can bring about an erroneous evaluation of DT-MRI parameters. In AD patients with brain atrophy the enlargement of cerebral ventricles might contribute to an increased MD (Kwong, et al., 1991). An increase
of the CSF area has also been observed in APP/PS1 mice by high-resolution MRI volumetry (Delatour, et al., 2006). From our VBA, we detected an increase in MD and RD and a decrease in FA in voxels along the hippocampal CA3 region and left and right ventricles. It is possible that the increase in ventricle size and atrophy of the fimbria of the hippocampus might have influenced these results, due to partial volume effects. The anatomical differences between groups and the influence of image deformation was however reduced by the use of a study-specific template and by the non-linear diffeomorphic transformation applied for the spatial normalization of the individual datasets. This makes it unlikely that partial volume effects near CSF areas have influenced the results in the hippocampal sub-regions.

In conclusion, in this paper we describe a method to acquire and process high-resolution DT-MRI images in mice with a voxel-based approach. This methodology was applied to evaluate changes in water diffusivity in both white and gray matter occurring in 12 months old APP<sub>sw</sub>71/PS1<sub>ΔE9</sub> mice, with a spatial characterization of the effects not limited to predetermined neuroanatomical locations. As changes in DT-MRI parameters have been associated with progression in neuronal and myelin damage, the methodology and results presented here provide the basis both for further studies on the biological cause of diffusion changes and for evaluation of the efficiency of future treatment strategies in mice models for AD and other neurodegenerative disorders.
Resting-state functional connectivity changes in aging apoE-ε4 and apoE-ko mice: association between cerebral perfusion, structural integrity and synaptic density

Abstract

It is well-established that the cholesterol-transporter apolipoprotein ε (apoE) genotype is associated with the risk of developing neurodegenerative diseases. Recently, brain functional connectivity (FC) in apoE-ε4 carriers has been investigated by means of resting-state fMRI, showing a marked differentiation in several functional networks at different ages compared to carriers of other apoE isoforms. The causes of such hampered FC are not understood, but it has been suggested that vascular function and synaptic repair processes, which are both impaired in carriers of apoE-ε4, can trigger loss of FC during aging. To test this hypothesis, we integrated several different MRI techniques and immunohistochemical staining in a translational study using aging apoE-ε4 and apoE-knockout (-ko) mice. Compared to wildtype mice, we detected profound FC reduction in adult and elderly apoE-ko mice, concomitant with strongly reduced brain perfusion. In apoE-ε4 mice perfusion deficits appear only later in life, and no significant changes of FC were seen. In both mouse models, water diffusion changes commonly associated to axonal disconnection and disorganization are found in hippocampal areas, in agreement with reduced post synaptic density levels at different ages. In conclusion, we provide new evidence for a relation between apoE and brain connectivity, possibly mediated by vascular risk factors and by the efficiency of apoE as synaptic modulator in the brain. Our results show that FC assessment by resting-state fMRI is an excellent tool to investigate neuropathology and aging effects in translational research.
Resting-state functional connectivity changes in aging apoE-ε4 and apoE-ko mice

Introduction
The only gene that currently is associated to sporadic Alzheimer’s disease (AD) is the ε4 allele of the apolipoprotein E (apoE) (Corder, et al., 1993, Mahley and Rall, 2000, Strittmatter, et al., 1993). Several mechanisms by which the apoE-ε4 promotes AD have been proposed; one of these states that, compared to the other isoforms, the ε4 confers impairment in cerebrovascular function, which in turn alters synaptic and neuronal health (Liu, et al., 2013, Zlokovic, 2013). In the brain, apoE is produced after injury to transport cholesterol to the damaged neuronal and synaptic membranes; however, apoE4-ε4 carriers seem to be more susceptible to vascular brain damages (e.g. stroke, brain haemorrhage (Zlokovic, 2011)); at the same time, the repair and remodelling of damaged synapses appears to be less effective by apoE-ε4 than other isoforms (Mahley, et al., 2006, Verghese, et al., 2011). This, in the end, can result in a permanent loss of synaptic contacts, with gradual loss of neuronal connectivity (Bu, 2009, Verghese, et al., 2011). This abnormal connectivity in apoE-ε4 carriers has recently been investigated by resting-state functional magnetic resonance imaging (rsfMRI).

RsfMRI examines the temporal correlations of blood oxygen level dependent (BOLD) fluctuations between brain different regions during a resting condition, thought to reflect resting neuronal activity (Damoiseaux, et al., 2006, De Luca, et al., 2006). The temporal correlation of neuronal activity among different brain regions is often referred as functional connectivity (FC) (Biswal, et al., 1995). This MR technique has generated a great deal of interest among neuroscientists and has been widely used to investigate neurological disorders (Greicius, 2008).

Many studies have reported on a correlation between apoE-ε4, AD, and abnormalities in functional connectivity measured with rsfMRI; cognitively normal young apoE-ε4 carriers showed elevated resting-state activity in the default mode network and high hippocampal activation during memory tasks; both areas that are preferentially affected in early AD (Bookheimer, et al., 2000, Filippini, et al., 2009). This hippocampal hyper-activation is thought to represent a compensatory response, in which increased cognitive effort is required to achieve an equal level of performance to that of non-ε4 carriers (Bondi, et al., 2005). Such hyper-activation is followed by a rapid decline in FC and structural interconnectivity between several cortical regions at older age (Brown, et al., 2011, Machulda, et al., 2011, O’Brien, et al., 2010). In elderly apoE-ε4 carriers a reduced FC in brain networks compared to apoE-ε3 carriers is
shown, even in the absence of amyloid-β plaques (Sheline, et al., 2010). Despite an increasing amount of evidence for an association between apoE genotype and FC, the mechanisms underlying this relationship remain elusive (Verghese, et al., 2011).

Here we investigate, in a translational study, functional connectivity in relation with cerebral perfusion, brain tissue microstructure, analyzed by diffusion tensor MRI (DT-MRI), and staining for post synaptic density. We used target-replacement apoE-ε4 mice, as a model for hypercholesterolemia and AD vascular risk factors, and apoE-ko mice, as model for severe atherosclerosis. We hypothesize that a decrease in FC can be caused by cerebrovascular impairments due to the apoE-ε4 (or its absence), which subsequently triggers neuronal and synaptic dysfunction.

Materials and methods

Animals
The ApoE-ε4 founder mice were originally obtained from Taconic Transgenic Models (Hudson, NY, USA) and a colony was established at the Radboud University Nijmegen Medical Centre (RUNMC). ApoE-ε4 mice were created by targeting the murine APOE gene for replacement with the human apoE-ε4 alleles cultured in E14TG2a Embryonic Stem (ES) cells as described previously (Sullivan, et al., 1997). Resulting chimeras were backcrossed to C57BL/6J (B6) mice for 8 generations. The line was derived by embryo transfer and is maintained by incrossing homozygous mice. For the present study, male and female apoE-ε4 breeder mice were used to generate homozygous apoE-ε4 offspring (3rd generation).

The ApoE-deficient (B6.129P2-Apoetm1Unc/J) founders were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and a colony was established at the RUNMC. In ApoE KO mice the APOE fragment was targeted with an apoE-specific probe (a Sac I/Bgl II fragment) isolated from a mouse apoE cDNA clone. The strongly hybridizing phage clones obtained in this screening, a 7.8-kilobase (kb) EcoRI fragment was isolated and compared with the restriction map. Subsequently these targeted cells were cultured in E14TG2a Embryonic Stem (ES) and injected into C57BL/6J (B6) mice. Resultant chimeras were backcrossed for 11 generations and intercrossed to homozygosity. The line was derived by
embryo transfer and is maintained by incrossing homozygous mice (Piedrahita, Zhang et al. 1992). For the present work, male and female ApoE knockout (apoE-ko) breeder mice were used to generate homozygous apoE-ko offspring (3rd generation).

C57BL6/JOlaHsd wild-type mice, obtained from our colony at the RUNMC were used as controls. Throughout the experiment animals were housed in groups of 2-7 mice per cage in a controlled environment, homogenously illuminated by normal fluorescent room light at 60 lux, with room temperature at 21ºC, and an artificial 12:12h light:dark cycle (lights on at 7 a.m.). Food and water were available ad libitum.

The experiments were performed according to Dutch federal regulations for animal protection. The Veterinary Authority of the Radboud University Nijmegen Medical Centre (RUNMC), the Netherlands, approved all the protocols within this study.

**MR imaging**

Two cohorts of apoE-ε4, apoE-ko and wildtype male mice of 12 months of age (number of animals for each genotype (n) = 8, 10 and 9, respectively) and of 18 months of age (n= 12, 9, 10, respectively) were used for this study. To study genotype and aging related differences in brain function and structure, resting state functional MRI (rsfMRI), cerebral blood flow (CBF) and diffusion tensor imaging (DT-MRI) were measured in each cohort.

MRI measurements were performed on an 11.7 T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m and operated by a Paravision 5.1 software platform. We used a circular polarized volume resonator for signal transmission and an actively decoupled mouse brain quadrature surface coil for signal reception (Bruker BioSpin). During the MR experiments mice were anesthetized with isoflurane (3.5% for induction and ~1.5% for maintenance). The mice were placed in a stereotactic device in order to immobilize the head. Body temperature was measured using a rectal thermometer and maintained at 37° C using a heated air flow device.

After standard adjustments, gradient echo (GE) T2*-weighted images covering the entire mouse brain were acquired for anatomical reference. Subsequently, rsfMRI datasets were acquired using a single shot spin echo sequence combined
with echo-planar imaging (SE-EPI) sequence. Six hundred repetitions with a repetition time (TR) of 1.8s and echo time of 11.8ms were recorded for a total acquisition time of 17 minutes.

To study brain **perfusion** under resting conditions, a flow-sensitive alternating inversion recovery arterial spin labelling (FAIR ASL) technique was used (Kim, 1995). Fifteen images with increasing inversion times (TIs) (40 ms - 3000 ms) were obtained for the $T_1$ calculations, amounting to a total scan time of 12 minutes. Inversion recovery data from the imaging slice were acquired after selective inversion interleaved with non-selective inversion.

**Diffusion** of water was measured as described previously (Harsan, et al., 2010,Zerbi, et al., 2013). In short, 31 axial slices covering the whole brain were acquired with a four-shot SE-EPI protocol. B0 shift compensation, navigator echoes and an automatic correction algorithm to limit the occurrence of ghosts and artefacts were implemented. Encoding b-factors of 0 s/mm$^2$ (b0 images; 5×) and 1000 s/mm$^2$ were used and diffusion-sensitizing gradients were applied along 30 non-collinear directions in three-dimensional space. All other imaging parameters are listed in Table 1.

<table>
<thead>
<tr>
<th>Imaging sequence</th>
<th>Anatomical images</th>
<th>Resting-state fMRI</th>
<th>Cerebral blood flow</th>
<th>Diffusion tensor imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method</strong></td>
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<td>Spin Echo EPI</td>
<td>FAIR-ASL</td>
<td>4-shot Spin Echo EPI</td>
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<td>260×260×500 μm/pixel</td>
<td>234×234×1000 μm/pixel</td>
<td>156×156×500 μm/pixel</td>
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<td><strong>Acquisition time</strong></td>
<td>~8 min</td>
<td>~18 min</td>
<td>2 x 12 min</td>
<td>~18 min</td>
</tr>
</tbody>
</table>

*Table 1. Parameters used in each MR scan*
**Functional connectivity measurements**

The rsfMRI datasets were first realigned using a least squares method and rigid-body transformation with Statistical Parametric Mapping (SPM) mouse toolbox (SPM5, University College London, http://www.fil.ion.ucl.ac.uk/spm/)(Sawiak, et al., 2009). Individual EPI datasets were then spatially normalized to a study-specific template through linear affine and non-linear diffeomorphic transformation (ANTs. V1.9, http://picsl.upenn.edu/ANTS/). Briefly, a group-wise normalization procedure was employed on the mean SE-EPI image of each mouse. The default implementation was followed with four iterations, using mutual information as the initial affine similarity metric and cross-correlation as ‘greedy SyN’ diffeomorphic transformation metric.

In plane spatial smoothing (0.4×0.4mm) and temporal high-pass filtering (cut-off at 0.01Hz) were applied to compensate for small across-mouse misregistration and temporal low frequency noise. Functional connectivity (FC) maps were calculated using total correlation analysis implemented in the REST Matlab toolkit (Song, et al., 2011). Sixteen seeds (2×2 pixel in plane) were selected in left and right cornus ammonis 1 and 3 (CA1, CA3), dentate gyrus (DG), thalamus (Th), visual cortex (Vctx), auditory cortex (Auctx), motor cortex (Mctx) and somatosensory cortex (SSctx). For each animal, correlation analyses of the BOLD time series were carried out between the seeds and the whole brain and Pearson’s correlation values were Fisher transformed to Z-scores.

**Cerebral blood flow calculation**

For each mouse, the FAIR images with different TIs were realigned over the first TI using a rigid-body model, implemented in SPM. Determination of $T_1$ selective and $T_1$ non-selective was performed by fitting the averaged signal intensities in each region of interest (ROI) with a three-parameters monoexponential $T_1$ relaxation curve. CBF was determined in cortex, hippocampus, and thalamus using the following equation:

$$\frac{CBF}{\lambda} = \frac{T_{1\ text{non-selective}}}{T_{1\ blood}} \left(\frac{1}{T_{1\ selective}} - \frac{1}{T_{1\ non-selective}}\right)$$

where $\lambda$ is the blood/tissue partition coefficient for water, assumed to be 0.9 ml/g (Herscovitch and Raichle, 1985,Leithner, et al., 2010) and $T_1$ blood was assumed to be 2.75s at 11.7T (Lin, et al., 2012).
Diffusion tensor MRI parameter estimation and group comparisons
The calculation of the two commonly used DT-MRI parameters, mean diffusivity (MD) and fractional anisotropy (FA), was performed following a protocol as described previously (Zerbi, et al., 2013). Briefly, the diffusion images were first realigned with SPM mouse toolbox, and then spatially normalized to a study-specific template through linear affine and non-linear diffeomorphic transformation using ANTs. Following these pre-processing steps, the diffusion tensor was estimated for every voxel using the PATCH algorithm (Zwiers, 2010).
Regional differences between apoE-ε4 mice and wildtype, and between apoE-ko and wildtype in spatially normalized FA and MD maps were assessed voxel-wise using SPMS with the SPMMouse toolbox. In both 12-month-old mice and 18-month-old mice, two t-tests were performed to identify genotype differences in the framework of the general linear model (GLM). Statistical significance for voxels exceeding a minimum cluster size of 4, to achieve cluster size ≈ 0.05 mm³ as in (Dubois, et al., 2008), was established at p<0.05, uncorrected for multiple comparisons. The contrasts were then colour coded and overlaid onto averaged FA and MD images derived from the template image. In addition, ROI of several white matter (WM) and gray matter (GM) areas were drawn on the template image based on an anatomical atlas (Paxinos, 1997) and the resulting FA, MD, radial diffusivity (RD) and first eigenvalue ($\lambda_1$) were measured for further statistical analyses. Only significant differences between mice groups are reported.

Immunohistochemistry
Directly following the MR measurements at 12 and 18 months of age, mice were sacrificed by transcardial perfusion with 0.1M phosphate buffered saline (PBS). The perfused brains were collected and postfixed for 15h at 4°C in 4% paraformaldehyde fixative and thereafter stored in 0.1M PBS with 0.01% sodium azide at 4°C for immunohistochemical staining. Eight series of 30 µm coronal sections were cut through the brain using a sliding microtome (Microm HM 440 E, Walldorf, Germany) equipped with an object table for freeze sectioning at -60°C. The tissue was stained for postsynaptic density with PSD95 antibody using one complete series of brain sections. Immunohistochemistry was performed using standard free-floating labeling procedures, as described previously (Jansen, et al., 2013).
PSD95
Polyclonal rabbit anti-PSD95 (1:2000; Abcam) was used as a primary antibody. The sections were first pre-treated with 0.9% H₂O₂ in PBS to block endogenous peroxidise and then incubated overnight at room temperature on a shaker table. After incubation, the sections were rinsed three times with 0.1M PBS and incubated with the secondary antibody, donkey anti-rabbit biotin (1:1500; Jackson Immuno Research). After 90 minutes, the sections were rinsed three times again and transferred to a solution containing Vector ABC-elite (1:800; Vector Laboratories) for 90 minutes. Thereafter, visualization of postsynaptic density was achieved by incubation with DAB-Ni solution. Stained sections were mounted on gelatin-coated glass slides, dried overnight in a stove at 37 °C, dehydrated in alcohol series, cleared with xylol and mounted in Entellan.

Quantification
The stained sections were analyzed using a Zeiss Axioskop microscope equipped with hardware and software of Microbrightfield (Williston, VT, USA). Brain regions were based on the mouse brain atlas of Franklin & Paxinos (third edition, 2008) and quantified in five regions of the hippocampus: the inner molecular layer (IML), outer molecular layer (OML), cornus ammonis 1 (CA1), CA2 and CA3. Additionally, two regions in the cortex corresponding to the visual and somatosensory cortex were analyzed. The relevant regions were digitized at 100 times magnification with immersion oil using Stereo Investigator. The quantification of the photographs was performed using Image J (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA). Images were converted to 8-bit gray scale, followed by conversion to 16-bit gray scale; subsequently, the contrast was enhanced and the amount of tissue stained was measured with a threshold-based approach.

Statistics
For the statistical analysis, IBM SPSS 20 software (IBM Corporation, New York, NY, USA) was used. Since the setup of the current study was designed to determine the effect of aging and the extent to which apoE-ε4 and apoE-ko mice develop neuropathological traits of AD and not to study the effects of the apoE allele itself, statistical analyses were performed separately for the apoE-ε4 and apoE-ko mice (apoE-ε4 versus wild-type, and apoE-ko versus wild-type). Multivariate ANOVA (MANOVA) with Bonferroni corrections was conducted with
between-group-factors genotype and age of the animals. If the Bonferroni post hoc test indicated a significant interaction between genotype and age, the data were split for the concerning factor and thereafter analyzed again with the MANOVA. Statistical significance was set at \( p \leq 0.05 \). Correlation analyses between region-specific FC, perfusion, diffusion and post-synaptic density parameters were performed with the bivariate Spearman’s correlation method. To avoid false positive correlation, the statistical significance for the correlation analyses was set at \( p \leq 0.01 \). All values used are expressed as mean ± SEM.

Results

Resting-state fMRI - Functional connectivity
Averaged functional connectivity (FC) patterns in wildtype mice from seeds in DG, thalamus, auditory cortex, visual cortex, motor cortex and somatosensory cortex of the left hemisphere are shown in Figure 1b. Several differences can be observed in the FC patterns for each seed ROI; particularly, a strong bilateral connectivity is notable when seeding in hippocampal regions. Moreover, a widespread FC was measured between hippocampal and thalamic regions, across the whole brain. In the cortex, we detected a single band covering somatosensory, auditory and visual cortices, with highest degree of connectivity closely located to the seed region. Functional correlations between several areas are shown as functional connectivity matrices, colour-coded for Z-fisher values, which represent the correlation between 16 defined ROI (Figure 2 and Figure 3).

ApoE-ε4 vs wildtype
In both apoE-ε4 and wildtype mice, 18-month-old animals showed decreased FC levels compared to 12-month-old mice. Most striking aging differences are the decrease in FC between the motor cortex (left and right), the left visual, auditory and somatosensory cortex and the hippocampus (left and right) (Figure 2). Despite that FC values were visibly lower in apoE-ε4 compared to age-matched wildtype mice, the MANOVA revealed no significant overall genotype nor genotypexage interactions in all the ROI.

ApoE-ko vs wildtype
The multivariate ANOVA showed overall significant genotype and aging effects when comparing apoE-ko with wildtype mice. The pairwise comparison revealed
significant lower FC in apoE-ko mice, independent of age; this reduction affected the connectivity between auditory cortex and somatosensory cortex with the hippocampus and thalamus, bilaterally (Figure 3). A reduced FC was also found as an effect of age, independent of genotype, in the visual, auditory and somatosensory cortices, particularly in the left-brain hemisphere.

**Figure 1.** Averaged functional connectivity Z-Fisher maps in wildtype animals. a) Anatomical reference based on $T_2$-weighted images. b) Comparison of averaged Z-maps of the different seeds positioned in the left hemisphere revealed functionally connected areas. The spatial colour-coded Z-maps are overlaid on the SE-EPI image template. A higher z-score (yellow) represents a higher correlation between the time course of the seed voxel and the other voxels in the brain. In particular, a strong bilateral connectivity between right and left hippocampus is notable. In the thalamus, the highest degree of connectivity was unilaterally distributed and extended to the hippocampal area. In the cortex, high connectivity was found between auditory, visual and somatosensory cortices. In the latter, high connectivity with the hippocampus is also seen.
Figure 2. Functional connectivity (FC) group differences between selected areas in apoE-ε4 and wildtype mice. On the vertical axe, the region of interest in which the seed is placed is shown with its correlation between the other areas shown on the horizontal side. Connectivity matrices show strong FC reduction due to age in both genotypes, particularly between the hippocampus and the visual, auditory and motor cortex. No significant genotype differences could be detected instead. b indicates significant aging effects, p<0.05.
Resting-state functional connectivity changes in aging apoE-ε4 and apoE-ko mice

Figure 3. Functional connectivity (FC) group differences between selected areas in apoE-ko and wildtype mice. On the vertical axe, the region of interest in which the seed is placed is shown with its correlation between the other areas shown on the horizontal side. Connectivity matrices revealed reduced FC due to overall genotype and aging effects in these mice. Specifically, apoE-ko mice showed impaired FC between auditory and somatosensory cortex and hippocampal and thalamic regions. Aging effects were detected as a reduced FC, particularly evident between the visual, auditory and somatosensory cortex and hippocampus. a indicates significant genotype differences; b indicates significant aging effects; c indicates significant genotype and aging effects, p<0.05.
Cerebral blood flow

To study group-related differences on cerebrovascular health, we measured cerebral blood flow (CBF) with a flow-sensitive MRI technique (FAIR ASL). Three regions of interest (ROI) on the left and right brain hemispheres were analysed: cortex, hippocampus and thalamus. Since no intra-individual differences in CBF between right and left hemispheres were detected between mice groups (data not shown), values from both sides were averaged.

ApoE-ε4 vs wildtype

In the comparison of apoE-ε4 with wildtype, the MANOVA revealed a genotype×age interaction in the cortex and in thalamus ($p=0.027$ and $p=0.026$, respectively); after splitting the data for age and for genotype, we found that 18-month-old apoE-ε4 mice have significantly lower CBF in these ROI compared with wildtype at the same age ($p=0.005$ for the cortex and $p=0.002$ for the thalamus) and also compared with 12-month-old apoE-ε4 animals ($p=0.005$ and $p=0.002$, respectively) (Figure 4). In the 12-month-old mice we did not detect any difference between wildtype and apoE-ε4.

ApoE-ko vs wildtype

Compared to wildtype, the apoE-ko mice showed a significant lower CBF in the cortex ($p=0.022$), independent of age. In the hippocampus and in the thalamus a slight reduction of CBF was also seen, although it did not reach statistical significance ($p=0.117$ and $p=0.084$, respectively). No aging effects were seen in both genotypes.

Figure 4. Cerebral blood flow (CBF) was measured in apoE-ε4, apoE-ko and wildtype mice at 12 and 18 months of age in three different regions of interest (ROI): cortex, hippocampus and thalamus. To measure the CBF we employed a FAIR ASL MRI technique. A reduced CBF was found in the cortex in 18-month-old apoE-ε4 mice and in 12 and 18-month-old apoE-ko mice compared to wildtype. A similar trend was observed in the thalamus, although it was not significant for the apoE-ko mice. No significant differences were seen in the hippocampus.
Diffusion tensor magnetic resonance imaging (DT-MRI)

In DT-MRI the water diffusivity, assessed in multiple directions, is used to reconstruct an ellipsoid for every voxel to model the diffusion. The mean diffusivity (MD) describes the size of the ellipsoid, while its shape is quantified by the fractional anisotropy (FA). Because the water diffusion in the brain is restricted by cell membranes, microtubules and myelin sheaths, changes in size and shape of the diffusion ellipsoid may reveal unique details about tissue micro-architecture in both white matter (WM) and gray matter (GM) (Zerbi, et al., 2013). Differences of diffusion tensor derived indices were determined separately in each age group with an explorative voxel-based approach (VBA) and, with the MANOVA for age and genotype with a ROI-based approach. The VBA can detect the occurrence of diffusion changes at higher spatial resolution and is the option of choice when there is no prior knowledge about the expected changes; however, these changes must then be confirmed by a proper statistical analysis in a ROI-based approach. For the VBA, t-value maps (for a p-value < 0.05, and minimum voxel cluster size set at 0.05 mm$^3$) were overlaid with FA and MD template images (Figure 5 and 6).

ApoE-ε4 vs wildtype

Several differences in diffusion parameters were detected from the VBA in apoE-ε4 mice compared to wildtype (Figure 5). Voxels of the internal capsule (ic), the anterior commissure (aca), the cerebral peduncle (cp) and the posterior hypothalamic area displayed significantly lower FA at both ages compared to wildtype. In the cp and in other white matter areas such as the corpus callosum (cc) an increase in MD was also seen, particularly in the 18-month-old mice. The VBA demonstrated the presence of significant genotype differences also in gray-matter structures; in the dentate gyrus of the hippocampus, the FA was highly reduced in apoE-ε4 compared to the wildtype at both ages, while the MD did not change significantly. An increase of MD was seen instead in several cortical and thalamic areas, and this was more apparent in the 18-month-old animals. Furthermore, in the lateral ventricles several clusters of reduced MD and increased FA were found in the apoE-ε4 mice in both age groups. The MANOVA for the selected ROI confirmed these findings; an overall reduction in FA, independent of age, was found in the apoE-ε4 mice compared to wildtype in the aca (0.38 ± 0.01 vs 0.44 ± 0.01, $p=0.027$), cp (0.65 ± 0.01 vs 0.68 ± 0.01, $p=0.044$), dentate gyrus (DG) (0.14 ± 0.01 vs 0.16 ± 0.01, $p=0.004$), cornu ammonus 1 (CA1)
(0.18 ± 0.01 vs 0.2 ± 0.01, \( p=0.016 \)) and cornu ammonis 3 (CA3) (0.17 ± 0.01 vs 0.19 ± 0.01, \( p=0.001 \)). This decreased FA is driven by a reduced diffusion along the axons in aca, cp and in CA3, and by an increased radial diffusivity in DG and CA1 (data not shown). In addition, an increased FA was found as a result of aging, independent of genotype, in the body of corpus callosum (bcc) (0.52 ± 0.01 vs 0.54 ± 0.01, \( p=0.003 \)), genu of cc (gcc) (0.35 ± 0.01 vs 0.37 ± 0.01, \( p=0.016 \)), splenium of cc (scc) (0.53 ± 0.01 vs 0.56 ± 0.01, \( p=0.034 \)), and fimbria (fi) (0.43 ± 0.01 vs 0.47 ± 0.01, \( p=0.006 \)). This increased FA is mainly driven by a reduced diffusivity across the axons (or radial diffusivity (RD); data not shown). A reduced MD was also found as an effect of age in fi (8.64 ± 0.16 vs 8.14 ± 0.13, \( p=0.024 \)) and in the visual cortex (vctx) (7.36 ± 0.08 vs 7.10 ± 0.07, \( p=0.030 \)).

**ApoE-ko vs wildtype**

When comparing apoE-ko mice with wildtype we found a different pattern of changes in the VBA concerning the two ages analysed (Figure 6); at 12 months of age, apoE-ko displayed an increased FA and reduced MD in some white matter structures including the cc, the external capsule (ec) and the ic. A widespread reduction of MD is also found in other cortical and thalamic regions in the apoE-ko. In the hippocampus, a reduction of FA was also visible. In 18-month-old animals, the increased FA in the ec, in the fi and in some thalamic and hypothalamic areas was still visible in the apoE-ko, but no other major differences were detected in the cc. Similarly to the apoE4, also the apoE-ko at 18 months of age showed increased MD in some cortical areas, and a markedly increased FA and reduced MD in the lateral ventricle areas. The MANOVA for the selected ROI showed an overall reduced MD in the apoE-ko in the CA3, independent on the age (7.93 ± 0.12 vs 8.28 ± 0.13, \( p=0.044 \)), caused by a strong reduction in \( \lambda_1 \) (data not shown). Similarly to the comparison with apoE4 mice, also in apoE-ko we found an increased FA due to age in the bcc (0.54 ± 0.01 vs 0.56 ± 0.01, \( p=0.023 \)), scc (0.54 ± 0.01 vs 0.57 ± 0.01, \( p=0.020 \)) and aca (0.42 ± 0.01 vs 0.45 ± 0.01, \( p=0.044 \)), mainly due to a reduction of RD (data not shown).

**Postsynaptic density protein 95 (PSD-95)**

Levels of postsynaptic density were measured with polyclonal rabbit anti-PSD95 and are shown as relative values compared to wildtype mice (Figure 7). In the 12-month-old animal group, we did not detect differences in PSD-95 levels between
Resting-state functional connectivity changes in aging apoE-ε4 and apoE-ko mice

Figure 5. Voxel based analysis (VBA) indicate significant differences in fractional anisotropy (FA) and mean diffusivity (MD) in apoE-ε4 mouse brain compared to wild type at 12 and 18 months of age. Seven rostral to caudal averaged FA and MD maps are overlaid with voxels that showed a significant difference (p < 0.05, minimum voxel cluster size 0.05 mm³). The voxel colour indicates a negative or positive change in the apoE-ε4 mice compared to the wild type mice. At both ages, apoE-ε4 mice showed a widespread reduced FA in some white matter regions and in the hippocampus. An increase in MD is found amongst others in the corpus callosum (cc), in the cerebral peduncle (cp) and in the cortex. Increases in FA and reduced MD are found in the ventricle area.

apoE-ε4 and wildtype mice in any of the selected regions. Instead, we did find a significant reduction of PDS-95 staining in apoE-ko mice compared to wildtype in the IML (p=0.005) and in the CA3 (p=0.046) (Figure 7, c). In 18-month-old animals, reduced PSD-95 levels in both apoE-ko and apoE-ε4 mice compared to wildtype were seen in the IML and OML; however, these differences were significant only in the apoE-ε4 group (p=0.043 and p=0.039, respectively) (Figure 7, d). Because the staining of the two groups was not performed at the same time, we could not assess aging effects on the different genotypes. The two-tailed Spearman’s correlation test revealed a strong positive correlation between PDS-95 levels and CBF in both the hippocampal region (p=0.003) and in the cortex (p=0.006).
Figure 6. Voxel based analysis (VBA) indicate significant differences in fractional anisotropy (FA) and mean diffusivity (MD) in apoE-ko mouse brain compared to wild type at the two ages. Seven rostral to caudal averaged FA and MD maps are overlaid with voxels that showed a significant difference (p < 0.05, minimum voxel cluster size 0.05 mm$^3$). The voxel colour indicates a negative or positive change in the apoE-ko mice compared to the wild type mice. At 12 months of age, apoE-ko showed concomitant increased FA and reduced MD in some white matter structures including the corpus callosum (cc), the external capsule (ec) and the internal capsule (ic). A small reduction of FA was also visible in the hippocampus. In 18-month-old animals, the increased FA in the ec, in the fimbria (fi) and in some thalamic and hypothalamic areas was still visible in the apoE-ko, but not in the cc. Moreover, an increased MD in some cortical areas was detected. Similarly to apoE-ε4 mice, increased FA and reduced MD were found in the ventricle area.
Figure 7. Post-synaptic density 95 (PSD-95) staining performed on brain sections of apoE-ε4, apoE-ko and wild-type mice. a, b) Representation of PSD-95 immunoreactive staining in hippocampal and cortical areas at two different magnifications (5× and 100×, respectively). c) In 12-month-old animals we detected a significant decrease in PSD-95 levels in apoE-ko mice compared to wildtype in the inner molecular layer (IML) and CA3 of hippocampus. d) In 18-month-old animals we show reduced PSD-95 levels in the apoE-ε4 animals in the outer molecular layer (OML) and in the inner molecular layer (IML), while no significant differences were seen between wildtype and apoE-ko mice. Values represent the mean and SEM; CA = cornus ammonis. SC=somatosensory cortex; VC=visual cortex.
Discussion
In the present study, we assessed resting-state functional connectivity (FC) in combination with perfusion, diffusion and synaptic density measurements in aging wildtype, apoE-ε4 and apoE-ko mice. Target-replacement apoE-ε4 and apoE-ko mice are attractive models to investigate the role of vascular risk factors in relationship with AD-like pathological features, such as changes in brain FC. However, the technical challenges to obtain good-quality MR images and the lack of knowledge in the murine brain network system have been a strong limiting factor for these studies. Only recently, new dedicated hardware and methods for acquisition and data analysis enabled the analysis of resting-state fMRI in mice (Jonckers, et al., 2011). In the present study, a particular attention was given to the anaesthetic used; some studies in rats have used analgesic muscle relaxants, such as dexmedetomidine (Lu, et al., 2012) or medetomidine (Zhao, et al., 2008), to limit the sedation levels of the animals. More recently, a study demonstrated that reliable FC measures in mice could be detected using a regime of low-dose isoflurane (Guilfoyle, et al., 2013). For our experiments, low-dose isoflurane was used, lightly adjusted throughout the experiment to maintain a fast and stable breathing frequency (>130 bpm), minimizing the impact of anaesthesia on the resting-state fMRI signal. Despite the limited literature about resting-state FC in murine brains, the correlation maps obtained for wildtype animals match previous findings very well. Our results exhibited many similarities with the resting-state networks described in mice by means of independent component analysis (Jonckers, et al., 2011); in particular, we show the presence of strong bilateral hippocampal connectivity, while a unilateral component was seen in the thalamus. Furthermore, we revealed a high correlation between somatosensory, auditory and visual cortices; a similar network has been found in rats and identified as the default mode network (Lu, et al., 2012).

A significant result of our study was a reduced FC in the old animals (18-month-old) compared to adults (12-month-old). Interestingly, we saw this pattern in all the genotypes. Resting-state fMRI and FC measurements have been increasingly used as a tool for investigating the aging human brain in vivo. With the exception of few studies reporting no or little changes in FC due to aging, most of the rsfMRI studies reported aging-related decrements in FC, which have been recently reviewed (Ferreira and Busatto, 2013). Since normal aging is also associated with decline in cognition, the reduction in FC is thought to correlate
with a disruption of neuronal connections and a deterioration of brain functioning in elderly people (Hedden and Gabrieli, 2004, Whalley, et al., 2004). It is therefore plausible that the results obtained here reflect similar changes in the aging mice. To the best of our knowledge, this is the first report of age-related differences in FC in mice and might represent a novel way to study the neurobiology of aging.

**Resting-state FC and perfusion changes**
Cerebral blood flow (CBF) was measured to test whether changes in perfusion occur before, simultaneous or after alterations in FC. CBF is closely coupled with brain metabolism (Raichle, 1998) and is commonly used as an indirect measure of brain energy demand, also during resting state (Roy and Sherrington, 1890).

**ApoE-ε4**
Cross-sectional studies in young, middle-aged, and elderly human apoE-ε4 carriers have reported reductions in regional CBF and regional cerebral glucose metabolism over time, particularly significant in brain regions susceptible to pathological changes in AD (Scarmeas and Stern, 2006). In apoE-ε4 subjects, a faster decline of regional CBF during aging has been shown compared to apoE-ε3, suggesting its contribution to the increased risk of developing AD (Wierenga, et al., 2013). In good agreement with these human studies, we demonstrated that apoE-ε4 mice suffer from decreased cortical CBF at 18 months of age compared to wildtype, while no differences in CBF were seen at 12 months of age. By hypothesizing a correlation between CBF and FC, we would have expected to see a similar decline in cortical connectivity in the 18-month-old apoE4 mice. However, despite that the FC was lower in apoE-ε4 mice between all selected ROI, this was not significantly different from wildtype mice. Similarly, the reduction of FC due to aging seems to be stronger in the apoE-ε4 mice than in the wildtype, but again this was not significantly different between the groups. These results indicate that, despite the rapid decline of cerebral perfusion during aging from adult to senile, neural connectivity is not severely compromised in apoE-ε4 mice. Therefore it seems that in apoE-ε4 mice cerebrovascular impairments occur before FC changes, and might trigger brain connectivity disruption only in a later stage, as the pathology progresses. Future studies in older mice are warranted to validate this hypothesis.
ApoE-ko

In apoE-ko mice we found a concomitant reduction in CBF and resting-state connectivity at both ages. These mice develop abdominal aortic aneurysm and atherosclerotic plaques starting in the aorta from 3 months of age; the amount and the severity of the plaques spread in other arteries throughout their life, including in the carotids. Furthermore, the size of atherosclerotic plaques in the aorta has been associated to the development of endothelial dysfunction (Crauwels, et al., 2003), making the apoE-ko a commonly used model for severe atherosclerosis and vascular pathology (Trolleye, et al., 2011). No investigations on resting-state fMRI were previously performed in apoE-ko mice; however, it is known that, together with vascular deficits, these mice also develop a compromised synaptic plasticity (Blain, et al., 2006, Masliash, et al., 1995a), and defective cognitive performances (Gordon, et al., 1995, Krzywkowski, et al., 1999, Masliash, et al., 1995b, Oitzl, et al., 1997). We therefore hypothesise that the severe vascular pathology, which these mice spontaneously develop at early age, could have accelerated the occurrence of brain injuries by an overexposure to stress factors, such as cerebral hypoperfusion; the absence of apoE would have further aggravated the synaptic repair processes, leading to a permanent loss of neuronal connectivity. In support of this hypothesis, we found a positive correlation in cortex and hippocampus between CBF and averaged post-synaptic density (measured in sampled regions). Although the correlation of these two measures do not imply a cause-effect mechanism, it reinforces the idea that neuronal and vascular health are tidily interdependent. Indeed, the role of vascular defects in neurodegenerative processes is vastly described, and a direct causal relation between hypoperfusion and neurodegeneration, including AD, has been also suggested by many (Altman and Rutledge, 2010, de la Torre, 2000, Iadecola, 2004). Changes in cerebrovascular dynamics, such as an impaired coupling between neural activity and hemodynamic response, might have directly influenced BOLD signal fluctuations and consequently FC changes (Liu, 2013). However, the lack of direct correlations between FC measures and post-synaptic density and CBF suggests instead a non-uniform contribution of both neurovascular coupling and neural activity to the changes in connectivity.
Structural changes revealed by DT-MRI

In order to find a possible relation between FC changes and structural differences in the brain of apoE-ε4 and apoE-ko mice, we measured diffusion tensor MRI (DT-MRI).

ApoE-ε4

In this study we showed altered WM microstructure in apoE-ε4 mice compared to wildtype, specifically manifested as reduced FA and increased MD (due to increased radial diffusivity (RD)) in the anterior commissure (aca), corpus callosum (cc) and cerebral peduncle (cp). We also demonstrated that apoE-ε4 mice displayed significant FA reduction in the hippocampus and a sparse increased MD in the cortex. Most of these changes occurred similarly in both age groups. Despite the lack of DT-MRI studies in apoE-ε4 mice, similar findings have been described in human studies, where significant FA reductions in WM of apoE-ε4 carriers are shown (Heise, et al., 2011, Honea, et al., 2009, Nierenberg, et al., 2005); increased MD and RD have also been reported for analogous WM regions, particularly in the cc. In agreement with our study, these dissimilarities were consistent across different ages, suggesting that APOE affects WM microstructure proprieties from early adulthood, without directly reflecting the associated risk of developing AD (Westlye, et al., 2012). Two studies also reported diffusion differences in hippocampal structures in apoE-ε4 carriers, specifically as a reduction in FA in the left hippocampus (Persson, et al., 2006) and in the parahippocampal gyrus (Nierenberg, et al., 2005). These changes have been related to regional atrophy and to disruption of white matter integrity within the medial temporal lobe (e.g., hippocampal–parahippocampal connectivity). When considered together with evidence from previous studies, the pattern of changes observed here suggests that apoE-ε4 mice develop early in life alterations in neuronal architecture and organization. These structural modifications are therefore not related to the occurrence of vascular impairment, but may be linked to the role of apoE in synaptic development, dendrite formation and axonal guidance. However, these changes do not seem to induce drastic abatements in functional connectivity.

ApoE-ko

In the apoE-ko mice, we found different changes in diffusion proprieties for WM and GM structures; one unanticipated finding was the increased FA and the
reduced MD in the cc and external capsule (ec) of the apoE-ko mice, particularly at 12 months of age. Because it is unlikely that the apoE-ko mice have better white matter integrity (to which these diffusion changes are often referred to), these differences may be explained by a local reduction of crossing fibres and in fibres spreading from the cc towards cortical and subcortical regions. However, most of the differences seen in the VBA were not confirmed by the MANOVA analysis for selected ROI. Interestingly, the strongest result was a reduced diffusivity along the axons ($\lambda_1$) in the CA3 region in both age groups, suggesting a lack of axonal organization in the hippocampus. In accordance with these findings, one study demonstrated that adult apoE-ko mice show a reduction in number and an irregular shape in unmyelinated peripheral axons, while myelinated fibres were structurally normal (Fullerton, et al., 1998). A decrease in spine density and dendritic length is also reported in 12-month-old apoE-ko mice (Ji, et al., 2003). These studies together suggest that lack of apoE leave unmyelinated axons especially vulnerable to injury. This also is in agreement with our observations of FC and perfusion deficits in the apoE-ko mice. However, other studies did not show differences in neuronal morphology and spine density in adult apoE-ko mice compared to wildtype or apoE3 mice (Anderson, et al., 1998, Klein, et al., 2010). It is possible that changes in other protein levels and the severe hyperlipidemic phenotype of apoE-ko mice may have a role in compensating the lack of apoE, making it difficult to interpret and to compare the changes seen in different studies.

**Post-synaptic density**

Synaptic loss and disconnection are strongly correlated with cognitive decline in AD and may influence FC (Selkoe, 2002, Serrano-Pozo, et al., 2011). Compared to wildtype, we found reduced PSD-95 levels in 12-month-old apoE-ko mice, and also in the 18-month-old apoE-ε4 mice. While in the apoE-ε4 mice these changes mirror the hypoperfusion deficits observed, in the apoE-ko our results seem rather contradictory. Since cholesterol released from apoE-containing lipoprotein particles is used to support synaptogenesis and the maintenance of synaptic connections (Mauch, et al., 2001, Pfrieger, 2003, Poirier, et al., 1995), a decrease in postsynaptic density in apoE-ko mice was expected in older animals as well as in the adults. A relative increase in synaptic density might therefore reflect a compensatory mechanism in the apoE-ko mice in response to early functional synaptic failure or insults to the brain, like stroke, ischemia, or the measured
hypoperfusion (Arendt, 2001, Jansen, et al., 2012, Jin, et al., 2004, Li, et al., 2010, Mu and Gage, 2011, Wang, et al., 2008). This also accords with earlier observations, which showed enhanced neurogenesis in apoE-ko mice (Levi and Michaelson, 2007). Unfortunately, we could not analyse aging effects with these data to confirm this effect. Nevertheless, this compensatory mechanism does not seem to restore or improve FC in these mice.

In conclusion, with this study we provide new evidence for a relation between vascular risk factors, the apoE genotype and functional connectivity; we propose that vascular deficits are the triggering events that lead to synaptic disruption, and that both these factors contribute to resting-state functional connectivity changes. Despite the limitations and technical challenges, we demonstrate that rsfMRI can be used as a powerful tool to investigate neuropathology and aging effects in translational research.
Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics and plasticity in apoE4 carrier and apoE knockout mice

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Abstract
Lipid metabolism and genetic background together strongly influence the development of both cardiovascular and neurodegenerative diseases like Alzheimer’s disease (AD). A non-pharmacological way to prevent the genotype-induced occurrence of these pathologies is given by dietary behavior. In the present study, we tested the effects of long term consumption of a specific multi-nutrient diet in two models for atherosclerosis and vascular risk factors in AD: the apolipoprotein e4 (apoE4) and the apoE knockout (apoE ko) mice. This specific multi-nutrient diet was developed to support neuronal membrane synthesis and was expected to contribute to the maintenance of vascular health. At 12 month of age, both genotypes showed behavioral changes compared to control mice and we found increased neurogenesis in apoE ko mice. The specific multi-nutrient diet decreased anxiety-related behavior in the open field, influenced sterol composition in serum and brain tissue, and increased the concentration of omega-3 fatty acids in the brain. Furthermore, we found that wild-type and apoE ko mice fed this multi-nutrient diet showed locally increased cerebral blood volume and decreased hippocampal glutamate levels. Taken together, these data suggest that a specific dietary intervention has beneficial effects on early pathological consequences of hypercholesterolemia and vascular risk factors for AD.
Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics, and plasticity

Introduction

Diseases related to a sedentary lifestyle with high-fat dietary intake become a major problem in our aging western society. For example, cardiovascular diseases, like coronary heart disease (CHD), and neurodegenerative disorders, like Alzheimer’s disease (AD), are the most described in the current literature and share many risk factors such as obesity, diabetes mellitus and hypercholesterolemia (Breteler, 2000, Kivipelto, et al., 2001, Kivipelto, et al., 2005, LaRosa, et al., 1990, Skoog and Gustafson, 2006). Despite the growing awareness of the impact of a sedentary lifestyle and high-fat dietary intake, these risk factors are likely to increase in the future and extend to developing countries, which will have a huge impact on the global economy because of costs associated with screening, treatment and daily care services. These same risk factors are modifiable by the management of hypercholesterolemia; this reduces the risk of developing cardiovascular-related pathologies, including CHD (Gupta, et al., 2010). Epidemiological studies have associated the use of cholesterol lowering agents (statins) with diminished prevalence of AD (Haag, et al., 2009, Jick, et al., 2000) and with preserved cognitive functions (Sparks, et al., 2005), although more recent studies have shown no beneficial effects of statins on dementia or AD (Kandiah and Feldman, 2009, McGuinness, et al., 2009, Zhou, et al., 2007).

To which extent a compromised cholesterol homeostasis in the vascular system and in the brain is able to trigger cerebrovascular and neurodegenerative pathologies is still under debate. A potential key-factor in the relationship between lipid metabolism and vascular and neurodegenerative disorders is the cholesterol transporter apolipoprotein ε4 (apoE-ε4), which is a major genetic risk factor for hypercholesterolemia, vascular dementia and sporadic AD (Hirsch-Reinshagen, et al., 2009, Huang, 2006, Kim, et al., 2009). ApoE is the most prevalent brain lipoprotein and plays a fundamental role in neuronal maintenance and repair. In the human population three common isoforms termed apoEε2, apoEε3, and apoEε4 are expressed, which differ from each other in one to two amino acids (Weisgraber, 1994). ApoE has several functions in the body and in the brain, including anti-inflammatory and antioxidation (Huang, 2010, Lynch, et al., 2001, Miyata and Smith, 1996), but especially functions as a ligand in receptor-mediated endocytosis of lipoprotein particles, such as cholesterol. Cholesterol released from apoE-containing lipoprotein particles is
used to support synaptogenesis and the maintenance of synaptic connections (Mauch, et al., 2001, Pfrieger, 2003). Compared to the apoEε2 and apoEε3 isoforms, apoEε4 is a less functioning cholesterol transporter contributing to hypercholesterolemia and atherosclerosis (Davignon, et al., 1988, Mahley, 1988).

Interestingly, while in the general population 10-15% carries one ε4 allele, the occurrence in AD patients is 40-65%. In addition, ε4 allele carriers develop AD at a younger age than non-ε4 allele carriers (Corder, et al., 1993), and ε4-positive AD patients display higher levels of AD pathological hallmarks, such as amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs), than do corresponding non-ε4 AD patients (Nagy, et al., 1995, Ohm, et al., 1999, Schmechel, et al., 1993).

The non-pharmacological management of cholesterol levels by means of specific diets containing fish oil has been recently proposed to prevent and treat the negative effects of the apoE4 genotype in AD (Cole, et al., 2009, Schipper, 2011). Recent experiments on young apoE4- target replacement mice suggest a positive influence of fish-oil containing diets on behavioral and cognitive performances (Kariv-Inbal, et al., 2012). These beneficial effects have been explained by the capacity to prevent arrhythmias (Leaf, et al., 2003), lowering plasma triacylglycerols (Harris, 1997a, Harris, 1997b, Sacks and Katan, 2002), decreasing blood pressure (Geleijnse, et al., 2002), improving vascular reactivity (Goodfellow, et al., 2000, Harris, 1997b) and decreasing atherosclerosis (Okuda, et al., 2005) and inflammation (Calder, 2001). Furthermore, high levels of omega-3 (n-3) long-chain polyunsaturated fatty acids (lc-PUFAs) replace omega-6 (n-6) fatty acids and cholesterol from cell membranes leading to increased fluidity of the membrane, increased number of receptors, enhanced receptor binding and affinity, and better ion channel functionality (Bourre, et al., 1991, Bourre, et al., 1989, Farkas, et al., 2002). As a result, this leads to improved neurotransmission and signalling (Bourre, et al., 1989), which is important for optimal cognitive functioning (Fontani, et al., 2005). Other dietary components like B-vitamins and antioxidants have been shown to protect the brain from oxidative and inflammatory damage (Guerrero, et al., 1999, Joseph, et al., 1998, Socci, et al., 1995, Yamada, et al., 1999), and synaptic and neuronal loss (Fujii, et al., 1996, Shrivastava, et al., 2005).

Based on these findings, a specific multi-nutrient diet has been developed for the dietary management of AD’s risk factors (Scheltens, et al., 2010). To date, two randomized, double-blind controlled clinical trials demonstrated improved memory performance in patients with mild AD, taking this multi-nutrient
component diet supplementation on a daily base (Kamphuis, et al., 2011, Scheltens, et al., 2010, Scheltens, et al., 2012). The mechanisms by which these dietary nutrients influence the cholesterol metabolism and have protective properties against AD traits yet need to be elucidated. In this study, we investigate the extent to which a specific multi-nutrient diet, containing docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), phospholipids, uridine monophosphate (UMP), choline, B-vitamins and antioxidants, which are precursors and co-factors for membrane synthesis and maintenance (Kamphuis and Scheltens, 2010) and may help to support vascular health, may inhibit cerebrovascular flaws and neuronal and synaptic loss in apoE4-carrier and apoE knockout mice. ApoE4-carrier and apoE knockout mice represent relevant models to study the effects of compromised cholesterol homeostasis on the development of CHD and AD. While apoE4 carrier mice exhibit an increased risk of vascular disorders due to altered cholesterol metabolism, apoE knockout mice spontaneously develop severe hypercholesterolemia and atherosclerosis (Breslow, 1996, Knouff, et al., 1999).

Materials and methods

Animals, dietary intervention and housing conditions
The apoE4 founder mice were originally obtained from Taconic Transgenic Models (Hudson, NY, USA) and a colony was established at the Radboud University Nijmegen Medical Centre (RUNMC), the Netherlands. In short, mice were created by targeting the murine APOE gene for replacement with the human APOE4 alleles (4/4) in 129P2/OlaHsd-derived E14TG2a ES cells and injecting the targeted cells into blastocysts. Resultant chimeras were backcrossed to C57BL/6J for 8 generations. The line was derived by embryo transfer and is maintained by increasing homozygous mice. For the present study, male and female apoE4 breeder mice were used to generate homozygous apoE4 offspring (1st generation at the RUNMC).

The apoE-deficient (B6,129P2-Apoe<sup>tm1Unc</sup>/J) founders were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) and a colony was established at the RUNMC. In short, mice were created by targeting the apoE gene in 129P2/OlaHsd-derived E14TG2a ES cells and injecting the targeted cells into
C57BL/6J blastocysts. Resultant chimeras were backcrossed to C57BL/6J for 11 generations. The line was derived by embryo transfer and is maintained by incrossing homozygous mice (Piedrahita, et al., 1992). For the present work, male and female apoE knockout (apoE ko) breeder mice were used to generate homozygous apoE ko offspring (1st generation at the RUNMC).

C57BL/6J wild-type mice, which were the non-transgenic wild-type littermates of our colony of AβPPswe-PS1dE9 mice, were used as controls. In short, the AβPP-PS1swe-PS1dE9 founders were originally obtained from John Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) and a colony was established at the RUNMC (Jankowsky, et al., 2004, Jankowsky, et al., 2001). The AβPPswe-PS1dE9 line (line 85) was originally maintained on a hybrid background by backcrossing to C3HEJ×C57BL/6J F1 (so-called pseudo F2 stage).

For the present work, male AβPPswe-PS1dE9 breeder mice were backcrossed to female C57BL/6J×OlaHsd (Harlan Laboratories, Inc., Horst, the Netherlands) for 10 generations, and the non-transgenic C57BL/6J wild-type offspring were used for the current study.

Throughout the experiments animals were housed in groups of 2-7 mice per cage in a controlled environment, homogenously illuminated by normal fluorescent room light at 60 lux, with room temperature at 21ºC, and an artificial 12:12h light:dark cycle (lights on at 7 a.m.). Food and water were available ad libitum.

Male apoE ko, apoE4 and wild-type mice were fed either a standard Control diet (CO diet), or a specific multi-nutrient diet, called Fortasyn® Connect (FC diet). The diets differed in composition with regard to the fat blends used, as well as the number of supplemented nutrients as indicated in Table 1. Diets were isocaloric and were manufactured by Research Diet Services (Wijk bij Duurstede, the Netherlands). In order to minimize oxidation of the lc-PUFAs, the experimental diets were stored at -20°C in 2-day supply aliquots. At 2 months of age the mice were put on the diets for the remainder of the experiment. Animals underwent behavioral testing at 9 ± 1 month of age and MRI measurements at 11 ± 1 month of age (Fig. 1). In total 84 mice were used. Table 2 describes the number of mice in each experimental group. The experiments were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority of the Radboud University Nijmegen Medical Centre.
Behavioral analyses
Behavioral testing was performed at 9 ± 1 months of age, in the following order: First open field, followed by Morris water maze (MWM), and finally the reversal MWM (Fig. 1). All testing sessions were performed during the light phase (between 9 a.m. and 5 p.m.) and were recorded for computer-assisted analysis using Ethovision 7.0 software (Noldus Information Technology B.V., Wageningen, the Netherlands). All behavioral testing was performed in the same room, homogenously illuminated by normal fluorescent room light at 60 lux.

Open field
To analyze explorative and anxiety-related behavior mice were placed individually in the center of a square open field (50×50×50 cm) with white Plexiglas walls, and were observed for 30 min. The duration (seconds) of walking, wall leaning, rearing, sitting and grooming were scored and analyzed. These open field parameters were defined as described previously (Hooijmans, et al., 2009, Streijger, et al., 2005). In addition, total walking distance, mean velocity, and the time spent in the corners respectively the center of the open field were obtained from the recorded sessions. The center of the open field was defined as a square measuring 20×20 cm, and the corners of the open field were defined as the sum of all four 10×10 cm squared corners.

Morris water maze (MWM)
To investigate spatial learning abilities, mice were tested in the Morris water maze (MWM). Mice were placed in a pool (104 cm diameter) filled with water (21-22°C; made opaque by the addition of milk powder) at different starting positions and trained to find a submerged platform by using distant visual cues present on the four walls of the test room at a distance of 0.5 meter. The 8 cm diameter round platform was submerged 1 cm below the water surface and placed in the middle of the northeast (NE) quadrant at a distance of 26 cm from the wall. During all trials the researcher was present and always located at the same location in the room (close to the SW quadrant).
Table 1. Compositions of the experimental diets used, based on AIN-93M (Reeves, et al., 1993) with minor revisions. All diets were isocaloric, contained 5% fat and standard vitamin and mineral premix, providing recommended daily amounts of these nutrients. All amounts of nutrients are indicated in g/100g of diet. UMP = uridine monophosphate; SFA = saturated fatty acids; MUFA = mono unsaturated fatty acids; PUFA = poly unsaturated fatty acids.

<table>
<thead>
<tr>
<th>Source</th>
<th>Control (CO)</th>
<th>Fortasyn® Connect (FC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>35.57</td>
<td>33.12</td>
</tr>
<tr>
<td>Casein</td>
<td>14.00</td>
<td>14.00</td>
</tr>
<tr>
<td>Corn dextrin</td>
<td>15.50</td>
<td>15.50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Fibers</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Mineral mix (AIN-93M-MX)</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Soy oil</td>
<td>1.900</td>
<td>-</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>0.900</td>
<td>0.100</td>
</tr>
<tr>
<td>Corn oil</td>
<td>2.200</td>
<td>1.870</td>
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<tr>
<td>Fish oil</td>
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<td>3.030</td>
</tr>
<tr>
<td>Additions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0.180</td>
<td>0.180</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.250</td>
<td>0.250</td>
</tr>
<tr>
<td>Tert-butylhydroquinone</td>
<td>0.0008</td>
<td>0.0008</td>
</tr>
<tr>
<td>Pyridoxine-HCL</td>
<td>-</td>
<td>0.00328</td>
</tr>
<tr>
<td>Folic acid (90%)</td>
<td>-</td>
<td>0.00067</td>
</tr>
<tr>
<td>Cyanocobalamin (0.1% in mannitol)</td>
<td>-</td>
<td>0.00350</td>
</tr>
<tr>
<td>Ascorbic acid (100% pure)</td>
<td>-</td>
<td>0.160</td>
</tr>
<tr>
<td>dl-α-tocopheryl acetate</td>
<td>-</td>
<td>0.4650</td>
</tr>
<tr>
<td>UMP disodium (24% H₂O)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chloride (74.576%)</td>
<td>-</td>
<td>0.402</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>-</td>
<td>0.402</td>
</tr>
<tr>
<td>Sodium selenite (46% min)</td>
<td>-</td>
<td>0.00023</td>
</tr>
<tr>
<td>Energy (kcal/100g chow):</td>
<td>376.9</td>
<td>367.1</td>
</tr>
</tbody>
</table>

% Fatty acids:

<table>
<thead>
<tr>
<th></th>
<th>Control (CO)</th>
<th>Fortasyn® Connect (FC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total n3</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Total n6</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>Ratio n6/n3</td>
<td>20.3</td>
<td>1.0</td>
</tr>
<tr>
<td>SFA</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>MUFA</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>PUFA</td>
<td>46</td>
<td>44</td>
</tr>
</tbody>
</table>
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Table 2. Overview of the number of mice used in each experimental group. CO = Control diet; FC = Fortasyn® Connect diet; (r)MWM = (reverse) Morris water maze; MRS = magnetic resonance spectroscopy; CBV = cerebral blood volume; IHC = immunohistochemistry; BCH = biochemistry.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Total</th>
<th>Open Field</th>
<th>(r)MWM</th>
<th>MRS</th>
<th>CBV</th>
<th>IHC</th>
<th>BCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>CO</td>
<td>17</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7-8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>8-9</td>
<td>7</td>
</tr>
<tr>
<td>apoE4</td>
<td>CO</td>
<td>13</td>
<td>6</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>5-6</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>13</td>
<td>8</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>5-6</td>
</tr>
<tr>
<td>apoE ko</td>
<td>CO</td>
<td>13</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>5-7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>5-6</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 1. Time line of experimental design. At 2 months of age, the mice were put on either control or Fortasyn® Connect diet for the remainder of the experiment. Behavioral testing was performed at 9±1 months of age. In the first week of behavioral testing, animals were exposed to the open field once for 30 minutes. In the second week, animals were trained in the Morris water maze (MWM) for 4 days. In the third week, animals were trained in the reverse Morris water maze (rMWM) for 2 days. MR imaging was performed at 11±1 months of age. Animals were sacrificed immediately after MR imaging.
Acquisition (spatial learning): Mice were trained to find the location of the submerged escape platform in 4 acquisition trials (maximal swimming time 120 s; 30 s on the platform; inter-trial interval 60 min) per day during 4 consecutive days. The latency time (seconds) to find the hidden platform was scored. Starting positions during the 4 trials/day were: S, N, E, W. After the 2 min swim the mice were placed back in their home cage, and a paper towel was available inside the cage for additional drying.

Probe (spatial memory): All mice performed a single probe trial 60 min after the last trial on day 4, in which the platform was removed from the swimming pool. Mice were allowed to swim for 60 s and the time spent swimming and searching in the former platform quadrant and former platform location), total swimming distance, and mean velocity were recorded.

Reverse Morris water maze (rMWM)
Four days after the standard MWM probe trial, a simplified reversal MWM (Hooijmans, et al., 2009) was performed in which the platform location was changed to the southwest (SW) quadrant. In this procedure, earlier platform location needs to be encoded in the long-term memory. Memory retrieval needs to be selective for the most recently learned location, introducing an episodic like component in the spatial memory task (de Bruin, et al., 1994). Acquisition and probe sessions were performed similar to the standard MWM sessions, except that starting positions were E, W, S, and N, the target quadrant was SW, and training lasted only 2 days (4 trials/day).

Magnetic resonance imaging and spectroscopy
MRI measurements were performed at 11 ± 1 month of age on a 7T/300mm horizontal-bore magnet interfaced to a ClinScan console (Bruker Biospin, Ettlingen, Germany). An integrated circular polarized transmit $^1$H volume coil (200mm/154mm outer/inner diameter) was used for signal transmission and combined with a circular polarized mouse brain $^1$H surface coil for signal reception. Before the MR measurements, all mice received an intravenous tail vein catheter. During the experiments, mice were anesthetized with 2% isoflurane (Abott, Cham, Switzerland) in a mixture of oxygen and N$_2$O (2:1) through a nose cone and placed in a stereotactic holder. Body temperature was maintained at physiological levels with heated airflow and monitored with a
rectal optical temperature probe. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). Initially, multislice turbo spin echo images in the coronal, transversal and longitudinal orientations were acquired to visualize the anatomy and the morphology of the mouse brain structures. Imaging parameters were: field of view (FOV) = 25×25 mm, matrix size = 256×256, slice thickness = 0.5 mm, echo time (TE) = 46 ms, and repetition time (TR) = 3500 ms.

**Magnetic resonance spectroscopy (MRS)**

Metabolite concentrations in the hippocampus were determined using $^1$H MRS. A volume of interest (VOI) of 1.0×1.0×2.0 mm was positioned inside the left hippocampus between -2.18 and -2.46 posterior to bregma, according to the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). $^1$H-MRS spectra were acquired with stimulated echo acquisition mode (STEAM) sequence with imaging parameters: TR = 1500 ms, TE = 13 ms, and 1024 signal averages. Water suppression was performed with variable pulse power and optimized relaxation delays (VAPOR). For each $^1$H MRS spectrum, a water reference spectrum was acquired without water suppression in the same VOI with the same imaging parameters and 32 signal averages. Total acquisition time for $^1$H MRS was 27 min per animal.

Quantification of the metabolite concentration was performed using the Linear Combination model software package (LCModel$^\text{TM}$, S. Provencher, Oakville, Canada). Model metabolite spectra were simulated to match the magnetic field strength, sequence type and sequence parameters used for data acquisition. Metabolite concentrations are given as absolute values using the water signal in non-water suppressed spectra as a reference. The criteria to select the reliable metabolite concentrations were based on the Cramér-Rao lower bounds (CRLB), which are measures of the minimum standard deviation of the fit for each metabolite (Cavassila, et al., 2001) and are also determined by LCModel$^\text{TM}$. Only metabolites that had a CRLB ≤ 20% in more than 80% of the spectra were included. Five metabolites or metabolite combinations fulfilled the criteria: creatine + phosphocreatine (tCre), glutamate (Glu), myo-Inositol + glycine (mI+Gly), N-acetylaspartate (NAA) and taurine (Tau).
Relative cerebral blood volume (rCBV)

To assess differences in microvascular brain blood volume reserves, relative cerebral blood volume (rCBV) was determined with a susceptibility contrast enhanced MRI technique as described previously, with modifications (Zerbi, et al., 2012). In short, a multi GE FLASH sequence was performed prior to and 1 min after administration of an intravenous bolus injections of ultra small particles of iron oxide (USPIO – AMI-277, Sinerem®, Guerbet Laboratories, France) at the dose of 140µg Fe/mouse. The USPIO is a contrast agent that provides a valuable tool to characterize tissue vascularity since it remains intravascular for a prolonged period of time and strongly enhances the transverse water proton MR relaxation rate ($R_2^*$). Previous studies have shown an approximately linear relationship between the increase of relaxation rate ($\Delta R_2^*$) and rCBV fraction over the physiologically relevant range (Belliveau, et al., 1990, Boxerman, et al., 1995). This technique has been widely used to assess rCBV in mice (Dennie, et al., 1998, Kennan, et al., 1994). Imaging parameters were: FOV = 30×30×14 mm, matrix size = 192×192×88, total resolution = 0.16×0.16×0.16 mm, TEs = 5.13 – 7.28 – 9.40 ms, TR = 50 ms, resulting in a total scan time of 35 min per mouse.

Data pre-processing and cerebral blood volume calculation

For spatial normalization, the GE images before and after USPIO of all mice were co-registered with a study-specific template created from the averaged GE images of all animals using Advanced Normalization Tools (ANTs. V1.9.x, http://picsl.upenn.edu/ANTS/). Spatial normalization was achieved by using mutual information as the initial affine similarity metric and cross-correlation as ‘greedy SyN’ diffeomorphic transformation metric (Avants, et al., 2008). Spatially normalized and masked datasets were then imported in MatLab (R2008a, Mathworks, Natick, MA, USA) and, for each mouse, a pixel-by-pixel delta relaxation rate $\Delta R_2^*$ map was obtained using the formula:

$$\Delta R_2^* = \frac{1}{\text{TE}} \log \left( \frac{S_{\text{bef}}}{S_{\text{aft}}} \right)$$

where $S$ is the signal amplitude before-USPIO ($S_{\text{bef}}$ ) and after-USPIO ($S_{\text{aft}}$). Image sets from the three TEs were used for the calculation of three $\Delta R_2^*$ values and thereafter these values were averaged for $\Delta R_2^*$ maps. Histogram analysis of the $\Delta R_2^*$ images was used to mask voxels containing pial and large perforating arteries and veins and focus on the microvasculature compartment ($rCBV_{\text{micro}}$) as
described in detail elsewhere (Zerbi, et al., 2012). The analysis of genotype-wise and diet-wise differences in rCBV\textsubscript{micro} was performed with a voxel-based and regions of interest (ROIs)–based approaches.

**Voxel-based and ROI-based group comparisons**

Normalized rCBV\textsubscript{micro} values were measured in several ROIs drawn based on the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). These ROIs include: hippocampus, cerebral cortex (all cortical areas above the corpus callosum), olfactory bulb, cerebellum and thalamus. For the ROI-based approach, we evaluated significant interactions between genotype and diet. Significant differences in rCBV\textsubscript{micro} maps were additionally assessed voxel-wise using the SPM\textsubscript{Mouse} toolbox within statistical parametric mapping 5 (SPM5, Wellcome Department of Clinical Neurology, London) (Sawiak, et al., 2009). Spatially normalized rCBV\textsubscript{micro} datasets were smoothed with a 500µm isotropic Gaussian kernel to correct for imperfect registration and subsequently a two-group t test was performed to identify genotype-wise and diet-wise differences in the framework of the general linear model (GLM). Statistical significance for an individual voxel was established at $p<0.01$, uncorrected for multiple comparisons (Ashburner and Friston, 2000).

**Tissue sampling**

Directly following the MR measurements at 11 ± 1 months of age, half of the number of mice was sacrificed by cervical dislocation to collect brain tissue for biochemical analyses, and the other half was sacrificed by transcardial perfusion fixation with Somogyi’s fixative (4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer, PB, pH=7.3). Blood samples were collected via eye extraction, and subsequently processed to obtain blood serum. Blood serum was stored at -80°C before further biochemical processing. Non-perfused brains were snap frozen in liquid nitrogen and then stored at -80°C, before further biochemical processing. Perfused brains were collected and postfixed for 15h at 4°C in Somogyi’s fixative and subsequently stored in 0.1M PBS with 1% sodium azide at 4°C for immunohistochemical stainings.

**Immunohistochemistry**

Before cutting, the brain tissue was cryoprotected by immersion in 30% sucrose in PB. Six series of 40 µm coronal sections were cut through the brain using a
sliding microtome (Microm HM 440 E, Walldorf, Germany) equipped with an object table for freeze sectioning at -60°C. Presynaptic boutons were visualized with anti-synaptophysin antibody (monoclonal rabbit anti-synaptophysin clone EP1098Y, Abcam Inc., Cambridge, UK). Synaptophysin is localized in small synaptic vesicles of the presynaptic terminal and functions in the regulation of exocytosis (Jahn and Sudhof, 1993). Newly formed immature neurons were visualized with anti-doublecortin antibody (polyclonal goat anti-doublecortin (C18): sc-8066, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Doublecortin is a microtubule-associated protein that is exclusively found in somata and processes of migrating and differentiating neurons (Francis, et al., 1999, Gleeson, et al., 1999). Immunohistochemistry was performed using standard free-floating labeling procedures, and was carried out on a shaker table at room temperature as described in detail elsewhere (Jansen, et al., 2012). One complete series of brain sections, with 240 µm distance between the sections, was used for each staining. Brain sections were incubated overnight with either monoclonal rabbit anti-synaptophysin (1:20,000), or polyclonal goat anti-doublecortin (1:3000) as primary antibody. Either donkey anti-rabbit biotin (1:1500) or donkey anti-goat biotin (1:1500) was used as a secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA).

Quantification of presynaptic boutons and doublecortin-positive newly formed immature neurons was performed using a Zeiss Axioskop microscope, equipped with hardware and software from Microbrightfield (Williston, VT, USA). Appropriate sections were digitized and photomicrographed using a computer-assisted analysis system (Stereo Investigator). Brain regions were based on the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). All quantifications were performed by two independent raters who were blind to the experimental groups. Measurements were averaged to obtain a single value per animal for every region of interest (ROI).

Quantification of synaptophysin-immunoreactive presynaptic boutons
To determine the amount of synaptophysin-immunoreactive boutons (SIPBs), appropriate sections were digitized and photomicrographed at 100× magnification using an 100× oil immersion objective. SIPBs were analyzed in the hippocampus (cornu ammonis (CA)1, CA3 and dentate gyrus (DG) regions), and in the cortical regions prelimbic area (PLA) and anterior cingulate gyrus (ACg). These regions were chosen because of their large amyloid load in AD patients.
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and transgenic mouse models for AD and their importance in learning and memory (Irizarry, et al., 1997, Shukla and Bridges, 1999). In the hippocampus, SIPBs were analyzed in the stratum radiatum of the CA1 area (SR), stratum lucidum of the CA3 area (SL), and the inner molecular layer (IML) and outer molecular layer (OML) of the DG using one section per animal at -2.18 mm posterior to bregma. The ACg was quantified at level +0.98 mm anterior to bregma using one section per animal. The PLA was quantified at +1.94 mm anterior to bregma using one section per animal. For every ROI, two photomicrographs were taken at 100× magnification by each independent rater. Images were further processed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) for the quantification of the amount of SIPBs. All settings were kept identical for all analyses and background levels were equalized using a threshold. Shading correction was performed before measurement to correct for irregularities in illumination in the microscopic field. A differential contrast enhancement filter was applied to selectively enhance weak differences in contrast. To eliminate noise signal and to differentiate between possible artifacts and specific SIPBs, particles were classified based on size. Particles ranging between 0.1-4.5 µm² were considered to be normal sized SIPBs (Mulder, et al., 2007), and were included for statistical analyses. The amount of SIPBs was defined as the total number of SIPBs per µm².

Quantification of doublecortin-positive cells
For the assessment of the amount of newly formed immature neurons in the subgranular zone of the hippocampus as a measure for neurogenesis, three sections per animal (at -1.70, -2.18 and -2.46 posterior to bregma) were digitized and contours were drawn along the borders of the hippocampus at 5× magnification using Stereo Investigator software. These three sections were selected as representative slices containing the subgranular zone of the hippocampus. Doublecortin-positive (Dcx+) cells were counted at 20× magnification, and the values of the three sections were averaged to obtain a single value per animal.

Biochemical analyses
Serum and brain sterol analysis
Serum cholesterol levels and the cholesterol precursor lathosterol and its oxidative brain specific metabolite 24S-hydroxycholesterol were measured by
gas-chromatography-mass-spectrometry-selected ionmonitoring (GC-MS-SIM) as described in detail previously (Lutjohann, et al., 2002, Lutjohann, et al., 2004, Thelen, et al., 2006). Brains were homogenized and sterols were extracted overnight by chloroform/methanol trimethylsilylated prior to GC-MS-SIM analysis (Lutjohann, et al., 2002, Thelen, et al., 2006).

**Brain fatty acid analysis**
Fatty acid analyses were performed with a part of the homogenate (described above). Total lipid was extracted from brain homogenates by methanol and chloroform. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower phase (chloroform and lipids) was removed. Chloroform was added to the upper phase, samples were centrifuged again at 3000 rpm for 10 min and the lower phase was combined with the first one. The chloroform fractions were dried in a SpeedVac® and 2 ml methanol and 40 μl concentrated sulfuric acid were added to the dried extract. The samples were heated at 100°C for 60 min, and 2 ml hexane and 0.5 ml 2.5M sodium hydroxide solution were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the upper layer was collected and evaporated in a SpeedVac®. The fatty acids (FAs) were dissolved in 125 μl iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m × 0.25 mm id. 0.22 film thickness). The n6/n3 ration was calculated as a sum of analyzed n6 FAs divided by the sum of n3 FA.

**Statistical analysis**
Data are expressed as mean ± SEM and were analyzed with SPSS for windows 16.0 software (SPSS Inc. Chicago, IL, USA). Since the setup of the current study was designed to determine the effect of diets and the extent to which apoE4 and apoE ko mice develop behavioral and neuropathological traits of AD and not to study the effects of apoE itself, statistical analyses were performed separately for the apoE4 and apoE ko mice (apoE4 versus wild-type, and apoE ko versus wild-type). The repeated measures ANOVA was used for the acquisition phase of the MWM and rMWM (with the repeated measure: trial block), followed by a Bonferroni post hoc to analyze possible interactions between trial block, genotype and/or diet. If interactions between trial block, genotype or diet (between-group-factors) were present, the data were split for the concerning factor and thereafter analyzed again with the repeated measures ANOVA. Multivariate ANOVAs (MANOVA) were conducted with between-group-factors
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genotype and diet, to analyze possible differences between wild-type, apoE4 and apoE ko mice, and between the CO and FC diets in the open field parameters, the probe trials of the MWM and rMWM, the body weight, brain weight, metabolite concentrations, rCBV, immunohistochemical stainings and biochemical analyses. If the Bonferroni post hoc test indicated a significant interaction between genotype and diet (between-group-factors), the data were split for the concerning factor and thereafter analyzed again with the MANOVA. For clarity reasons, F-values are not displayed. Furthermore, only between-group interactions that reached statistical significance are specified in detail. Statistical significance was set at $p \leq 0.05$.

Results

Body and brain weight
All mice were weighed one week before starting the behavioral tests at the age of 9 ± 1 months and again on the day of the MR measurements at the age of 11 ± 1 months. Body weight was not affected by genotype or diet when animals were measured one week before starting the behavioral tests ($p > 0.05$). Overall mean body weight was 34.8 ± 0.8 g in the apoE ko mice, 33.8 ± 0.5 g in the apoE4 mice and 32.6 ± 1.0 g in the wild-type mice. Body weight was affected by genotype, but not by diet when animals were measured on the day of the MR measurement. At scanning, ApoE ko mice were significantly heavier than wild-type mice ($p < 0.001$), independent of diet. Overall mean body weight was 36.5 ± 0.7 g in the apoE ko mice, 33.3 ± 0.7 g in the apoE4 mice and 31.7 ± 0.8 g in the wild-type mice. Since absolute brain weight was not affected by genotype or diet ($p > 0.05$), we expect no confounding effects of the increased body weights in ApoE ko mice on the MR parameters measured. Overall mean brain weight was 0.49 ± 0.01 g in apoE ko mice, 0.48 ± 0.01 g in both apoE4 and wild-type mice.

Behavioral analyses

Open Field
In the open field, locomotion activity and active exploration parameters (walking, sitting, wall leaning, rearing) and grooming are scored for 30 minutes. In addition, total walking distance, mean walking velocity, and the time spent in the
corners respectively the center of the open field were obtained from the recorded sessions. ApoE4 mice, but not apoE ko mice, were more active in the open field than wild-type mice. ApoE4 mice walked more (Fig. 2A; \( p=0.016 \)) and sat less (Fig. 2B; \( p=0.004 \)), although the total distance moved (Fig. 2F; \( p=0.140 \)) and mean walking speed (\( p=0.141 \); wild-type 6.1 ± 0.3 cm/s, apoE4 7.0 ± 0.5 cm/s, apoE ko 6.3 ± 0.4 cm/s) did not differ significantly between apoE4 and wild-type mice. Dietary intervention with the FC diet had no effect on these parameters (\( p>0.05 \)). There was a significant genotype\(\times\)diet interaction for the time spent grooming (\( p=0.032 \)). ApoE4 mice on CO diet spent slightly less time grooming than wild-type mice on CO diet (Fig. 2E; \( p=0.062 \)), whereas no difference was found between apoE4 and wild-type mice on the FC diet, or between apoE ko and wild-type mice (\( p=0.411 \)). No differences were observed between the apoE4, apoE ko and wild-type mice in the time spent rearing (Fig. 2C) and wall leaning (Fig. 2D). ApoE ko animals spent significantly less time in the center (Fig. 2G; \( p=0.001 \)) and significantly more time in the corners of the open field (Fig. 2H; \( p=0.025 \)) than wild type mice, independent of diet. Overall MANOVA analysis revealed significant genotype\(\times\)diet interactions for the time spent in the center (\( p=0.007 \)) and corners (\( p=0.015 \)) of the open field when comparing apoE4 and wild-type mice. Only in wild-type mice, the FC diet increased the time spent in the center (\( p=0.001 \)), and decreased the time spent in the corners (\( p=0.002 \)) of the open field compared to the CO diet (Fig. 2G, H). The FC diet had no effect on these parameters in the apoE4 mice (\( p=0.360 \) and \( p=0.532 \) respectively). Furthermore, an overall effect of the FC diet on time spent in the center (\( p<0.001 \)) and corners (\( p<0.001 \)) was found comparing the apoE ko and wild-type mice. Again, the FC diet increased the time spent in the center and decreased the time spent in the corners of the open field compared to the CO diet (Fig. 2G, H).

Altogether these data show increased activity in apoE4 mice, whereas the apoE ko mice did not differ from the wild-types on the parameters related to locomotion. Instead, apoE ko mice show increased anxiety-related behavior, indicated by the increased time spent in the corners and decreased time spent in the center of the open field (Hooijmans, et al., 2009, Simon, et al., 1994). The FC diet decreased anxiety-related behavior in both apoE ko animals and wild-type mice.
Morris water maze (MWM)

The Morris water maze is designed to test spatial learning by training the mice to find a hidden platform (acquisition phase). Spatial memory is tested in a trial in which the platform is removed from the maze (probe trial) directly following the acquisition phase. ApoE4, apoE ko and wild-type mice showed a decrease in escape latency during training (Fig. 3A; \( p < 0.001 \)). No significant trial block×genotype, trial block×diet or trial block×genotype×diet interaction was observed, indicating that all animals learned the position of the hidden platform equally well. Escape latencies did not differ between apoE4, apoE ko and wild-type mice, nor did they differ between the CO and FC diet (\( p > 0.05 \)).

During the probe trial, apoE4 mice spent less time in the target NE quadrant (Fig. 3C; \( p = 0.020 \)) than wild-type mice. Furthermore, both apoE ko and wild-type mice performed well above 25% chance level, indicating good memorization of the platform quadrant, whereas the apoE4 mice did not significantly deviated from 25% chance performance (\( p = 0.057 \)). However, no differences were observed between apoE4, apoE ko and wild-type mice in the time spent in the exact platform area (Fig. 3D), reflecting good memorization of the platform location.

The FC diet had no effect on the spatial acquisition and the spatial memory parameters. Overall analysis comparing apoE4 and wild-type mice indicated no effects of the FC diet on swim distance (\( p = 0.402 \)) and velocity (\( p = 0.403 \)). However, when comparing apoE ko and wild-type mice, the FC diet significantly decreased swim distance (Fig. 3B; \( p = 0.049 \)) and swim velocity (\( p = 0.049 \); CO diet 18.2 ± 0.6 cm/s, FC diet 16.3 ± 0.8 cm/s), suggesting that the effects of the FC diet on those parameters are found mainly in the apoE ko mice.

Reverse Morris water maze (rMWM)

In the reverse Morris water maze, mice have to learn to find a novel position for the hidden platform. This task is considered to be a test for new learning abilities, in which a previous successful strategy must be discarded and a new strategy should be developed. ApoE4, apoE ko and wild-type mice showed a decrease in escape latency during training (Fig. 4A; \( p < 0.001 \)).

No significant trial block×genotype, trial block×diet or trial block×genotype×diet interaction was observed, indicating that all animals learned the new position of the hidden platform equally well. Escape latencies did not differ between apoE4, apoE ko and wild-type mice, nor did they differ between the CO and FC diet.
Figure 2. Open Field behavior in 9 ± 1 months old apoE4, apoE ko and wild-type mice on control (CO) and Fortasyn (FC) diet. Different open field parameters were measured within a 30 min period. A) duration of walking, B) duration of sitting, C) duration of rearing; i.e. standing on the hind paws and exploring the environment, D) duration of wall leaning, E) duration of grooming, F) total distance moved, G) duration spent in the center of the open field, and H) duration spent in the corners of the open field. Values represent the mean and SEM. a * different from wild-type mice p<0.05; b # different from wild-type CO p=0.062; c * different from control (CO) diet p<0.05.
Figure 3. Spatial learning and memory in the Morris water maze (MWM). Spatial learning was measured in a 4 day acquisition phase in 9 ± 1-month-old apoE4, apoE ko and wild-type mice on control (CO) and Fortasyn (FC) diet, by determining the latency to find a hidden platform (A). The spatial memory was tested in the probe phase in which the % of time spent in the north east (NE) target quadrant was measured (C), the % of time spent in the platform area (where formerly the platform had been located; D), and the total distance moved (B). Values represent the mean and SEM. a * different from wild-type mice p<0.05.

During the probe trial, no differences were observed between the groups in the swim distance (Fig. 4B) or in the mean swim velocity (wild-type 18.5 ± 0.5 cm/s, apoE4 18.5 ± 0.9 cm/s, apoE ko 17.9 ± 1.0 cm/s). No differences were observed between the groups in time spent in the exact platform area (Fig. 4D) or in the target SW quadrant (Fig. 4C). All animals spent well above 25% chance level in the SW target quadrant, indicating good memorization of the platform quadrant.
Figure 4. Spatial learning and memory with an extra episodic memory component in the reversal Morris water maze (rMWM). Spatial learning was measured in a 2 day acquisition phase in 9 ± 1-month-old apoE4, apoE ko and wild-type mice on control (CO) and Fortasyn (FC) diet, by determining the latency to find a hidden platform in the south west (SW) quadrant (A). The spatial memory was tested in the probe phase in which the % of time spent in the SW target quadrant was measured (C), the % of time spent in the platform area (where formerly the platform had been located; D), and the total distance moved (B). Values represent the mean and SEM. No significant differences were observed.
Magnetic resonance imaging and spectroscopy

Magnetic resonance spectroscopy (MRS)

To determine the effects of the FC diet on hippocampal metabolite concentrations, $^1$H MRS with a single voxel acquisition method (i.e. STEAM) was used in wild-type, apoE4 and apoE ko mice (Fig. 5). ApoE4 mice showed significantly higher levels of taurine (Tau) compared to wild-type animals, independent of diet ($p=0.030$). All the other metabolites levels showed no genotype differences or diet effects ($p>0.05$). In apoE ko and wild-type mice on FC diet, a significant decrease in glutamate (Glu) was observed compared to the same groups on CO ($p=0.021$). No genotype or diet effects were seen in other hippocampal metabolites levels ($p>0.05$).

Figure 5. Hippocampal metabolite levels measured with $^1$H MRS. Multivariate ANOVA revealed an overall increase in taurine (Tau) in apoE4 mice compared to wild-type, independent of dietary intake. Compared to control (CO) diet, wild-type and apoE ko animals fed the Fortasyn (FC) diet showed an overall reduction in glutamate (Glu) levels. Values represent the mean and SEM. a* different from wild-type mice CO $p<0.05$, b* different from apoE ko CO $p<0.05$, c* different from wild-type FC $p<0.05$. 

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Relative cerebral blood volume (rCBV)

To measure changes in rCBV, susceptibility-induced contrast MR imaging was used. Relaxation rate maps were obtained from \( T_2^* \)-weighted images before and after i.v. injection of USPIO. The contribution of large cerebral vessels was excluded by histogram analysis, to shift the sensitivity of \( \Delta R_2^* \) images towards the microvascular compartment (rCBV\(_{\text{micro}}\)) (Zerbi, et al., 2012).

Figure 6. Measurement of the microvascular cerebral blood volume (rCBV\(_{\text{micro}}\)) in several ROIs revealed no significant genotype differences between wild-type, apoE4 and apoE ko mice at 12 months of age. Values represent the mean and SEM (A). In (B) and (C), VBA results are shown superimposed on anatomical images. Voxels with decreased/increased rCBV\(_{\text{micro}}\), corresponding to \( p<0.01 \) for a two-tailed test, are highlighted in the t-maps. No relevant differences between the three genotypes were detected.
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**Figure 7.** VBA was assessed between the two diet groups, split by genotypes, to reveal possible diet×genotype interactions not seen in the ROI analysis. No significant effects were seen in wild-type and apoE4 mice in response to dietary intervention (A). Instead, in wild-type and apoE ko the Fortasyn diet significantly increased the rCBV\textsubscript{micro} in a relatively large area of the midbrain, in anatomical correspondence to the small branches of the circle of Willis, and in other cortical areas (B). Voxels showing decreased and increased rCBV\textsubscript{micro} due to dietary treatment correspond to \( p < 0.01 \) for a two-tailed test.

**ROI-based analyses.** The analysis focused on the hippocampus, cerebral cortex, olfactory bulb, cerebellum and thalamus. No significant differences between genotypes or diet \( (p > 0.05) \) were found in any ROIs between apoE4, apoE ko and wild-type mice (Fig. 6A).

**Voxel-based analyses.** To determine the occurrence of rCBV\textsubscript{micro} changes at higher spatial resolution, we applied a voxel-wise statistical approach to the co-registered brains. No clear dissimilarities in rCBV\textsubscript{micro} were seen between all the genotypes, with few voxels indicating significant positive or negative differences sparse throughout the brain (Fig. 6B,C). In wild-type and apoE ko mice on FC diet, several voxels in the midbrain – at the anatomical location of the first and second branches of the circle of Willis and of the posterior cerebral artery - revealed a significantly increased rCBV\textsubscript{micro} compared to their respective control diet groups. A moderate increase in rCBV\textsubscript{micro} was also seen in the parieto-temporal cortex for the same groups on FC diet (Fig. 7). No distinct effects of FC diet were detected for the apoE4 mice.
Immunohistochemistry
Immunohistochemical stainings were performed using standard free-floating labeling procedures on brain sections from 11 ± 1 month-old transgenic apoE4 and apoE ko, and wild-type mice fed either the CO diet or the FC diet for 9 ± 1 months.

Synaptophysin-immunoreactive presynaptic boutons (SIPBs)
The amount of presynaptic boutons (size ranging from 0.1-4.5 µm²) was visualized using a monoclonal antibody against synaptophysin (Fig. 8A, B). Synaptophysin-immunoreactivity was determined in cortical regions prelimbic area (PLA) and anterior cingulate gyrus (ACg), and in hippocampal regions stratum radiatum of area CA1 (SR), stratum lucidum of area CA3 (SL), and inner (IML) and outer (OML) molecular layer of the dentate gyrus. No significant differences were found in the amount of SIPBs between apoE4, apoE ko and wild-type mice (Fig. 8C) in any of the regions analyzed (p>0.05). Furthermore, the FC diet had no effect on the amount of presynaptic boutons (p>0.05).

Doublecortin-positive immature neurons
Newly formed immature neurons were visualized with a polyclonal antibody against doublecortin (Fig. 9A). Doublecortin-positive (Dcx+) cells were counted in the subgranular zone of three alternating hippocampal sections as a measure for neurogenesis. ApoE ko mice showed increased amount of Dcx+ cells in the hippocampus compared to wild-type mice (Fig. 9B; p=0.045), independent of diet, suggesting increased neurogenesis in apoE ko animals. ApoE4 mice did not differ from wild-types (p=0.163). Furthermore, the FC diet had no significant effect on the amount of newly formed immature neurons (p>0.05). However, it appears that the FC diet increased the amount of Dcx+ cells in apoE4 animals, but due to the large variation within this group, it did not reach statistical significance in the overall analysis (p=0.087), and no genotype×diet interactions were found.
Figure 8. Synaptophysin immunohistochemical staining performed on brain sections from 11±1 month-old apoE4, apoE ko and wild-type mice on control (CO) and Fortasyn (FC) diet. A) Representative image of the synaptophysin-immunoreactive presynaptic boutons (SIPBs) in the hippocampus. Image taken using an 5× objective. Scale bar=100 µm. B) Representative image of the SIPBs in the stratum radiatum (SR). Image taken using an 40× objective. Scale bar=100 µm. C) Amount of SIPBs (between 0.1-4.5 µm²) per µm² in hippocampal and cortical areas. Multivariate ANOVA did not reveal any significant differences between the different genotype and diet groups. Values represent the mean and SEM. IML=inner molecular layer, and OML=outer molecular layer (of the dentate gyrus); SR=stratum radiatum (CA1 area); SL=stratum lucidum (CA3 area); PLA=prelimbic area; ACg=anterior cingulate gyrus.
Figure 9. Doublecortin immunohistochemical staining performed on brain sections from 11±1 month-old apoE4, apoE ko and wild-type mice on control (CO) and Fortasyn (FC) diet. A) Representative image of the doublecortin-positive (Dcx+) cells in the subgranular zone of the hippocampus. Image taken using an 10× objective. Scale bar=100 µm. B) Amount of Dcx+ cells in the subgranular zone of the hippocampus. Multivariate ANOVA revealed an overall increase in the amount of newly formed immature neurons in apoE ko mice compared to wild-type animals. Values represent the mean and SEM. a* different from wild-type mice p<0.05.

Biochemical analyses

Serum and brain sterol analysis

As shown in Table 3, brain cholesterol levels did not change due to genotype or the FC diet (p>0.05). However, serum cholesterol levels were approximately 5.5× higher in apoE ko mice than in wild-type animals (p<0.001), whereas no differences were found between apoE4 and wild-type mice (p=0.917). Furthermore, serum cholesterol levels decreased due to the FC diet in wild-type and apoE4 animals (p=0.002). Overall analysis did not reveal such an effect of the FC diet comparing apoE ko and wild-type mice (p=0.112). In addition, serum lathosterol, a main cholesterol precursor in the “de novo” synthesis pathway, significantly decreased in FC fed wild-type and apoE4 mice (p=0.046). Again, overall analysis did not reveal such an effect of the FC diet comparing apoE ko and wild-type mice (p=0.833). Serum lathosterol levels were approximately 30× higher in apoE ko mice than in wild-type animals (p<0.001), and approximately
1.5× higher in apoE4 animals compared to wild-type mice (p=0.005), independent of diet. In contrast, brain lathosterol levels were decreased in apoE ko animals (p=0.001), whereas no differences were observed between apoE4 and wild-type mice (p=0.771).

The cholesterol elimination rate from the brain, measured by serum 24S-OH-cholesterol levels decreased in FC fed wild-type and apoE4 mice (p=0.001). No diet effects were found on serum 24S-OH-cholesterol levels when comparing apoE ko and wild-type mice (p=0.380). ApoE ko animals displayed a 5-fold increase in serum 24S-OH-cholesterol levels (p<0.001), whereas no differences were observed between apoE4 and wild-type mice (p=0.304). In contrast, brain 24S-OH-cholesterol levels did not change between apoE ko and wild-type mice (p=0.755), whereas apoE4 animals showed increased brain 24S-OH-cholesterol levels compared to wild-type mice (p=0.001), independent of dietary intake.

The relative concentrations of different fatty acids (FA) in brain tissue of 12-month-old wild-type, apoE4 and apoE ko mice are shown in Table 4. Wild-type, apoE4 and ApoE ko mice fed the FC diet showed a shift in the balance between n3 and n6 fatty acids. The relative amount of n3 FA in FC fed mice increased compared to the CO diet (p<0.001), independent of genotype, whereas the relative amount of n6 decreased (p<0.001), resulting in a pronounced shift in the n3/n6 ratio in FC fed mice in favor of the n3 FA (p<0.001). The reduction of the relative n6 content was mainly caused by a decrease in arachidonic acid (20:4n6; p<0.001), while the higher n3 content originated from an increase in relative DHA (22:6n3; p<0.001) in all genotypes. Furthermore, the relative concentration of OA (18:1n9) was increased in the FC fed mice compared to the CO diet (p<0.001), independent of genotype.

Overall analysis comparing apoE4 and wild-type mice indicated that the FC diet decreased the relative concentration of PUFA (p=0.020), which was not observed when comparing apoE ko and wild-type mice (p=0.112), suggesting that this effect of the FC diet is found mainly in the apoE4 mice.
Table 3. Serum and brain concentration of sterols. a=different from wild-type (CO); b=different from wild-type (FC); c=different from apoE4 (CO)

Table 4. Relative fatty acid composition % of the lipid fraction of brain homogenates of 12-month-old wild-type, apoE4 and apoE ko mice on Control (CO) and Fortasyn (FC) diet. SFA = saturated fatty acids; MUFA = mono unsaturated fatty acids; PUFA ; poly unsaturated fatty acids; DHA = docosahexaenoic acid; AA = arachidonic acid; OA = oleic acid. a=different from wild-type (CO); b=different from apoE4 (CO); c=different from apoE ko (CO); # trend, p=0.058.
Discussion
The present study aimed to investigate the extent to which a specific multi-nutrient diet can modulate cerebral hemodynamics, behavior, cognition, brain metabolism, and plasticity in the apoE4-carrier and apoE-knockout mice, which are models for risk factors in cardiovascular disorders and Alzheimer’s disease. To this end, we performed behavioral testing, MRI and MRS approaches and immunohistochemical stainings to determine to which extent these transgenic animal models develop behavioral and neuropathological traits of AD and to determine the effect of dietary intervention. An overview of the main results found in the current study is shown in Table 5.

ApoE4 mice were hyperactive in the open field, while apoE ko mice showed more anxiety-related behavior. The FC diet appears to have a general anxiolytic effect on open field behavior
Our results show that while apoE4 mice are more active in the open field, apoE ko mice show more anxiety-related behavior, as indicated by the increased time spent in the corners (i.e. anxious mice prefer the borders of the open field) and decreased time spent in the center of the open field (Hooijmans, et al., 2009, Pugh, et al., 2007, Simon, et al., 1994). These findings are in line with previous studies in adult (6-13 months old) apoE4 and apoE ko mice showing increased measures of anxiety in the elevated plus maze and higher acoustic startle responses to unavoidable anxiety-provoking stimuli compared to wild-type mice (Hartman, et al., 2001, Raber, et al., 2000, Robertson, et al., 2005). Furthermore, both apoE4 and apoE ko mice required more time to habituate to a novel environment compared to wild-type mice, suggesting increased “emotional reactivity” (Hartman, et al., 2001, Raber, et al., 2000). Consistent with their transgenic apoE mouse data, Robertson and colleagues also reported higher anxiety scores in probable AD patients with ε4/ε4 compared to those with ε3/ε3 (Robertson, et al., 2005). Anxiety symptoms and restlessness may occur in up to 70% of AD patients during the course of their illness and are significantly correlated with impairments in activities of daily living (Ferretti, et al., 2001, Grossberg, 2003, Patterson, et al., 1990, Teri, et al., 1999). Furthermore, our results might suggest that the FC diet could have an anxiolytic effect, since it increased the time spent in the center and decreased the time spent in the corners of the open field in both apoE ko animals and wild-type mice.
Table 5. Overview of the main results found in the current study in 12-month-old apoE4 and apoE ko mice supplemented with a specific multi-nutrient diet for 10 months. $^1$H MRS = proton magnetic resonance spectroscopy; rCBV = relative cerebral blood volume; n3 = omega-3 fatty acids; n6 = omega-6 fatty acids; OA = oleic acid (18:1n9); n.s. = not significant.
This is in line with studies showing that rats fed an n-3 PUFA deficient diet display increased anxiety-related behavior in the open field and elevated plus maze tasks as compared to animals fed an n-3 PUFA adequate diet (Bhatia, et al., 2011, Takeuchi, et al., 2003). Supplementation with n-3 PUFAs led to substantial reduction in the anxiety levels of n-3 PUFA deficient mice (Carrie, et al., 2002) and rats (Naliwaiko, et al., 2004, Takeuchi, et al., 2003). Schipper et al. showed that the combination of n-3 lc-PUFAs, phospholipids and B-vitamins (similar to our FC diet) completely abolished anxiety-related behavioral responses, increased social behavior and facilitated fear extinction recall in serotonin transporter knockout (SERT-ko) rats (Schipper, et al., 2011). Supplementation with n-3 lc-PUFAs has also shown beneficial effects on depressive symptoms and agitation in patients with mild to moderate AD (Chiu, et al., 2008, Freund-Levi, et al., 2008).

Spatial learning in the MWM and rMWM was unaffected, although apoE4, but not apoE ko mice, showed impaired spatial memory in the MWM. The FC diet had no effect on spatial learning and memory performance. In line with results from previous studies (Bour, et al., 2008, Grootendorst, et al., 2005), no differences were observed between apoE4, apoE ko and wild-type mice in their ability to learn the position of a submerged platform during acquisition training in the MWM and rMWM. However, the performance of the apoE4 mice during the MWM probe trial was worse than the performance of wild-type and apoE ko mice, independent of diet. The time apoE4 mice spent in the target quadrant did not exceed chance level, and was significantly shorter compared to wild-type mice, indicating spatial memory impairment. These results are consistent with previous reports of impaired memory retention performance in apoE4 mice in several spatial memory tasks (Bour, et al., 2008, Grootendorst, et al., 2005, Hartman, et al., 2001, Raber, et al., 1998). Moreover, several studies have shown a direct correlation between the apoE4 allele and cognitive decline in healthy elderly individuals (Caselli, et al., 1999, Mayeux, et al., 2001, Savitz, et al., 2006).

Contrary to expectations, no cognitive impairment could be observed in the apoE ko mice in the current study. Previous studies have shown that under basal naive conditions, apoE ko mice are severely impaired in water maze performance (Gordon, et al., 1995, Krzywkowski, et al., 1999, Masliah, et al., 1995, Oitzl, et al.,...
However, this cognitive deficit was abolished when apoE ko mice were repeatedly exposed to stressful stimuli. Stressed apoE ko mice performed the MWM tasks as good or even better than stressed wild-type mice, and this effect appeared to be mediated by corticosterone (Grootendorst, et al., 2001a,Grootendorst, et al., 2001b,Grootendorst, et al., 2002,Zhou, et al., 1998). In the current study, animals were not experimentally exposed to repeated stressful stimuli. However, the normal daily care, like handling the animals, cleaning cages and transport for behavioral testing, can activate the stress responsive system especially in the apoE ko mice, and might have positively affected their performance in the MWM task (Claassen, 1994,Holscher, 1999,Kaneto, 1997).

Previous studies have shown that n-3 lc-PUFA intake may improve cognition in both mice and rats (Fedorova and Salem, 2006,Hooijmans, et al., 2009,Tanabe, et al., 2004). Besides n-3 lc-PUFAs, other nutrients can also affect spatial memory performance. Supplementing CDP-choline or UMP and choline to rats improved spatial memory in the water maze (De Bruin, et al., 2003,Teather and Wurtman, 2003). In addition, transgenic mice fed vitamin E (Conte, et al., 2004) or vitamin B3 (Green, et al., 2008) showed normalized escape latency in the water maze. However, in agreement with our current results, there are also some reports showing no effect of n-3 PUFA intake and other nutrients on learning and memory performance (Arendash, et al., 2007,Fedorova and Salem, 2006,Oksman, et al., 2006). Moreover, most intervention studies based on supplementation with n-3 PUFAs have failed to show any protective effect on AD (Chiu, et al., 2008,Jicha and Markesbery, 2010,Kotani, et al., 2006) or cognitive decline (Dangour, et al., 2010,Rogers, et al., 2008,van de Rest, et al., 2008), except in some AD patients with very mild cognitive impairment (Freund-Levi, et al., 2006,Kotani, et al., 2006,Yurko-Mauro, et al., 2010). Furthermore, apoE4 carriers have been reported to lack risk reduction from n-3 PUFA consumption (Barberger-Gateau, et al., 2011,Huang, et al., 2005,Schaefer, et al., 2006,Whalley, et al., 2008), and to be less sensitive to the protective effects of n-3 PUFA supplementation on cognition (Cole and Frautschy, 2010,Jicha and Markesbery, 2010), although the exact mechanisms underlying this gene-by-diet interaction remain unclear. Further research is needed to explain the differential effect of n-3 PUFA on AD according to apoE genotype.
The amount of presynaptic boutons was unaffected by genotype and dietary treatment. The amount of newly formed immature neurons was increased in apoE ko mice, which might reflect a compensatory mechanism in response to functional synaptic and neuronal failure or insults to the brain. Neurogenesis appeared unaffected by the FC diet

Synaptic loss is a pathological hallmark of AD, and it is the best correlate of cognitive impairment (DeKosky, et al., 1996; Scheff and Price, 2003; Selkoe, 2002; Terry, et al., 1991). However, synaptic loss does not appear to correlate with the APOE genotype (ε2, ε3, ε4) (Scheff, et al., 2007; Scheff, et al., 2006). Synaptophysin is a widely used marker for quantification of presynaptic terminals, but conflicting results have been reported on synaptophysin immunoreactivity (SYN-IR) both in AD patients (Hamos, et al., 1989; Leuba, et al., 2008; Scheff and Price, 1998; Scheff, et al., 1996) and AD mouse models. SYN-IR in transgenic mice that develop Aβ plaques has been reported to decline, increase, or not change, apparently depending on the interplay of mouse strain, transgene, age, and disease progression (Dong, et al., 2007; Jansen, et al., 2012; King and Arendash, 2002; Masliah, et al., 1996; Mucke, et al., 2000; Rutten, et al., 2003; Yao, et al., 2005). In the current study, no changes were found between apoE4, apoE ko and wild-type mice in the amount of presynaptic boutons in hippocampal and cortical regions, which is in line with previous studies (Levi, et al., 2005; Rijpma, et al., 2013), reporting no differences in presynaptic density in the hippocampus of male apoE4, apoE ko and wild-type mice. Veinbergs and colleagues did find a decrease in presynaptic density in the hippocampus and frontoparietal cortex of 12-month-old apoE ko mice as compared to wild-type mice, while presynaptic density was preserved in 12-month-old apoE4 mice (Veinbergs, et al., 1999). Since cholesterol released from apoE-containing lipoprotein particles is used to support synaptogenesis and the maintenance of synaptic connections (Mauch, et al., 2001; Pfrieger, 2003; Poirier, et al., 1995), a decrease in presynaptic density in apoE ko mice could be expected. However, no differences in brain cholesterol levels between wild-type, apoE4 and apoE ko were observed in the current study, which might explain the lack of differences in amount of presynaptic boutons. It should be noted that we used female C57BL/6J breeder mice from Harlan Laboratories, Inc. (Horst, the Netherlands) to generate male wild-type offspring for the current study. It is known that these mice carry a gene mutation resulting in an alpha-synuclein gene deletion (Specht and Schoeppfer, 2001). The alpha-synuclein deletion has
some effects on synapse function related to neurotransmitter mobilization (Gureviciene, et al., 2007, Yavich, et al., 2006, Yavich, et al., 2004). However, basal synaptic neurotransmission is unimpaired (Gureviciene, et al., 2007), and there are no indications of an altered number of (pre)synapses or neurogenesis in C57BL/6J OlaHsd mice. Furthermore, alpha-synuclein is not essential for spatial learning tasks, such as the Morris water maze (Chen, et al., 2002). Thus, although we should keep this limitation in mind when interpreting our results, the use of the C57BL/6J wild-type offspring as control mice is not expected to significantly alter the outcomes of the experiments.

In line with a previous study by Levi and Michaelson, (Levi and Michaelson, 2007), apoE ko mice displayed enhanced neurogenesis, which might reflect a compensatory mechanism in response to functional synaptic failure or insults to the brain, like stroke or ischemia. (Arendt, 2001, Jansen, et al., 2012, Jin, et al., 2004b, Li, et al., 2010, Mu and Gage, 2011, Wang, et al., 2008). Both in AD patients and in AD and apoE mouse models, conflicting results have been found regarding neurogenesis. Most studies showed a decrease in neurogenesis in both AD patients (Boekhoorn, et al., 2006, Lazarov and Marr, 2010, Lazarov, et al., 2010) and in APP and PS1 mouse models (Demars, et al., 2010, Hamilton and Holscher, 2012, Taniuchi, et al., 2007). In contrast, some studies found an increase in AD patients (Jin, et al., 2004b) and in AD transgenic animal models (Jin, et al., 2004a, Yu, et al., 2009). It has been suggested that neurogenesis, as well as synaptogenesis, may have a compensatory role in the early stages of AD which can counteract the effect of impaired synaptic and neuronal function, neurodegeneration and cell death (Abrous, et al., 2005, Gould, et al., 1999, Jin, et al., 2004b, Mu and Gage, 2011). Accordingly, activation of apoptotic mechanisms and neuronal death during acute and chronic insults, such as ischemia, Huntington’s disease and AD, is indeed accompanied by compensatory increases in hippocampal neurogenesis (Briones, et al., 2005, Curtis, et al., 2003, Greenberg and Jin, 2006, Kee, et al., 2001, Li, et al., 2010, Mu and Gage, 2011, Sutula, et al., 1989, Wang, et al., 2008, Yagita, et al., 2001).

In the current study, the FC diet had no significant effect on the amount of presynaptic boutons or the amount of newly formed immature neurons, although in the latter case one might notice the slight, but non-significant, increase in the amount of newly formed immature neurons in the apoE4 animals.
on the FC diet. In line with our results, previous studies failed to demonstrate an
effect of n-3 PUFA diets on synaptophysin levels; instead, n-3 PUFAs seem to
exert their beneficial effects more by improving synaptic function rather than by
synaptogenesis (Arsenault, et al., 2011,Cole and Frautschy, 2006,Kariv-Inbal, et
al., 2012,Savelkoul, et al., 2012). Other studies have shown that omega-3 fatty
2009), folic acid (Das, 2008,Zhang, et al., 2012), and vitamins and antioxidants
(Bonnet, et al., 2008,Cuppini, et al., 2002) may promote neurogenesis. However,
the majority of the studies reporting a positive effect of n-3 PUFAs, folic acid, and
vitamins and antioxidants on neurogenesis were conducted in nutrient-deficient
animals (Bonnet, et al., 2008,Kawakita, et al., 2006). These results could imply
that beneficial effects of dietary interventions might become apparent only when
neurogenesis is severely compromised in response to a dietary deficiency. Since
our apoE4 and apoE ko mice were not nutrient-deficient and did not show
compromised neurogenesis at 11 ± 1 months of age, this might explain the lack
of differences due to dietary intervention in the current study.

**ApoE4 and apoE ko mice did not show differences in CBV compared to wild-type
mice. The FC diet increased CBV in wild-type and apoE ko mice next to the brain
feeding arteries**

Among other proprieties, apolipoprotein is very important in the maintenance of
brain vascular health, and in the repair of blood brain barrier (BBB) after injury. A
list of studies reported a number of neurovascular pathologies associated with
apoE4 genotype, such as increased small vessel arteriolosclerosis and micro
infarcts of the deep nuclei (Yip, et al., 2005), cerebral amyloid angiopathy
(Premkumar, et al., 1996) and inflammation triggering blood brain barrier (BBB)
breakdown (Bell, et al., 2012). These pathologies, together with gross
cardiovascular problems, are present in higher extent in mice lacking apoE, and
their conditions are worsened if the animals were fed a high fat / high cholesterol
apoE ko mice are shown to precede neuronal dysfunction and can initiate
neurodegenerative processes (Bell, et al., 2012). We tested here if brain vascular
pathology is detectable in apoE4 and apoE ko mice with MR imaging and if the
specific multi-nutrient diet has beneficial effects on the neurovascular system.
Our analysis revealed an increased rCBV due to the FC diet in apoE ko and wild-
type mice in the midbrain and in the parieto-temporal cortex.
The lack of genotype differences is in contrast with our initial expectations of a diminished cerebral blood volume due to atherosclerosis in apoE4 and in apoE ko mice (Plump, et al., 1992). However, a micro-CT imaging study showed that apoE ko mice from 16 to 80 weeks develop atherosclerotic plaques in the aorta, pulmonary artery and carotid artery, but not in cerebral arteries (Langheinrich, et al., 2007). The presence of atherosclerosis in the systemic circulation may have a detrimental effect on the cerebral perfusion, without directly compromising the brain vascular structure. The absence of genotype differences found in this study seems to confirm this hypothesis. Microcirculation deficits would then be triggered by a direct interaction between apoE and the neurovascular unit, rather than from the accumulation of atherosclerotic plaques in cerebral vessels. A recent study proposed the proinflammatory cyclophilin-a as a possible mediating factor between apoE genotype and brain microcirculation (Bell, et al., 2012).

In the mid-brain, adjacent to the circle of Willis and the posterior cerebral artery, we found voxels of increased rCBV in apoE ko and wild-type mice fed with the multi-nutrient FC diet, which may reflect an improvement in brain perfusion. Such improved cerebrovascular circulation is possibly influenced by both the n-3 PUFA components and by the B-vitamins in the FC diet and is consistent with other animal studies (Hooijmans, et al., 2009,Tsukada, et al., 2000). The beneficial effects on the cardiovascular system by n-3 lc-PUFAs, important components in our FC diet, have been widely demonstrated in the last decade. Several epidemiological studies and controlled trials showed a correlation between n-3 PUFA and improvement in autonomic function, lowered blood pressure, reduced atherosclerosis and enhanced microvasculature endothelium-dependent vasodilation processes (Artham, et al., 2008,Lavie, et al., 2009). All these factors may have contributed to improve the functionality of the brain vascular system, particularly in apoE ko mice, where these pathologies are accelerated. Furthermore, it has been shown that deficiency of folate and B-vitamins, which are instead supplemented in the FC diet, reduced brain capillary density in mice (Troen, et al., 2008). Finally, all mice fed with FC diet showed significantly decreased serum total cholesterol levels, which may have an additional effect on the fluid dynamics of the blood, lowering plasma viscosity and facilitating blood perfusion (Ercan, et al., 2003,Isik, et al., 2012,Tous, et al.,
2004). A direct measure of the cerebral blood flow with other techniques (e.g. arterial spin labeling MRI) would help to quantitatively determine perfusion deficits in these mice and the effects of diets on brain hemodynamics.

ApoE4 mice displayed increased levels of Tau in the hippocampus. The FC diet reduced the hippocampal Glu levels in all genotypes

$^1$H hippocampal MR spectroscopy revealed increased Tau levels in the apoE4 mice and a decrease in Glu levels in wild-type and apoE ko mice by supplementation with the FC diet. Tau is an amino acid that is reported to have a number of physiological and pharmacological functions, including osmoregulation, cell volume adjustment, antioxidation and modulation of the action of neurotransmitters (Oja and Saransaari, 1996). Although the effects of taurine on brain metabolism are still unclear, some studies reported beneficial effects of taurine on brain function (Ito and Azuma, 2004). Moreover, it has been reported that taurine prevents the formation of atherosclerotic plaques in apoE ko mice (Kondo, et al., 2000). Taurine is mainly obtained by dietary intake, but it can also be synthesized in the brain from other sulphur-containing amino acids in response to stressful events. Taurine levels increases in both rats and mice as a consequence of various cell-damaging conditions, including ischemia (Lekieffre, et al., 1992, Saransaari and Oja, 1997). The increase in taurine in the apoE4 mice may therefore reflect a spontaneous response of the brain cells to an increased stress conditions / pathological status in these mice. The mechanism by which apoE4 genotype, and not the absence of apoE, induces excessive taurine production is however far from being clear and requires further studies.

In our experiment, we found decreased levels of Glu due to the specific multi-nutrient diet supplementation. Glutamate, and glutamate receptors, play a fundamental role in neuronal grown and differentiation, synaptic plasticity and in regulating neurotransmission, by being the major mediator of excitatory signals in the central nervous system. However, excess in glutamate production is potentially harmful due to its neurotoxic proprieties. In many neurodegenerative diseases, such as AD, glutamate levels are found to be higher than in normal aging. Amyloid-β peptides have been shown to increase glutamate release, inhibit clearance of glutamate by astrocytes and affect glutamate receptor activity (Revett, et al., 2013). Furthermore, glutamate is the major mediator of excitotoxic neuronal death following cerebral ischemia. Under hypoxic or
ischemic conditions, glutamate transporters can functionally reverse to release glutamate, thereby inducing further neuronal injury (Kato and Kogure, 1999;Noga, et al., 1997;Wang, et al., 2013). Although the interpretation of these data need needs to be made carefully, our results suggest that the FC diet can modulate the production / uptake of glutamate, and this is possibly linked to a better brain perfusion with an amelioration of neuronal function.

**Conclusion**
The multi-nutrient Fortasyn Connect^®^ (FC) diet consists of nutrients which are precursors and co-factors for membrane synthesis and maintenance, and also contains various nutrients that may be expected to support cardio- and cerebrovascular health. In the present study, we showed that the supplementation of FC diet is able to change fatty acid composition in the brains of all our mice. We showed that 11 month-old apoE4 mice display increased activity and slightly reduced memory compared to wild-type mice, which may be a consequence of hypercholesterolemia and AD. The lack of substantial pathological status in these mice may explain why we could detect only minor effects of the specific multi-nutrient diet. Instead, in the apoE ko mice, a more severe model for atherosclerosis and hypercholesterolemia, we measured several beneficial effects due to the FC diet: decreased anxiety-related behavior, a reduction in hippocampal glutamate levels, and increased rCBV. We hypothesize that these features reflect a better response from neuronal cells to sustain ischemic driven events, due to an amelioration of cardiovascular health and possibly due to improving the cell membrane properties. Taken together, these results suggest that this specific multi-nutrient diet has the potential to ameliorate AD-related and ischemia-related pathologies, by positively influencing both the vascular system and the cell membrane composition.
Impact of a multi-nutrient diet on cognition, brain metabolism, hemodynamics, and plasticity
Multi-nutrient diets improve cerebral perfusion and neuroprotection in an Alzheimer mouse model

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Abstract

Nutritional intervention may retard the development of Alzheimer’s disease (AD). In this study we tested the effects of two multinutrient diets in an AD mouse model (APP<sub>swe</sub>/PS1<sub>dE9</sub>). One diet contained membrane precursors such as omega-3 fatty acids and uridine monophosphate (DEU), while another diet contained cofactors for membrane synthesis as well (Fortasyn); the diets were developed to enhance synaptic membranes synthesis, and contain components that may improve vascular health. We measured cerebral blood flow (CBF) and water diffusivity with ultra-high-field MRI, as alterations in these parameters correlate with clinical symptoms of the disease. APP<sub>swe</sub>/PS1<sub>dE9</sub> mice on control diet showed decreased CBF and changes in brain water diffusion, in accordance with findings of hypoperfusion, axonal disconnection and neuronal loss in AD patients. Both multi-nutrient diets were able to increase cortical CBF in APP<sub>swe</sub>/PS1<sub>dE9</sub> mice and Fortasyn reduced water diffusivity, particularly in the dentate gyrus and in cortical regions. We suggest that a specific diet intervention has the potential to slow AD progression, by simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms.
Introduction
Alzheimer’s disease (AD) is the leading cause of dementia worldwide. The disease is clinically characterized by cognitive impairment, memory loss, anoma, apraxia, anosognosia and alteration of personality. Pathological hallmarks of AD include the presence of neurofibrillary tangles (NFTs), amyloid-beta (Aβ) plaque formation and neurodegeneration (Sennvik, et al., 2000). The development of AD also involves several associated processes all of which are described in AD pathophysiology, such as: synaptic loss, chronic brain inflammation, white matter degeneration, mitochondrial dysfunction and cerebrovascular defects. After many years of extensive research, the idea that a single event can trigger AD pathogenesis is fading, replaced by the belief that all these events combined lead to a progressive damage of neuronal function, loss of synaptic integrity and cognitive decline. The failure of several anti-AD drugs in phase 3 clinical trials has been related to the fact that these medicines targeted a single trait of the disease, already in a stage when neuronal damage had become too widespread (Kozauer and Katz, 2013); this emerging idea emphasizes the importance of combining new therapeutic approaches that act in parallel on multiple AD risk factors in an early stage of the disease (Iqbal and Grundke-Iqbal, 2010). One such approach currently available is the use of nutrients and dietary modifications.

Aside from aging, many risk factors of AD, including atherosclerosis and hypercholesterolemia, seem to be related to the development of a less healthy lifestyle in the western population. The consumption of high cholesterol and high caloric food typical of the western diet, resulting in overweight with its associated disorders, is proposed as one of the main causes of the increased AD prevalence, largely because of their negative effect on the vascular system (Puglielli, et al., 2003)(Casserly and Topol, 2004,Luchsinger, et al., 2005)(Wolozin, et al., 2006)(Canevari and Clark, 2007). A beneficial impact on AD onset and development has been suggested via healthy dietary behaviour instead. Among a long list of nutrients that are considered potential candidates for interventions to delay AD, omega-3 (n-3) long-chain polyunsaturated fatty acids (LCPUFA) from fish oil are the most studied. With the discovery of the therapeutic effects of n-3 LCPUFA supplementation against cardiovascular pathologies (Lee, et al., 2008), the idea arose about influencing AD development by attenuating vascular risk factors. Furthermore, high levels of n-3 LCPUFA replace omega-6 (n-6) fatty acids
and cholesterol from cell membranes leading to increased fluidity of the membrane, increased number of receptors, enhanced receptor binding and affinity, and improved neurotransmission and signalling (Bourre, et al., 1989), which is important for optimal cognitive functioning (Bourre, et al., 1991,Farkas, et al., 2002,Fontani, et al., 2005). Several epidemiological studies associated a reduced risk of developing dementia, including AD, with an increased dietary intake of n-3 LCPUFA from fish oil, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Kalmijn, et al., 1997,Schaefer, et al., 2006),(Barberger-Gateau, et al., 2007,Beydoun, et al., 2008,Eskelinen, et al., 2008). However, evidence from clinical randomized controlled trials failed to confirm the promising findings of their efficacy as AD treatment (Cunnane, et al., 2009,Yurko-Mauro, et al., 2010,Quinn, et al., 2010).

One of the reasons behind these inconclusive results may lie in the concomitant presence of multiple phenotypic traits in AD. While n-3 LCPUFA alone improve the functionality of the neural and vascular system and have a beneficial effect on cardiovascular disease prevention, this might not be sufficient to avert neurodegenerative processes and synaptic loss as occurring in AD. Several preclinical studies showed that specific combinations of nutrients, rather than a single component, can synergistically act to express a better therapeutic outcomes; animals fed with a combination of uridine monophosphate (UMP), DHA and choline, which are key nutritional precursors to form phosphatidylcholine (Kennedy and Weiss, 1956), but not DHA alone, showed increased levels of brain phospholipids, dendritic spines and neurite outgrowth, with beneficial effects on cognition (Cansev and Wurtman, 2007,Sakamoto, et al., 2007,Wurtman, et al., 2006). Recently, a novel combination diet that also includes other precursors and cofactors in membrane synthesis (such as phosphatidylcholine, B-vitamins and antioxidants), called Fortasyn, has been proposed for the dietary management of AD (Kamphuis and Scheltens, 2010). To date, two randomized controlled clinical trials have shown improvements in the delayed verbal recall task and better cognitive performance in mild AD patients supplemented with a Fortasyn-containing diet (Cummings, 2012,Scheltens, et al., 2010,Scheltens, et al., 2012). The mechanisms by which these dietary nutrients influence the pathophysiology of AD yet need to be elucidated, and there is the need for more studies to confirm their efficacy. In this study we tested the hypothesis that the multi-nutrient Fortasyn diet, and a diet consisting of DHA,
Multi-nutrient diets improve cerebral perfusion and neuroprotection

EPA and UMP without additional nutrients, have a positive effect in delaying AD pathology progress both by improving cerebral perfusion and by protecting against neuronal degeneration. With this aim, we evaluated cerebral blood flow (CBF) and gray and white matter integrity with MR imaging at ultra-high field in a 12-month-old double transgenic mouse model for genetic AD (APP<sub>swe</sub>/PS1<sub>dE9</sub>).

Materials and methods

Animals
The APP<sub>swe</sub>/PS1<sub>dE9</sub> founder mice were originally obtained from John Hopkins University, Baltimore, MD, USA (Borchelt, et al., 1997), and a colony was established at the Central Animal Facility at Radboud University Nijmegen Medical Center, the Netherlands. In short, 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 promotor elements. The two transfected genes cointegrate and co-segregate as a single locus (Jankowsky, et al., 2001). This line (line 85) was originally maintained on a hybrid background by backcrossing to C3HeJ × C57BL6/J F1 mice (so-called pseudo F2 stage). For the present work, the breeder mice were backcrossed to C57BL6/J for 13 generations to obtain mice for the current study. To reduce the variability of the data, only male mice were used for the experiment. Throughout the experiments, the animals were housed in a controlled environment, with room temperature at 21°C, and an artificial 12:12h light:dark cycle (lights on at 7 a.m.). Food and water were available ad libitum. The experiments were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority of Radboud University Nijmegen Medical Center.

Diets
From two months of age, male transgenic mice (n=27) and wild-type littermates (n=43) were assigned to three different diet groups until the end of the experiment (Table 1). One group of mice was used as a control group and received a standard normal rodent chow with 5% fat percentage obtained from a combination of corn oil (2.2%), soy oil (1.9%) and coconut oil (0.9%). In the second group the fat composition in the food was replaced by fish oil (3.0%),
corn oil (1.9%) and coconut oil (0.1%). In addition, uridine monophosphate (UMP) was added (1%). This diet will be called DEU (DHA+EPA+UMP) throughout the article. The third group received the same DEU diet with additional nutrients such as choline, phospholipids, vitamins B6, B12, C and E, folic acid and selenium. This multi-nutrient diet will be called Fortasyn. The composition of the diets is shown in Table 1. All three diets were isocaloric. All diets were manufactured and pelleted by Research Diet Services (Wijk bij Duurstede, NL) and stored at -20°C until use.

<table>
<thead>
<tr>
<th>Oils</th>
<th>Control [g/100g]</th>
<th>DEU [g/100g]</th>
<th>Fortasyn [g/100g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>% soya oil</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% coconut oil</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>% corn oil</td>
<td>2.2</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>% fish oil</td>
<td>-</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additives</th>
<th>Control [g/100g]</th>
<th>DEU [g/100g]</th>
<th>Fortasyn [g/100g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>% pyridoxine-HLC</td>
<td>-</td>
<td>-</td>
<td>0.00033</td>
</tr>
<tr>
<td>% folic acid (90%)</td>
<td>-</td>
<td>-</td>
<td>0.00067</td>
</tr>
<tr>
<td>% cyanocobalamin</td>
<td>-</td>
<td>-</td>
<td>0.00350</td>
</tr>
<tr>
<td>% ascorbic acid</td>
<td>-</td>
<td>-</td>
<td>0.16</td>
</tr>
<tr>
<td>% dl-α-tocopheryl acetate</td>
<td>-</td>
<td>-</td>
<td>0.465</td>
</tr>
<tr>
<td>% uridine monophosphate (UMP)</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>% choline chloride</td>
<td>-</td>
<td>-</td>
<td>0.402</td>
</tr>
<tr>
<td>% soya lecithin</td>
<td>-</td>
<td>-</td>
<td>0.412</td>
</tr>
<tr>
<td>% sodium selenite</td>
<td>-</td>
<td>-</td>
<td>0.00023</td>
</tr>
</tbody>
</table>

Table 1. Oil sources and additives in the experimental diets.
MRI protocol

MRI measurements were performed at the age of 12 months on a 11.7 T BioSpec Avance III small animal MR system (Bruker BioSpin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m and operated by a Paravision 5.1 software platform. We used a circular polarized volume resonator for signal transmission and an actively decoupled mouse brain quadrature surface coil with integrated combiner and preamplifier for signal receiving (Bruker BioSpin). For the MR imaging procedure, the animals were anesthetized with isoflurane (3.5% for induction and 2% for maintenance) in a 2:1 oxygen and N₂O mixture and placed in a stereotactic holder to prevent unwanted movement during the scanning. Body temperature was maintained at 37˚C with heated airflow and monitored with a rectal temperature probe. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA).

After standard adjustments, gradient echo (GE) images in three orthogonal directions were acquired to determine an anatomical reference of the mouse brain. Image parameters: echo time (TE)=5 ms; repetition time (TR)=630 ms; flip angle=16°; field of view=4 cm; matrix size=512×512; spatial resolution=0.078×0.078×0.34 mm/pixel; total scan time=8 min.

Brain perfusion was measured under resting conditions using an established arterial spin labelling (ASL) method with flow-sensitive alternating inversion recovery (FAIR) technique (Kim, 1995, Kwong, et al., 1991). A spin-echo planar imaging sequence preceded by a 180° hyperbolic secant (sech80) RF inversion pulse was used. Imaging parameters: TE = 11.8 ms; TR = 13.75 s; image matrix = 128×128; field of view = 30×30 mm; spatial resolution = 0.234×0.234×1 mm/pixel. Inversion parameters: inversion slab thickness = 6 mm; slice margin = 1.5 mm. Twenty-five images with increasing inversion times (TIs) (40 ms - 3000 ms) were obtained for the T₁ calculations, amounting to a total scan time of 24 minutes per mouse. Inversion recovery data from the imaging slice were acquired after selective inversion interleaved with non-selective inversion.

Diffusion of water was measured by the protocol of Harsan et al., which we modified as described previously (Harsan, et al., 2010, Zerbi, et al., 2013). In short, 31 axial slices covering the whole brain were acquired with a spin-echo planar imaging protocol (SE-EPI). B₀ shift compensation, navigator echoes and an automatic correction algorithm to limit the occurrence of ghosts and artefacts were implemented. Encoding b-factors of 0 s/mm² (b₀ images; 5×) and 1000
s/mm² were used and diffusion-sensitizing gradients were applied along 30 non-collinear directions in three-dimensional space. Other imaging parameters were: TE = 21.4 ms; TR = 7750 ms; time between the application of diffusion gradient pulses Δ = 10 ms; diffusion gradient duration δ = 4 ms; number of segments = 4; spatial resolution 0.156×0.156×0.5 mm/pixel; total scan time of 18 minutes for each mouse.

Cerebral blood flow calculation
For each mouse, the FAIR images with different TIs were realigned and resliced over the first TI using a rigid-body model, implemented in the Statistical Parametric Mapping 8 tool (SPM8, University College London, http://www.fil.ion.ucl.ac.uk/spm/).

Determination of $T_1$ selective and $T_1$ non-selective was performed by fitting the averaged signal intensities in each ROI with a three-parameters monoexponential $T_1$ relaxation curve. The perfusion was calculated from both $T_1$ values using the equation:

$$\frac{CBF}{\lambda} = \frac{T_1\text{non-selective}}{T_1\text{blood}} \left( \frac{1}{T_1\text{selective}} - \frac{1}{T_1\text{non-selective}} \right)$$

where $\lambda$ is the blood/tissue partition coefficient for water, assumed to be 0.9 ml g⁻¹ (Leithner, et al., 2008) and $T_1\text{blood}$ was assumed to be 2.75s at 11.7T (Lin, et al., 2012). Regional perfusion was evaluated in the cerebral cortex (all cortical regions above corpus callosum), hippocampus, thalamus and in the entire brain according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). All algorithms were implemented in MATLAB R2008a (Mathworks, Natick, MA, USA).

Diffusion tensor MRI (DT-MRI) parameter estimation
By measurement of the water diffusivity in multiple directions, DT-MRI reconstructs an ellipsoid to model the diffusion in every voxel. The diffusion tensor is characterized by the magnitude of the diffusivity over its three axes (eigenvectors). The mean diffusivity (MD) is the average of these diffusivities and describes the size of the tensor. The axial diffusivity ($\lambda_1$) is the diffusion aligned to the primary diffusion direction and the radial diffusivity (RD) represents the diffusivity perpendicular to this main direction. The shape of the diffusion tensor is quantified by the fractional anisotropy (FA), which is an index between 0 and 1.
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that indicates to what degree diffusivity is different over the three axes of the tensor.

The pre-processing steps consisted in individual realignment and reslice of the diffusion images (SPM8), followed by spatial normalization and tensor estimation. For spatial normalization, a study-specific template from the MR diffusion images was created of all WT and transgenic animals using Advanced Normalization Tools (ANTs. V1.9, http://picsl.upenn.edu/ANTS/). A group-wise normalization procedure (SyGN, as implemented in the buildtemplateparallel.sh script V0.0.13) was employed on the mean diffusion image of each mouse (Avants et al., 2010). The default implementation was followed with four iterations, using mutual information as the initial affine similarity metric and cross-correlation as ‘greedy SyN’ diffeomorphic transformation metric. A mask that included the entire brain was manually defined in the normalized coordinate space and transformed to each animal’s native space for use in subsequent processing. The diffusion tensor was estimated for every voxel using the PATCH algorithm (Zwiers, 2010). This method incorporates realignment and is robust against both regional artefacts (e.g. cardiac motion) and slice-wise artefacts (e.g. bulk motion) by providing a weight for every voxel that reflects the probability of being an outlier in the tensor fitting. From the eigenvalues of the diffusion tensor, FA, MD, RD and λ₁ were calculated. The resulting volumes were spatially normalized to the template space using the affine parameters and deformation field obtained at the creation of the template.

Region of interest analysis

The maps of FA, MD, RD and λ₁ were evaluated for significant genotype differences with a region of interest (ROI) approach. For the ROI-based approach, nine white matter tracts were selected bilaterally across the mouse brain based on an atlas (Franklin K, 1997,Franklin and Paxinos, 1997). These regions include the anterior commissure (aca), body (bcc), genu (gcc) and splenium of the corpus callosum (scc), cerebral peduncle (cp), external capsule (ec), fimbria of the hippocampus (fi), and fornix (fo). The hippocampus and cerebral cortex (all cortical areas above the corpus callosum) were also defined approximately at -1.22 up to -2.54 posterior to bregma.
**Voxel based analysis (VBA)**

Regional differences in spatially normalized FA and MD maps between APPswe/PS1<sub>de9</sub> mice and WT littermates and between the diet groups were additionally assessed voxel-wise using MATLAB R2008a (Mathworks, Natick, MA, USA) and Statistical Parametric Mapping 5 (SPM5, Wellcome Department of Clinical Neurology, London, UK) with the SPMMouse toolbox (Sawiak, et al., 2009). Two t-tests were performed to identify genotype-wise differences in the framework of the general linear model. VBA was further assessed between the control diet and DEU and Fortasyn diet groups, to reveal diet effects not seen in the ROI analysis. Statistical significance for an individual voxel was established at p<0.05, uncorrected for multiple comparisons. The locations of significant voxels exceeding a minimum cluster size of 4 (to achieve cluster size ≈ 0.05 mm<sup>3</sup> as in (Dubois, et al., 2008)) were determined and overlaid onto images derived from the template image.

**Tissue sampling**

Directly following the MR measurements, half of the mice were sacrificed by cervical dislocation to collect brain tissue for biochemical analyses, and the other half were sacrificed by transcardial perfusion fixation with saline followed by 4% paraformaldehyde. Brains were removed from the skull after decapitation. Brains for biochemical analyses were snap frozen in liquid nitrogen and stored at -80°C. Perfused brains were postfixed for 15h at 4°C in 4% paraformaldehyde and subsequently stored in 0.1M PBS with 1% sodium azide at 4°C for immunohistochemical stainings.

**Amyloid-β staining**

Amyloid-β deposits were visualized using WO-2 antibody (mouse anti-human Aβ<sub>4-10</sub> from K. Beyreuther, Centre for Molecular Biology, University of Heidelberg, Germany). In short, 40μm thick coronal sections were pre-treated with sodium citrate solution 0.05M at 85°C for 30 min and incubated for 18h at 4°C with primary antibody mouse anti-human Aβ<sub>4-10</sub> (1:20,000 in TBS-T). The next day sections were rinsed in TBS-T and incubated for 90 min with the secondary antibody donkey-anti mouse biotin (1:1500, Jackson ImmunoResearch). Thereafter, sections were transferred to a solution containing Vector ABC-Elite 1:800 (Vector laboratories, Burlingame, CA, USA). The sections were incubated in DAB-Ni solution with perhydrol for 10 min and mounted on gelatin-coated slides.
After dehydration in alcohol series, slices were cleared with xylol and mounted in Entellan. The amount of β-amyloid was determined in appropriate sections digitized using a Carl Zeiss Axioskop microscope, equipped with hardware and software of Microbrightfield (Williston, USA). Extracellular Aβ load was quantified with a computer-assisted analysis system (Stereo Investigator, Microbrightfield, Williston, USA) using Cavalieri’s probe. Contours along the whole hippocampus, along three hippocampal regions (cornu Ammonis area 1 and 3 (CA1, CA3) and dentate gyrus (DG)) and in the frontal prelimbic area (PLA) and anterior cingulate gyrus (ACg) were drawn. Measurement of Aβ load was defined as the percentage of area covered by Aβ. Serial images with 20× magnification were used for the quantification by two investigators.

**Brain fatty acid analysis**

Fatty acid analyses were performed on homogenized brain tissues – including frontal lobe and olfactory bulb - and total lipid was extracted by adding methanol and dichloromethane. Subsequently, samples were centrifuged at 3000 rpm for 10 min and the lower layer (dichloromethane and lipids) was collected. Chloroform was added to the upper phase, samples were centrifuged again at 3000 rpm for 10 min and the lower phase was combined with the first one. The chloroform fractions were dried in a SpeedVac®, and 2 ml methanol and 40 μl concentrated sulfuric acid were added to the dried extract. The samples were heated at 100°C for 60 min, and 2 ml hexane and 0.5 ml 2.5M sodium hydroxide solution were added. After vortexing and centrifuging the samples for 5 min at 3000 rpm, the upper layer was collected and evaporated in a SpeedVac®. The fatty acids were dissolved in 125 μl iso-octane and analyzed on a GC-FID with a CP-SIL88 column (50 m × 0.25 mm id. 0.22 film thickness). The n6/n3 ration was calculated as a sum of analyzed n6 fatty acids divided by the sum of n3 fatty acids.

**Statistics**

For the statistical analysis, SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) for Windows was used. The univariate ANOVA test with diet as between-group-factor was used to evaluate regional differences in Aβ- load in APP<sub>swe/</sub>PS1<sub>△E9</sub> mice. In all other cases, multivariate two-way ANOVAs (MANOVA) with genotype and diet as between-group-factors were conducted to analyze possible differences.
between APP<sub>swe</sub>/PS1<sub>dE9</sub> and wild-type mice, and between the control, DEU and Fortasyn diets. If the Bonferroni post hoc test indicated a significant interaction between genotype and diet (between-group-factors), the data were split for the concerning factor and thereafter analyzed again with the MANOVA. For clarity reasons, F-values are not displayed. Furthermore, only between-group interactions that reached statistical significance are specified in detail. If the overall statistical analysis showed a significant diet effect, we used the post-hoc Tukey’s HSD test on the separate groups. Correlation analyses between perfusion, diffusion parameters and Aβ load in the whole hippocampus were performed with the bivariate Pearson’s correlation method. Statistical significance was set at \( p \leq 0.05 \). All values used are expressed as mean ± SEM.

**Results**

**Body weight**

All mice were weighed before the MR measurements. Body weight was affected by genotype, with mean body weight being slightly higher in the APP<sub>swe</sub>/PS1<sub>dE9</sub> mice compared to wildtype mice (38.5 ± 1 g and 35.4 ± 0.7 g, respectively; \( p=0.008 \)). We did not observe differences in the body weight by the various diets.

**Cerebral blood flow**

To study genotype and diet intervention effects on cerebral hemodynamic, we measured cerebral blood flow (CBF) with a flow-sensitive MRI technique (FAIR ASL) in 12-month-old APP<sub>swe</sub>/PS1<sub>dE9</sub> mice and wildtype littermates on the three different diets. Four regions of interest (ROI) were analyzed: entire brain, cortex, hippocampus and thalamus (Figure 1, c). Since no intra-individual differences in CBF between right and left hemispheres were detected between mice groups (data not shown), values from both sides were averaged. Compared to wildtype, the APP<sub>swe</sub>/PS1<sub>dE9</sub> mice showed a significant reduced CBF, independent of diet, in the entire brain (\( p<0.001 \)) and in the hippocampus (\( p=0.001 \)). In the cortex and in the thalamus, the two-way MANOVA revealed genotype×diet interactions; after splitting the data for age and for genotype, we found that APP<sub>swe</sub>/PS1<sub>dE9</sub> mice on control diet had a lower CBF also in both regions (\( p<0.001 \)). The APP<sub>swe</sub>/PS1<sub>dE9</sub> mice fed with the DEU diet also showed a reduced CBF in the cortex compared to wildtype (\( p=0.008 \)), while transgenic mice fed with the Fortasyn diet had significant reduced CBF compared to wildtype littermates only in the thalamus (\( p=0.011 \)).
Figure 1. To measure the cerebral blood flow (CBF) we employed a FAIR MRI technique; from a series of EPI-images (a) we were able to acquire high-resolution CBF images of the mouse brain (b). CBF was measured in APPswe/PS1dE9 and wildtype mice on three different diets (Control, DEU and Fortasyn) in four different regions of interest (ROI): Entire brain, cortex, hippocampus and thalamus (c).

By comparing the different dietary groups, we found significant CBF differences in the transgenic mice; specifically, in APPswe/PS1dE9 mice on DEU diet, we measured an increased CBF in the cortex and in the thalamus compared to the APPswe/PS1dE9 mice on control diet (p=0.045 and p=0.021). Similarly, the APPswe/PS1dE9 mice on Fortasyn diet showed increased perfusion in the cortex (p=0.046) and a slight increase in the thalamus (p=0.056), when compared to
transgenic animals on control diet. No differences in CBF by the diets were found in the wildtype mice.

**Figure 2.** In the cerebral cortex, the two-tailed Pearson’s correlation test revealed a significant positive correlation between diffusion fractional anisotropy (FA) and cerebral blood flow (CBF).

**Diffusion tensor imaging**

*Region of interest based analysis*

Quantitative assessment of diffusion tensor derived indices was performed for ROIs drawn in white and gray matter regions to assess genotype and diet effects in transgenic and non-transgenic mice (Figure 3c, 4c and 5b). Because of severe artifacts in the diffusion-weighted images, two mice have to be excluded from this analysis. Independent of diet, the APP<sub>swe/PS1<sub>dE9</sub></sub> mice showed a significantly lower axial diffusivity (λ<sub>1</sub>) compared to wildtype in several white matter tracts, such as aca (p=0.05), scc (p=0.034), bcc (p=0.027) and fo (p=0.014). In the latter two regions, a decreased mean diffusivity (MD) was also present in transgenic mice, possibly linked to the reduced λ<sub>1</sub> (p=0.05 and p=0.036, respectively). A reduced λ<sub>1</sub> in APP<sub>swe/PS1<sub>dE9</sub></sub> compared to wildtype mice was also measured in the cortex (p=0.046). Overall, decreased mean fractional anisotropy (FA) values and increased radial diffusivity (RD) values were found in transgenic animals in most
of the ROIs analyzed, although they did not reach statistical significance. In the fimbria of the hippocampus, a significant genotype × diet interaction for all different diffusion parameters allowed to split these data and revealed beneficial effects of dietary intervention (not shown). In the APPsw/PS1deg mice on control diet we found a reduced FA ($p=0.049$) and increased RD ($p=0.022$) compared to wildtype; these differences were not seen in the DEU and Fortasyn groups. The APPsw/PS1deg on DEU diet showed a reduced RD ($p=0.023$) compared to the control diet and increased FA and $\lambda_1$ compared to the Fortasyn diet ($p=0.019$ and $p=0.035$). In the cerebral cortex, we found that the Fortasyn diet, independent of genotype, reduced the MD and RD ($p=0.043$ and $p=0.019$, respectively) compared with control diet.

The two-tailed Pearson’s test showed a positive correlation in the cortex between the FA and the CBF ($p=0.004$, Figure 2) and between the $\lambda_1$ and the CBF ($p=0.032$) in both wildtype and APPsw/PS1deg animals. A positive correlation was also found between FA and CBF in the hippocampus for wildtype mice ($p=0.028$), but not for APPsw/PS1deg ($p=0.956$). These results suggest that structural integrity and anisotropy, as reflected by $\lambda_1$ and FA, in gray matter are associated with a better perfusion.

**Voxel-based Analysis**

To determine the occurrence of diffusion changes at a higher spatial resolution and to reveal possible genotype and diet interactions not seen in the ROI analysis, we applied a voxel-based statistical approach (VBA) to the co-registered diffusion maps. As the VBA allowed only two-group comparisons, these analyses were conducted between the two genotypes separated by diet groups, and between the control diet group and the DEU and the Fortasyn diet groups. For the VBA, $p$-value maps ($p$-value thresholded at 0.05, minimum voxel cluster size set at 0.05 mm$^3$) were overlaid with an anatomical template. The VBA in Figure 3 and Figure 4 indicates areas in which diffusion parameters FA and MD differ significantly between the two genotypes. Voxels that differ significantly between the selected groups are lit up, with the color-coding indicating the magnitude of difference. In the APPsw/PS1deg mice on control diet, areas of the corpus callosum and fimbria (fi) showed significantly lower FA and higher MD compared to wildtype. Similar changes were detected in other areas, like the internal capsule (ic) and the anterior commissure, posterior (acp) (not shown).
Figure 3. Representative image of fractional anisotropy (FA) of a mouse brain in three orthogonal slices is shown (a). The voxel based analysis (VBA) revealed gray and white matter FA changes in APP\textsubscript{swe}/PS1\textsubscript{dE9} mice (b); in the transgenic animals on control diet we detected reduced FA in the corpus callosum, fimbria and cortex, as indicated by arrows. In mice on DEU diet, the reduced FA in the cortex of transgenic mice was still present, but no differences were seen in other brain regions. These genotype differences were absent in the mice on Fortasyn diet. The regions of interest (ROI) based approach revealed a reduction of diffusivity along the axons ($\lambda_1$) in the APP\textsubscript{swe}/PS1\textsubscript{dE9} mice in several white matter tracts and in the cortex (c). ROI include the anterior commissure anterior (aca), genu (gcc) – body (bcc) – splenium of the corpus callosum (scc), fimbria of the hippocampus (not shown), fornix (fo), cerebral cortex (ctx) and hippocampus (hc).
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Figure 4. Representative image of mean diffusivity (MD) of a mouse brain in three orthogonal slices is shown (a). The VBA revealed MD changes in APP_swe/PS1_{dE9} mice on control diet, particularly in the dentate gyrus of the hippocampus (b, arrows). These genotype differences were not present in the mice groups on DEU and Fortasyn diet. The region-of-interest based approach revealed reduced mean diffusivity (MD) in the APP_swe/PS1_{dE9} mice in the body of corpus callosum (bcc) and in the fornix (fo), likely due to the decreased $\lambda_1$ in this same regions (c).
Figure 5. VBA between the DEU and Fortasyn diet groups, compared to the control diet (a). Mice on DEU and Fortasyn diet showed increased FA and decreased MD in several white and gray matter regions. Particularly, a widespread reduced MD in the cortex and hippocampus is notable by the Fortasyn diet, suggesting an enhanced neuroprotective action of this diet. The region-of-interest based approach revealed a reduced mean diffusivity (MD) in the cortex in both wildtype and APP<sub>swd/PS1<sub>ΔE9</sub></sub> mice on Fortasyn diet, compared to the control diet; this effect is likely to follow the decreased radial diffusivity in the same region (not shown) (b).
Similar to previous findings in the same mouse strain, the VBA demonstrated the presence of significant genotype differences in gray-matter structures (Zerbi, et al., 2013); in the dentate gyrus the MD was highly increased in transgenic mice compared to the wildtype, while the FA did not change significantly. Furthermore, in the visual, auditory and somatosensory cortex, several clusters of decreased FA were also found in the $\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}$ mice on control diet. Genotype differences between mice on DEU diet were less frequent and evident. Among them, a decreased FA in the cortex was still present, similar to the findings in the control diet group. In the Fortasyn diet group, no notable genotype differences were detected, suggesting an amelioration of white and gray matter structural integrity in the transgenic mice.

Data were further analyzed comparing the different dietary groups, to visualize the overall effects of DEU and Fortasyn intervention (Figure 5). In the optic tract, fi and some areas of the ic we detected higher FA and lower MD in animals fed with the experimental diets, compared to the ones fed with the control diet. Interestingly, Fortasyn and DEU diets both increased FA and decreased MD in the hippocampal and in several other cortical regions, when compared to the control diet. All these effects are more evident in the Fortasyn diet (Figure 5-b) than in the DEU diet (Figure 5-a).

**Amyloid plaque load**

Among the regions of interest analyzed, the highest amount of plaque load in the transgenic animals was found in the DG of the hippocampus (12.1±0.6 % of $\text{A}\beta$ per area), in the GC (11.3±1.2 % of $\text{A}\beta$ per area) and PRL (9.6±1.1 % of $\text{A}\beta$ per area) compared to the CA1 (4.5±0.3 % of $\text{A}\beta$ per area) and CA3 regions (1.9±0.3 % of $\text{A}\beta$ per area) (for all comparisons, $p<0.001$). $\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}$ mice on DEU diet showed reduced amyloid plaque burden in hippocampal area CA1 when compared to the Fortasyn diet and slightly, but not significantly, compared to the control diet (3.35±0.48 % of $\text{A}\beta$ per area for DEU; 5.38±0.2 % of $\text{A}\beta$ per area for Fortasyn, $p=0.028$ and 4.98±0.54 % of $\text{A}\beta$ per area for control, $p=0.060$). However, these differences were not significant when we considered the whole hippocampal area (6.78±0.31 of $\text{A}\beta$ per area for control; 5.48±0.6 % for DEU; 6.59±0.66 % for Fortasyn, $p=0.203$). In the other analyzed regions of interest, no effects of the diets were detected. No significant correlations between $\text{A}\beta$ load and CBF in the hippocampus, and between $\text{A}\beta$ load and hippocampal diffusion parameters were found.
Brain fatty acids

The relative amounts of different fatty acids in the lipid fraction of the brain homogenates are displayed in Table 2. No significant differences between brain fatty acid profiles of APP<sub>swe</sub>/PS1<sub>de9</sub> transgenic and wildtype mice were noted. In mice on DEU and Fortasyn diets the relative amount of n3 fatty acids, including DHA, increased compared to the control diet (p<0.001), whereas the relative amount of n6, including arachidonic acid, decreased significantly (p<0.001). This resulted in a pronounced increase in the n3/n6 ratio in DEU and Fortasyn fed mice (p<0.001).

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<th>DHA</th>
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<th>MUFA</th>
<th>PUFA</th>
<th>Σn6</th>
<th>Σn3</th>
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<tr>
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<td>20.7±1.3</td>
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<tr>
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<td>19.3±0.3</td>
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Table 2. Relative fatty acid composition expressed in percentage of the lipid fraction of brain homogenates in wildtype and APP<sub>swe</sub>/PS1<sub>de9</sub> on three different diets. DHA = docosahexaenoic acid; AA = arachidonic acid; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; *overall diet effect: different from control. p<0.001. No genotype effects were seen.
Discussion
In this study we tested whether a novel combination of nutrients including DHA, EPA, phospholipids, UMP, choline, vitamins and antioxidants, which are factors for membrane synthesis and maintenance via the Kennedy cycle (Kennedy and Weiss 1956), prevents the occurrence of AD pathologies in an AD mouse model. We show that the APPswe/PS1dE9 mouse model, which is a model for β-amyloidosis, displays two of the pathological features that are often found in AD patients: a reduced cerebral blood flow, which reflect vascular disturbances, and water diffusion abnormalities in several brain regions, which reflect neuronal and axonal degeneration. In particular, we show that in these mice specific multi-nutrient diets are able to enhance cerebral perfusion and to protect against neurodegenerative processes in both gray- and white matter.

Perfusion
Epidemiological studies have shown that cardiovascular disorders and other vascular-related diseases that lead to chronically diminished cerebral blood flow (CBF) are associated with increased risk of AD (de la Torre, 2002),(de Toledo Ferraz Alves, et al., 2010). The CBF is therefore considered to be a good biomarker to investigate the development of AD. Measurement of CBF in small animals is challenging because of smaller volumes of interest and higher blood velocity compared to humans. Most of the techniques for measuring CBF in rodents are invasive (e.g. laser-Doppler after cranial windowing) or require the injection of a contrast agent (e.g. PET with intravenous $^{15}$O water, $^{14}$C-IAP autoradiography or dynamic susceptibility contrast perfusion MRI with gadolinium compounds). In contrast, MRI arterial spin labelling (ASL) techniques, whereby arterial blood is magnetically tagged before it enters the tissue of interest, are fully non-invasive, as they do not require the injection of contrast agents (Calamante, et al., 1999). The feasibility of the FAIR-ASL technique for the quantitative assessment of CBF has been proven in clinical and pre-clinical studies (Kim, 1995),(Oosterlinck, et al., 2011). Particularly in mouse studies, the use of ultra-high magnetic field, strong gradients and dedicated coils is required to compensate the relatively low signal-to-noise ratio (SNR) and to prolong the lifetime of the magnetic labelling (Zheng, et al., 2010). In the present study we fulfilled these requirements and we were able to acquire high-resolution CBF maps of a mouse brain: the CBF values of control wildtype animals resemble those previously published for mice with the
same anaesthetic setting (Leithner, et al., 2008, Weidensteiner, et al., 2009, Zheng, et al., 2010). We demonstrated that 12 months old \(\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}\) mice on control diet suffer from a decreased brain perfusion compared to their wild type littermates in all the measured ROIs. The reduced CBF found in these mice is of particular interest, as it mirrors the brain hypoperfusion status found in the majority of AD patients (Alsop, et al., 2000, Ruitenberg, et al., 2005, Schreiber, et al., 2005). A recent study in AD patients confirmed that CBF imaging with MRI is a valuable tool to detect functional changes in the prodromal and more advanced stages of AD and can be used as a marker for disease severity (Binnewijzend, et al., 2013). The absence of a correlation between CBF deficits and Aβ plaque burden suggests that Aβ plaque deposition is not the primary cause of this vascular impairment. However, still other Aβ species, particularly soluble Aβ1-40, may be involved in damaging the vessel walls. This can result both in a reduced vasodilation activity, caused by a brain endothelial dysfunction with diminished production of NO by endothelial cells (E. Farkas and P.G. Luiten, 2001, Niwa, et al., 2000, Price, et al., 2001), and in a reduced vessel lumen, caused by Aβ accumulation in the blood vessel walls (Shin, et al., 2007).

In addition to the decreased CBF detected in the \(\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}\) mice on control diet, our data revealed that the multi-nutrient diets are able to enhance the brain perfusion in the transgenic mice. In the \(\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}\) mice on DEU and on Fortasyn diet we measured an increased blood supply in the cortex and in the thalamus, compared to transgenic mice on control diet. It is however important to mention that, in the hippocampus, the CBF was instead significantly impaired in the \(\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}\) mice, independent of diet; this brain region is linked to memory and cognitive processes and is among others one of the earliest areas affected by AD pathology and amyloid plaque deposition in \(\text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}}\) mice (Garcia-Alloza, et al., 2006), as well as in patients (Braak and Braak, 1995). It is therefore plausible to conclude that these dietary supplementations have the potential to significantly slow the occurrence of brain perfusion failure caused by AD pathology development, particularly in an early stage of the pathology, before brain damages become too big to be averted.

We believe this effect is determined by two mechanisms. First, the n-3 components in the diets might have a beneficial effect on systemic circulation. Several clinical trials demonstrated the cardiovascular protective effect of n-3 LCPUFA diets, which are nowadays recommended for patients with a history of cardiovascular disease (Lee, et al., 2008). Omega-3 fatty acids reduce the risk of
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atherosclerosis through interacting with platelet aggregation and might also effectively prevent hypertension, with beneficial effect on perfusion (de Wilde, et al., 2002,E. Farkas and P.G.M. Luiten, 2001). Multi-nutrient diets containing n-3 LCPUFA also ameliorate microvasculature endothelium-dependent vasodilation processes and protect from cerebral microcirculatory abnormalities (Morgan, et al., 2006), often seen in AD patients (de la Torre, 2004) and in transgenic AD mouse models (Meyer, et al., 2008, Zerbi, et al., 2012). Second, the increased CBF might indirectly reflect an improved neuronal health in APP_{swe}/PS1_{dE9} mice on the DEU and Fortasyyn diets. The functional coupling between regional CBF and local glucose metabolism has been largely demonstrated in animal and human studies; glucose utilization in turn mainly reflects neuronal pre-synaptic activity (Jueptner and Weiller, 1995, Kida and Hyder, 2006). Our CBF data therefore might indicate that the DEU and the Fortasyn diets maintained cortical neuronal and synaptic health in the APP_{swe}/PS1_{dE9} mice on a physiological level, possibly by slowing down the neurodegenerative processes that occur, instead, in the transgenic mice on control diet (Zerbi, et al., 2013). Although not directly measured in this study, diets providing specific phospholipid precursors have previously shown improvements in the generation of new synaptic connections, such as dendritic spine growth, neurite branching, neurite protein synthesis and neuritogenesis (Knott, et al., 2006, Pooler, et al., 2005, Sakamoto, et al., 2007). The positive correlation found in cortex between CBF and diffusion markers of axonal integrity (e.g. FA and \lambda_1) further suggests a dual effect of these diets on both neuronal and vascular health. Although the correlation of these two measures do not imply a cause-effect mechanism, it reinforces the idea that neuronal and vascular health are tidily interdependent and are both affected by the genotype and by the dietary intervention. This relationship seems lost in the hippocampus of the APP_{swe}/PS1_{dE9}, possible due to the advanced stage of AD pathology in this area.

Diffusion

Diffusion tensor MRI (DT-MRI) measures the incoherent motion of water molecules in every voxel from multiple directions and reconstructs the geometry of their diffusion into an ellipsoid (Basser, et al., 1994, Beaulieu, 2002). This technique is widely used to study neurological disorders, like AD, because water molecule diffusion patterns can reveal details about tissue micro-architecture, either normal or in a diseased state, which are not measurable by other imaging techniques (Kantarci 2011). In AD patients the changes in DT-MRI parameters are
consistent, showing decreased fractional anisotropy (FA) in white matter regions and increased mean diffusivity (MD) in gray matter regions (Hanyu, et al., 1998, Kantarci, 2011, S.-K. Song, et al., 2004b). Interestingly, similar alterations have been reported in mouse models for AD (Song, et al., 2005, Sun, et al., 2008). These changes have been correlated with loss of myelin, decreased axonal density and connectivity in white matter (S.K. Song, et al., 2004, Chen, et al., 2011) and neuronal loss in gray matter (Sykova, et al., 2005). The results obtained here from the APP_swe/PS1<sub>de9</sub> mice on control diet are in good agreement with these previous findings. In white matter areas of anterior commissure (aca), fornix (fo) and splenium of corpus callosum (scc) we found a reduced axial diffusivity (λ<sub>1</sub>) in the transgenic mice compared to wildtype. The fimbria (fi) of APP_swe/PS1<sub>de9</sub> mice on control diet showed decreased FA and increased RD. Decreases in FA and λ<sub>1</sub> and increases in RD are typically associated with axonal injury and myelin degradation, respectively. Such white matter changes would effectively constrain the diffusion of water molecules in the direction of the fiber bundle, while increasing the diffusivity perpendicular to it (S.-K. Song, et al., 2004a).

In the APP_swe/PS1<sub>de9</sub> mice we also measured a decrease in λ<sub>1</sub> in the cortex, which is possibly linked to the reduced FA visible in the VBA comparison. Furthermore, the VBA highlighted an increased MD in the hippocampus, specifically in the dentate gyrus. We previously showed that the APP_swe/PS1<sub>de9</sub> mice display several differences in gray matter diffusion compared to wildtype; these include a significant increase in water diffusivity in the molecular layer of the dentate gyrus, which was associated with neuronal loss by visualizing myelin and neurons with Klüver-Barrera staining (Zerbi, et al., 2013). Few other studies have been published on gray matter diffusion using DT-MRI in AD mice. However, there have been a number of human studies that reported increased gray matter MD in AD patients, which has been ascribed to neuronal degeneration and cell loss (Nakata, et al., 2009). Such changes in tissue structure would lead to less hindrance for water to diffuse. There are indications about the increased MD in gray matter being a better indicator of disease progression in mild cognitive impaired and healthy subjects than hippocampal volumetry (Fellgiebel, et al., 2004)(Kantarci, et al., 2005).

The analysis of DT-MRI parameters further revealed that dietary intervention interferes with the structural damage occurring in both white and gray matter in the transgenic mice. In the fi, the changes in RD and FA measured in the
Multinutrient diets improve cerebral perfusion and neuroprotection

APP<sub>swe/PS1<sub>de9</sub> mice on control diet were reverted by both experimental diets. Furthermore, compared to the control-fed mice, Fortasyn-fed mice displayed a significant reduction of MD and RD in the cortex. The effects of dietary intervention in diffusion parameters became even more striking when we applied the VBA on the separate diet groups. In the mice on DEU diet we found fewer differences between genotypes, although differences in the FA of the cortex were still present. In mice on Fortasyn diet, almost all the differences between transgenic and non-transgenic animals faded, indicating that Fortasyn reduced the impact of the transgenicity. By analyzing the diet effects on both genotypes, we confirmed that both the DEU and Fortasyn diets similarly increased the FA in the cortex and hippocampus and in other white matter regions, such as the cp and fi; the Fortasyn diet also highly reduced the MD in the cortex. Together, these data indicate a beneficial effect of the diets, and particularly Fortasyn, in preventing axonal and neuronal damage in several brain regions, which are otherwise severely compromised in transgenic AD mice. In line with these results, another study reported a reduced neocortical neurodegeneration in 6-month-old wildtype and female APP<sub>swe/PS1<sub>de9</sub> mice fed with Fortasyn, observed by the Amino Cupric Silver staining (Broersen, et al., 2013). In AD patients, electroencephalography (EEG) measures showed that Souvenaid® (Nutricia N.V., Zoetermeer, The Netherlands), a medical food containing Fortasyn, have an effect on brain functional connectivity, supporting the underlying hypothesis of an amelioration of neuronal health and synaptic activity (Scheltens, et al., 2012). In the light of our results, future studies on brain diffusion-related parameters are suggested to confirm the efficacy of Fortasyn supplementation in a clinical setting.

One explanation for the results found may be linked to the n-3 components in the experimental diets, and particularly DHA. Many studies revealed that DHA is neuroprotective via multiple mechanisms: by reducing arachidonic acid and its metabolites (Oksman, et al., 2006); by increasing neuroprotectin D1, which down-regulates pro-apoptotic mediators (Bazan, 2005); by increasing the production of brain-derived neurotrophic factor (Rao, et al., 2007) and by promoting antioxidant defence (Akbar, et al., 2005). N-3 fatty acids also replace n-6 fatty acids and cholesterol from cell membranes increasing fluidity of the membrane, enhancing receptor binding and affinity, and improving the function of ion channels (Bourre, et al., 1991, Bourre, et al., 1989, Farkas, et al., 2002). It has also been shown that DHA supplementation was able to alleviate age-related
neurogenesis decreases in the hippocampal areas CA1 and dentate gyrus (Dyall, et al., 2010), promoting hippocampal neuronal development and synaptic functioning (Cao, et al., 2009). The different outcome of the two diets, particularly in cortical diffusion, suggests a better maintenance of cortical neuronal health by the Fortasyn diet, possibly determined by the inclusion in this diet of additional nutritional components that may synergistically improve neuronal protective mechanisms. In this experiment, the DEU and the Fortasyn diets were equally effective in raising brain n3 fatty acids levels; however, the addition of antioxidants like vitamins B, E and C is suggested to improve the neuroprotective effects of DHA(Cole, et al., 2009). Previous studies also showed that vitamins and antioxidants are necessary to support the biochemical processes involved in the Kennedy cycle for phospholipid synthesis (Kennedy and Weiss, 1956,Wurtman, et al., 2009) by increasing the availability of the membrane precursors (van Wijk, et al., 2012a,van Wijk, et al., 2012b).

Taken together, these data seem to confirm our initial hypothesis of a higher efficacy, in term of neuronal protection, of a multi-nutrient approach. In line with this suggestion, is has been recently shown that Fortasyn protected the central cholinergic system against Aβ1-42 toxicity in rats infused with Aβ1-42 (De Wilde, Penke, van der beek 2011). This specific nutrient combination also showed improved searching behavior and swim efficiency in AβPP/PS1 mice during the morris water maze cognitive test (Wiesmann et al., 2013).

**Amyloid-β pathology**

Contrarily to our expectations, only the DEU diet reduced the amyloid plaque burden in the CA1 hippocampal region, and this was only significant compared to the Fortasyn diet. In addition, when considering the whole hippocampus, this effect vanished, showing no significant differences between control, DEU and Fortasyn diets. Previously, various studies have examined the effects of different combination of nutrients and dietary supplementation on brain amyloid in many transgenic models for AD, with mixed outcome. In a similar mouse model for AD, reduced hippocampal amyloid burden due to the supplementation of DHA have been reported, albeit not consistently (Arendash, et al., 2007,Green, et al., 2007,Perez, et al., 2010,van de Rest, et al., 2009). In a more recent study, 6-month-old female APPswed/PS1de9 mice showed significantly lower levels of soluble Aβ1-40, Aβ1-42 and plaque load when fed with Fortasyn compared to a control diet and compared to a DHA and UMP containing diet (Broersen, et al., 2013). The
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different outcomes between this and our study may derive from the gender difference and the age at which the immunohistological examination is performed. In our experiment, male 12-month-old mice were used; at this age, severe plaque deposition is already present in the hippocampus, covering approximately 6-7% of the total hippocampal area compared to the 0.4-0.5% measured by Broersen and colleagues (Broersen, et al., 2013). This difference seems to indicate that (1) the effects of DHA on Aβ accumulation/production mechanisms depend on its dietary context and (2) a multi-nutrient dietary intervention could effectively interact against Aβ production and aggregation when the pathology is mild and not yet fully developed. A similar hypothesis has been suggested from the Souvenir I clinical trial, which reported the greatest effect of Souvenaid in the population of patients with very mild AD (mean baseline minimal mental state examination (MMSE) = 25.61 (Scheltens, et al., 2010). These observations, together with epidemiological data of dietary patterns on risk reduction of AD (Mi, et al., 2013), suggest a greater opportunity of intervention in the very early stage of AD. Nevertheless, in this study the amount of plaques did not correlate with the severity of the diffusion changes, nor with the perfusion changes. Taken together, these data suggest that nutritional intervention can be effective in reducing AD-related pathology, such as enhancing perfusion and neuroprotection, and cognitive function (Wiesmann, et al., 2013), without necessarily affecting Aβ plaque levels.

The data presented here is encouraging for planning future clinical studies; to confirm the impact on AD development of these specific Fortasyn-based multi-nutrient diets, measures of brain perfusion and diffusion, next to standard cognitive tests, are strongly recommended. Indeed other variables would require special considerations when designing clinical trials with n-3 LCPUFA-including supplements; in particular, it would be interesting to study the effects of the APOE genotype in relation with dietary intervention. The apolipoprotein E (apoE) exerts a key role in the transport of cholesterol and other lipids involved in brain composition and functioning (Frieden and Garai, 2012). It has been recently suggested that the action of lipid-based diets on AD-risk factors, such as atherosclerosis (Minihane, et al., 2000), on the risk of developing dementia (Barberger-Gateau, et al., 2011) and the incorporation of n3-LCPUFA from the diet might differ according to apoE polymorphism (Samieri, et al., 2013).
In conclusion, we showed that specific multi-nutrient diets are able to enhance brain perfusion and protect against neuronal structural degeneration in our mouse model for AD. These results were independent from the amount of Aβ plaque deposits and were more evident particularly on structural integrity measures in animals on Fortasyn diet. This is in line with the background hypothesis of this study: the supplementation of all the nutrients necessary to improve cerebral perfusion and promote neuronal membranes and synapse formation, rather than single/few components, seems to be a better strategy to protect against progression of AD.
Multi-nutrient diets improve cerebral perfusion and neuroprotection
Summary

Nederlandse samenvatting
Summary
Alzheimer’s disease (AD) is the leading cause of dementia in human. More than 35 million people suffered from AD in 2010 worldwide, and this number is expected to increase in the coming years. It is therefore of the highest priority to find a cure against this disease, or a way to prevent it, before it will become an unsustainable social and economic burden. A recent study from the US Alzheimer’s Association predicted that by delaying the onset of AD by just 5 years, the costs for the society related to the disease would decrease approximately of 50% (http://www.alz.org/documents_custom/trajectory.pdf).

This ambitious plan however requires more research (1) on the mechanisms via which the pathology origins and progresses, (2) on the development of novel biomarkers that can reveal the occurrence of AD in its early stage, and (3) on novel therapeutic approaches that could significantly interrupt or delay the pathology progress in the majority of AD patients. With the work presented in this thesis, we experimentally addressed specific questions related to these three research lines.

In the first experimental work, described in chapter 2, we aimed to comprehensively characterize the occurrence of vascular impairment in a double transgenic mouse model for AD (APP\textsubscript{swe}/PS1\textsubscript{dE9}) during aging. As vascular disorders can either be cause or consequence in the pathophysiology of AD, it was relevant for us to understand how and when these deficits occur in relation with amyloid-β (Aβ) accumulation. With this aim, we developed a novel method to obtain microvascular relative cerebral blood volume (rCBV\textsubscript{micro}) maps from gradient echo MR imaging by histogram evaluation and we applied a voxel-wise approach to detect rCBV\textsubscript{micro} changes at the highest possible spatial resolution (0.16 millimeter isotropic). At 8 months of age, a decreased rCBV\textsubscript{micro} appeared in some cortical regions and in the thalamus, which spread over several sub-cortical areas and the hippocampus at 13 months of age. Additionally, we showed that hippocampal rCBV\textsubscript{micro} in 13-month-old wild type and APP\textsubscript{swe}/PS1\textsubscript{dE9} mice correlated strongly with capillary density measured with immunohistochemical staining. However, no difference in capillary density could be detected between the genotypes, indicating no loss of vessels. The rCBV\textsubscript{micro} values showed no significant correlation with Aβ plaque deposition, nor with presence of Aβ in bloodvessel walls, nor with biochemically measured levels of Aβ\textsubscript{1-40}, Aβ\textsubscript{1-42}.
oligomers and fibrillar forms. These results suggested that rCBV\textsubscript{micro} reduction is possibly caused by an impaired vasoactivity of capillaries and arterioles, which is not directly correlated with the amount of A\textbeta deposition in parenchyma or bloodvessel walls.

In the second experiment (chapter 3), we investigated gray and white matter water diffusivity changes in the same \textit{APP}\textsubscript{swe}/PS1\textsubscript{dE9} mice with diffusion-tensor MRI (DT-MRI) at ultra-high field. Changes in DT-MRI parameters are thought to reflect structural alterations in neural tissue, and may represent a novel way to follow noninvasively the severity of neurodegenerative processes. In this experiment, we found substantial changes in water diffusion parallel and perpendicular to axonal tracts in several white matter regions like corpus callosum and fimbria of the hippocampus in the brains of \textit{APP}\textsubscript{swe}/PS1\textsubscript{dE9} mice. These results matched with previous findings of axonal disconnection and myelin degradation in AD patients. Moreover, we found a significant increase in diffusivity in specific hippocampal sub-regions, which is supported by neuronal loss as visualised with the Klüver-Barrera staining. This work demonstrated the potential of ultra-high field DT-MRI as a non-invasive modality to describe white and gray matter structural changes in mouse models for neurodegenerative disorders.

In the third experiment (chapter 4) we investigated the association between the cholesterol-transporter apolipoprotein ε (apoE) genotype and the risk of developing neurodegenerative diseases. Recently, brain functional connectivity (FC) in apoE-ε4 carriers has been investigated by means of resting-state fMRI, showing a marked differentiation in several functional networks at different ages compared to carriers of other apoE isoforms. The causes of such hampered FC are yet not understood; it is suggested that vascular function and synaptic repair processes, which are both impaired in carriers of apoE-ε4, can trigger loss of FC during aging. To test this hypothesis, we integrated several different MRI techniques and immunohistochemical staining in a translational study using two models for atherosclerosis and vascular risk factors in AD: the apoE-ε4 and the apoE-ko mice. Compared to wildtype mice, we detected profound FC reduction in adult and elderly apoE-ko mice, concomitant with strongly reduced brain perfusion. In apoE-ε4 mice perfusion deficits appear only later in life, and not significant changes of FC were seen. In both mouse models, water diffusion
changes commonly associated to axonal disconnection and disorganization are found in hippocampal areas. Reduced post synaptic density levels are also measured in the hippocampus in both models, at different ages. In conclusion, we provide new evidence of a relation between apoE and brain connectivity, possibly mediated by vascular risk factors and by the efficiency of apoE as synaptic modulator in the brain. Our results showed that FC assessment by resting-state fMRI is an excellent tool to investigate neuropathology and aging effects in translational research.

All these three studies mentioned here provided valuable knowledge and the necessary tools to investigate novel AD prevention strategies in translational research. In the second part of this thesis, we applied these methodologies to evaluate the effects of specific nutrient diets as a non-pharmacological way to prevent genotype-induced AD traits. In chapter 5, we tested the effects of a multi-nutrient diet in apoE-ε4 and the apoE-ko mice as models for AD vascular risk factors. This specific multi-nutrient diet was developed to support neuronal membrane synthesis (and concomitant synapse synthesis) and was expected to contribute to the maintenance of vascular health. At 12 months of age, both genotypes showed behavioral changes compared to control mice and we found increased neurogenesis in apoE-ko mice. The specific multi-nutrient diet decreased anxiety-related behavior in the open field, influenced sterol composition in serum and brain tissue, and increased the concentration of omega-3 fatty acids in the brain. Furthermore, we found that wild-type and apoE-ko mice fed this multi-nutrient diet showed locally increased cerebral blood volume and decreased hippocampal glutamate levels. Taken together, these data suggested that a specific dietary intervention has beneficial effects on early pathological consequences of hypercholesterolemia, atherosclerosis and other vascular risk factors for AD.

To investigate the effects of nutritional intervention as a strategy to retard the development of AD-like features, we tested the effects of two multinutrient diets in 12-month-old APPswe/PS1dE9 (chapter 6). We measured cerebral perfusion and water diffusivity with MRI at ultra-high field, as alterations in these parameters correlate with clinical symptoms of the disease. APPswe/PS1dE9 mice on control diet showed decreased cerebral blood flow and changes in water diffusion in several brain regions, in accordance to findings of hypoperfusion, axonal
disconnection and neuronal loss in AD patients. The multi-nutrient diets were able to restore the cerebral blood flow of the \( \text{APP}_{\text{swe}}/\text{PS1}_{\text{de9}} \) mice to wildtype levels. Animals on the multi-nutrient diets also showed improved structural integrity, particularly in the dentate gyrus and in cortical regions. We concluded that a specific diet intervention has the potential to slow AD progression, by simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms.

Overall, the work presented in this thesis proved the utility of advanced MR neuroimaging tools for detecting changes in brain structure and function with respect to dietary intervention.
Nederlandse samenvatting

De ziekte van Alzheimer (AD) is de meest voorkomende oorzaak van dementie bij de mens. In 2010 leden er meer dan 35 miljoen mensen wereldwijd aan deze ziekte, en naar verwachting zal dit aantal toenemen in de komende jaren. Het heeft dan ook de hoogste prioriteit om een remedie te vinden tegen AD, of een manier om het te voorkomen, voordat het een onhoudbare sociale en economische last zal worden. Een recent onderzoek van de US Alzheimer’s Association heeft voorspeld dat de kosten voor de samenleving met ongeveer 50% zou afnemen als de ontwikkeling van AD met slechts 5 jaar kan worden uitgesteld (http://www.alz.org/documents_custom/trajectory.pdf).

Dit ambitieuze plan vergt echter meer onderzoek (1) naar de mechanismen die bijdragen aan het ontstaan en het verloop van de pathologie, (2) naar de ontwikkeling van nieuwe biomarkers die de ziekte van AD al een in vroeg stadium kunnen aantonen, en (3) naar nieuwe therapeutische interventies die het verloop van de ziekte aanzienlijk zouden kunnen verstoren of vertragen in de meerderheid van de Alzheimer patiënten. In de experimenten beschreven in dit proefschrift, hebben we specifieke vragen gesteld met betrekking tot deze drie onderzoekslijnen.

De eerste studie, beschreven in hoofdstuk 2, heeft als doel om de vasculaire stoornissen tijdens veroudering in kaart te brengen in het dubbele transgene muismodel voor AD (APPswe/PS1dE9). Aangezien vasculaire aandoeningen ofwel een oorzaak ofwel een gevolg kunnen zijn in de pathofysiologie van AD is het relevant om te begrijpen hoe en wanneer deze stoornissen zich voordoen in relatie tot de ophoping van amyloid-β (Aβ). Om deze vraag te beantwoorden hebben we een nieuwe methode ontwikkeld om microvasculaire relatieve cerebrale bloedvolume (rCBV micro) kaarten te verkrijgen van gradiënt echo MR imaging door middel van histogram analyse. Om veranderingen in de rCBV micro met de hoogst mogelijke ruimtelijke resolutie (0.16 millimeter isotroop) te kunnen detecteren, hebben we gebruik gemaakt van een voxel-wijze benadering. Op de leeftijd van 8 maanden was er een verminderde rCBV micro in bepaalde corticale gebieden in het APPswe/PS1dE9 muismodel. Op de leeftijd van 13 maanden was de verminderde rCBV micro verspreid over meerdere subcorticale gebieden en de hippocampus. Daarnaast hebben we aangetoond dat de rCBV micro in de hippocampus van 13 maanden oude wild type en APPswe/PS1dE9 muizen
sterk correleerde met de capillaire dichtheid, zoals bepaald met een immunohistochemische kleuring. Er was echter geen verschil te vinden in de capillaire dichtheid tussen de genotypen, wat suggereert dat er geen verlies van bloedvaten is opgetreden. De rCBV\textsubscript{micro} waarden correleerden niet met de dichtheid van Aβ plaques, noch met de aanwezigheid van Aβ in de wanden van de bloedvaten, of met de biochemisch gemeten niveaus van Aβ\textsubscript{1-40} en Aβ\textsubscript{1-42} oligomeren en fibrillaire vormen. Deze resultaten suggereren dat een afname in de rCBV\textsubscript{micro} mogelijk wordt veroorzaakt door een verminderde vasoactiviteit van haarvaten en arteriolen, dat niet direct gecorreleerd is met de hoeveelheid Aβ ophoping in het brein of in de bloedvatwand.

In het tweede experiment (hoofdstuk 3) onderzochten we veranderingen in de water diffusiviteit van de grijze en witte stof in dezelfde APP\textsubscript{swe}/PS1\textsubscript{de9} muizen met diffusie-tensor MRI (DT-MRI). Het wordt gedacht dat veranderingen in DT-MRI parameters structurele afwijkingen in het hersenweefsel weerspiegelen. DT-MRI zou daarom een nieuwe, niet-invasieve, manier kunnen bieden om de ernst van neurodegeneratieve processen te volgen. In dit experiment vonden we substantiële veranderingen in de water diffusiviteit, evenwijdig en loodrecht op de axonbanen, in verschillende witte stof gebieden zoals het corpus callosum en fimbria van de hippocampus in APP\textsubscript{swe}/PS1\textsubscript{de9} muizen. Deze resultaten komen overeen met eerdere bevindingen van axonale disconnectie en myeline afbraak in Alzheimer patiënten. Daarnaast vonden we een significante toename van water diffusiviteit in specifieke subgebieden van de hippocampus, wat duidt op neuronaal verlies zoals ook gevisualiseerd is met de Klüver-Barrera kleuring. Dit werk laat de mogelijkheden zien van DT-MRI bij ultrahoge veldsterkte, om op een niet-invasieve manier structurele veranderingen in witte en grijze stof te bepalen in muismodellen voor neurodegeneratieve ziekten.

In het derde experiment (hoofdstuk 4) onderzochten we de relatie tussen het cholesterol-transporteiwit apolipoproteïne ε (apoE) genotype en het risico op het ontwikkelen van neurodegeneratieve aandoeningen. Onlangs is de functionele connectiviteit (FC) in het brein van apoE-ε4 dragers onderzocht door middel van resting-state fMRI. Hieruit bleek een duidelijke differentiatie in verschillende functionele netwerken op verschillende leeftijden in vergelijking met dragers van de andere apoE isovormen. De oorzaken van deze belemmerde FC zijn nog niet bekend; het wordt gesuggereerd dat vasculaire functie en synaptische
herstelprocessen, die beiden verstoord zijn in apoE-ε4 dragers, mogelijk het verlies van FC tijdens veroudering kunnen veroorzaken. Om deze hypothese te testen hebben we verschillende MRI technieken en een immunohistochemische kleuring geïntegreerd in een translationele studie met behulp van twee muismodellen voor arteriosclerose en vasculaire risicofactoren in de ziekte van Alzheimer: de apoE-ε4 en de apoE-ko muizen. In vergelijking met wildtype muizen was er een uitgesproken vermindere FC in volwassen en oudere apoE-ko muizen, alsmede een sterk verminderde hersendoorbloeding. In apoE-ε4 muizen verschenen stoornissen in de doorbloeding pas op latere leeftijd, en waren er niet-significante veranderingen in FC waarneembaar. In beide muismodellen werden er veranderingen in water diffusiviteit, die vaak geassocieerd worden met axonale disconnectie en desorganisatie, gevonden in subgebieden van de hippocampus. Beide muismodellen hadden ook een verminderde postsynaptische dichtheid in de hippocampus, op verschillende leeftijden. Concluderend, wij lieten nieuwe bewijzen zien van een relatie tussen apoE en breinconnectiviteit, wat mogelijk gemedieerd wordt door vasculaire risicofactoren en de efficiëntie van apoE als synaptische modulator in het brein. Onze resultaten toonden aan dat FC bepaling met behulp van resting-state fMRI een uitstekend hulpmiddel is om het effect van neuropathologie en veroudering te onderzoeken in translationele studies.

Alle drie experimenten die hierboven zijn genoemd verstreken waardevolle kennis en de benodigde tools om nieuwe preventiestratiegeën tegen AD te kunnen onderzoeken in translationele studies. In het tweede deel van dit proefschrift hebben we deze methodieken toegepast om de effecten van specifieke diëten te evalueren, om op een niet-farmacologische manier de genotype-geïnduceerde kenmerken van AD te voorkomen. In hoofdstuk 5 hebben we de effecten van een multi-nutriënt dieet getest in de apoE-ε4 en apoE-ko muizen, als modellen voor vasculaire risicofactoren in AD. Het specifieke multi-nutriënt dieet was ontwikkeld om de neuronale membraan aanmaak te ondersteunen (alsmede de aanmaak van synapsen), en ook werd verwacht dat dit dieet bij zou kunnen dragen aan het in stand houden van de vasculaire gezondheid. Op de leeftijd van 12 maanden vertoonden beide genotypen gedragsveranderingen in vergelijking met controle muizen, en we vonden verhoogde neurogenese in de apoE-ko muizen. Het specifieke multi-nutriënt dieet verminderde angst-gerelateerd gedrag in het open veld, beïnvloedde de
samenstelling van sterolen in het serum en in het brein, en verhoogde de concentratie van omega-3 vetzuren in de hersenen. Verder toonden we aan dat het specifieke multi-nutriënt dieet zorgde voor een lokaal verhoogde cerebrale bloedvolume en verminderde hippocampale glutamaat niveaus in wild-type en apoE-ko muizen. Samengevat, deze data suggereren dat deze specifieke dieetinterventie gunstige effecten heeft op de vroege pathologische gevolgen van hypercholesterolemie, atherosclerose en andere vasculaire risicofactoren voor AD.

Om de effecten van voedingsinterventie te onderzoeken als een strategie om de ontwikkeling van AD-achtige kenmerken te vertragen, hebben we twee multi-nutriënt diëten getest in 12 maanden oude APPswe/PS1De9 muizen (hoofdstuk 6). We hebben de cerebrale perfusie en de water diffusiviteit gemeten met behulp van MRI bij ultrahoge veldsterkte, aangezien veranderingen in deze parameters correleren met klinische symptomen van de ziekte. In overeenstemming met de bevindingen van hypoperfusie, axonale disconnectie en neuronal verlies in Alzheimer patiënten, lieten APPswe/PS1De9 muizen op een controle dieet een verminderde cerebrale doorbloeding zien, almede veranderingen in de water diffusiviteit in verschillende hersengebieden. De multi-nutriënt diëten waren in staat om de hersendoorbloeding van APPswe/PS1De9 muizen te herstellen tot het niveau van wildtype muizen. Dieren op de multi-nutriënt diëten lieten ook een verbeterde structurele integriteit zien, met name in de gyrus dentatus en in corticale gebieden. We concludeerden dat een specifieke dieetinterventie de mogelijkheid heeft om het verloop van de ziekte van Alzheimer te vertragen, door tegelijkertijd de cerebrovasculaire gezondheid te verbeteren en neuroprotectieve mechanismen te stimuleren.

In conclusie, het werk beschreven in dit proefschrift toont het nut aan van geavanceerde MR neuroimaging technieken voor het detecteren van veranderingen in hersenstructuur en breinfunctie met betrekking tot dieetinterventie.
General discussion
and concluding remarks
The role of preclinical research in AD. Challenges and limitations

Identification of novel imaging biomarkers of Alzheimer’s disease (AD) is crucial for an early and accurate diagnosis of the disorder. The need for developing imaging techniques is pressing, and it is easy to foresee that in the near future neuroimaging methods would allow monitoring disease progression and distinguishing purely symptomatic treatments from disease-modifying therapies. However, while some AD markers are based on direct imaging of pathological hallmarks, such as Aβ plaque imaging with PET, many others are surrogate biomarkers that identify changes in brain structure and function. For a definite diagnosis and to expand the knowledge on AD-related changes, it is therefore of the highest importance: (1) to fully understand the biological basis of the changes reflected by surrogate biomarkers; (2) to determine the time-course in which these pathological events occur and study their interrelationship.

Preclinical research, mainly based on the use of transgenic mouse models of AD, is an emerging field that may have the potential to answer all these questions. The use of animal models, specifically developed to homogenously reproduce AD brain lesions, allows studying the relationship between a biomarker and its biological value, circumventing biases and limitations of human studies. Detecting and quantifying the AD pathological processes in the rodent brain might be viewed as a challenge, because the mouse brain is approximately 3000-fold smaller than the human brain. The use of high-field MRI has nevertheless allowed to bypass these technical limits and increases the possibilities to discover and validate biomarkers of the disease in preclinical research.

There are, however, other obstacles to overcome in achieving this goal, both technical and scientific; for example, while most of the human MRI systems operate at 1.5 or 3 Tesla, in small animal MR scanners, the field strength can vary from 4.7 to 11.7 Tesla or higher. Although the higher field strength clearly results in a higher signal-to-noise ratio, which enables higher resolution acquisition, a large variety of artefacts and limitations related to the specific absorption rate represent significant challenges in these experiments (Bernstein, et al., 2006). Among them, chemical shift- and susceptibility-related artefacts are especially common at high field strengths and need to be considered. The broad field strength range in the animal systems also involve different relaxation times T₁, T₂ and $T_2^*$; this makes the comparison of the results obtained in different laboratories more difficult, and limits the reproducibility and the translational
aspect of the research. Furthermore, in animal research different types of anaesthesia are used, which must be properly chosen and calibrated to the specific requirements posed by the animal and the experiment, avoiding interference with biological processes (Lukasik and Gillies, 2003); this is especially true for cerebral blood flow/blood volume and fMRI studies (Kannurpatti and Biswal, 2004). Finally, due to the relatively novelty of high-field small animal imaging, translational research still lack a standardized platform to process mouse neuroimaging data, and most of the methods described in the literature are developed in-house and often difficult to replicate.

Besides these technical limitations, during the development of the work presented in this thesis, we encountered also several biomedical challenges. The strongest limitation was the difficulty in comparing our results with other studies in different mouse strains. Currently, many mouse models for familial AD, mainly expressing familial mutations of the human amyloid precursor protein (APP) and presenilins (PS1 or/and PS2), have been proposed (Braidy, et al., 2012); each of these models exhibit some of the key aspects of the disease, such as amyloid plaques deposition, deficits in cognitive tasks, and abnormal cerebral metabolism (Higgins and Jacobsen, 2003, Hsiao, et al., 1996). However, in most of the cases these models vary between each other in severity and type of pathology that they express, and also differ in the age in which they develop traits of AD. In addition, the information gained from models of familial genetic forms of AD may not translate to sporadic (or late onset) forms of the disease, the latter of which comprises more than 90% of the cases (Hoyer, 2000, Mattson, 2004). In fact, the only way to study sporadic AD is to use animal models that mimic one or more of the risk factors. All these limitations must be taken into account when designing an experiment, when drawing conclusions about the clinical relevance of the results obtained, and when comparing the findings with other models.

To overcome these limitations, some possible routes should be followed: one is to convince the scientific community to standardize as much as possible the experimental conditions and the use of animal models in their studies. Ideally, this would require imposing the same housing/living condition of the animals and limiting the number of mouse models used in AD research to one or two strains. This last option however is at the moment a too strong limiting factor, since none of the existing transgenic models of AD fully recapitulate all of the pathological features and behavioural changes found in human patients (see however (Braidy, et al., 2012)). Alternatively, it is desirable to exert longitudinal studies with a
multi-modality approach, including different MR imaging techniques, to characterize the development of the pathology in the selected model in relation with its specific mutations as good as possible. It is therefore important to develop state-of-the-art methodologies for the acquisition and processing of mouse MR neuroimaging datasets, providing all the information needed to be used by multiple centres and in different mouse models. These protocols need to respect certain criteria of data quality, and must prove their usefulness to identify macroscopic/microscopic lesions in these species, as well as functional alterations reminiscent of AD pathology. The assessment of these protocols is one of the aims of the work presented in this thesis.

In the first part of this thesis (chapter 2-3-4) we developed and optimized methodologies to characterize changes in brain structure and function in animal models for familial AD (APP\textsubscript{swe}/PS1\textsubscript{de9}) and for risk factors for both vascular disorders and AD (apoE4 and apoE-ko). Four MRI methods that are commonly used in human studies for the detection of relevant surrogate biomarkers of AD are discussed: (1) the assessment of whole-brain cerebral blood volume, with an approach that enhances the sensitivity of the measure towards the microvascular compartment. (2) the assessment of a fast and high-resolution diffusion tensor imaging of the whole brain, that includes a robust tensor estimation and automatic artefact corrections. The use of an arterial spin labelling technique to measure cerebral blood flow and (4) the assessment of resting-state functional connectivity measurements in mice. Each of these techniques was designed to require no more than 20-30 minutes for the acquisition, making it possible to combine all these different imaging modalities in a single MR session. The amount of information acquired could then provide the basis both for studying the biological cause of structural and functional brain changes in relation with AD and for evaluating the efficiency of future treatment strategies for AD and other neurodegenerative disorders.

**Vascular disorders in AD: cause or consequence?**
There is now overwhelming evidence, also from studies presented in this thesis, that vascular disease plays an important role in the development of late-life cognitive dysfunction and dementia. But when exactly do vascular impairments occur in different mouse models, and to what extent are they related to other...
pathological hallmarks of the disease? And how do they influence the progression of the pathology?
To answer these questions it was necessary to perform longitudinal studies in different mouse strains. First, we used the APP_{swe}/PS1_{de9} mice as a model of brain amyloidosis and familial AD, to study the effects of amyloid-β on the vascular system (chapter 2 – 6). In this thesis, we demonstrated that APP and PS1 mutations cause microvascular cerebral blood volume reduction in cortical regions and in the thalamus starting at 8 months of age. These deficits in the vasculature spread over several sub-cortical areas and the hippocampus at 13 months of age (chapter 2); at this age, a strong reduction in cerebral perfusion was also detected (chapter 6). The reduced CBV and CBF found in these mice is of particular interest, as it mirrors the brain hypoperfusion status found in the majority of AD patients (Alsop, et al., 2000), (Ruitenberg, et al., 2005, Schreiber, et al., 2005). Importantly, these vascular impairments did not correlate with the amount of Aβ plaques in the parenchyma nor in the blood vessel walls. We therefore hypothesise that these vascular deficits are caused by other Aβ species, which may be involved in damaging the structure and the function of vessel walls. In agreement with these findings, it has been shown that soluble Aβ_{1-40} can reduce capillary vasodilation activity, caused by a brain endothelial dysfunction with diminished production of NO by endothelial cells (Farkas and Luiten, 2001, Niwa, et al., 2000, Price, et al., 2001).
Because of the strong association between perfusion deficits and cognitive impairments, we believe that once these cerebrovascular deficits are triggered, the whole pathology aggravates. Following the “vascular hypothesis” cascade by de la Torre, such a decrease in cerebral perfusion could lead to increased oxidative stress and decreased energy metabolism (de la Torre, 2000b). All these events consequently contribute to a vicious circle of the progression of neurodegenerative traits typical of AD, such as synaptic loss, increased Aβ and NFTs production, increased brain inflammation, brain tissue atrophy and cognitive decline (de la Torre, 2000a, de la Torre, 2000c, de la Torre and Stefano, 2000). Following this interpretation of the results, it is also interesting to include in this discussion the correlation found in 12-month-old APP_{swe}/PS1_{de9} mice between hippocampal CBF and diffusion anisotropy measure with DT-MRI (chapter 6). The correlation of these two measures reinforces the idea that neuronal and vascular health are tidily interdependent. The relevance of this dependency is clearly supported by the findings illustrated in chapter 3, where
we showed that diffusion parameters changes in these mice reflect neuronal loss and damage of the brain tissue at microstructural level.

We then addressed the question “how vascular risk factors can influence the development of pathological traits of late-onset AD?”. To this end, we used target-replacement apoE-ε4 and apoE-ko mice as models of respectively hypercholesterolemia and atherosclerosis. In these mice, we measured CBV and CBF in relation to functional connectivity, brain tissue microstructure, behaviour, cognition and neuropathology (chapter 3 – 4).

In the apoE-ε4 mice the results obtained were mixed; we showed no CBV and CBF deficits in adult mice (12-month-old), while a reduced cortical and thalamic perfusion was found only in older animals (18-month-old). These findings are in agreement with human studies in apoE-ε4 subjects, showing a fast decline of regional CBF during aging (Wierenga, et al., 2013). These perfusion deficits are hence suggested to contribute to the increased risk of developing AD. However, no consistent results were found from behavioural and cognitive tests in our experiments, nor could we detect significant differences in functional connectivity, compared to wildtype mice. Overall, these results indicate that cerebrovascular impairments in apoE-ε4 mice occur before other AD-like pathological changes become evident, and might trigger brain dysfunction only in a later stage, as the pathology progresses. To validate this hypothesis, studies in older apoE-ε4 mice are required; the use of very old mice (> 18 months of age), however, could introduce biases in the results due to aging-related pathologies, making more complex to study the effect of vascular risk factor with this model.

In the apoE-ko model, we found as expected the strongest vascular pathology, specifically manifested as decreased cortical perfusion in both adult (12-month-old) and old mice (18-month-old) (chapter 4). These deficits are likely to be caused by atherosclerosis, aortic aneurysm and endothelial dysfunction that these mice spontaneously develop from 3 months of age (Crauwels, et al., 2003, Trollope, et al., 2011). Simultaneously to cerebrovascular hypoperfusion, we detected: increased anxiety, increased serum cholesterol, reduced postsynaptic density and reduced functional connectivity in apoE-ko mice; all factors attributable to AD-like pathology progression. Interestingly, we also noted a relative increase in synaptic density in old apoE-ko mice, which may reflect a compensatory mechanism in response to early functional synaptic failure or brain insults, such as stroke, ischemia, or the measured hypoperfusion (Arendt,
2001, Jansen, et al., 2012, Jin, et al., 2004, Li, et al., 2010, Mu and Gage, 2011, Wang, et al., 2008). This also accords with the enhanced neurogenesis shown in apoE-ko mice as described in chapter 5.

In conclusion, the work presented in this thesis fully supports the idea that vascular defects are both cause and consequence of AD pathology. The evidence that this thesis provides implicate that AD prevention should focus more on keeping hemodynamic parameters in a physiological range. Alterations in these parameters should be properly evaluated for the diagnosis of the disease, following-up its progression and for assessing the effects of disease-modifier therapies.

**Dietary intervention as therapeutic approaches against AD**

There is increasing recognition that neuroimaging tools should also be implemented in studies towards the development of new therapeutic approaches. In the second part of this thesis (chapter 5 - 6), we assessed brain structure and function with MRI to investigate the effects of nutrition as a preventive strategy against AD.

In the current literature, a mismatch exists between epidemiological data on the role of nutrition in AD and the lack of clinical efficacy in nutritional intervention studies (reviewed in (de Wilde, et al., 2011)). Several reasons behind these inconclusive results may be hypothesised; first, despite the potential utility of imaging techniques in monitoring treatment effects, most of the clinical studies on nutritional intervention in AD still rely on cognitive measurements as the only outcome. Hence, further investigation is warranted to confirm whether nutritional approaches impact on other surrogate biomarkers of the disease, such as cerebral perfusion, functional connectivity and tissue microstructure. These findings would then possibly help the physicians to understand the mechanisms of action of the diets, and help them to determine which patient can best benefit from the dietary intervention. Second, most of these studies were performed in patients with a diagnosis of MCI or AD; in the latter group, patients are often in an advanced stage of neurodegeneration, and therefore less resilient to therapies. Also, the definite neuropathological confirmation of MCI and early AD diagnosis in the patients is very difficult to obtain in these populations. Third, the interventions consisted in most of the cases of single nutrient supplementation added to their normal diet; because of the
concomitant presence of multiple phenotypic traits in AD, it is unlikely that one single nutrient can have beneficial effects on all the different aspects of the disease.

In this thesis we tried to overcome these issues by employing a range of different MR imaging methods and neuropathological investigations on \( \text{APP}_\text{swe}/\text{PS1}_{\text{de9}} \) (chapter 6), apoE-ε4 and apoE-ko mice (chapter 5) to evaluate the efficacy of several diets. We fed these mice with multi-nutrient diets specifically designed to synergistically enhance the synthesis of synaptic membranes (Kamphuis and Scheltens, 2010) throughout their life. Some of the components in these diets, such as omega-3 (n-3) long-chain polyunsaturated fatty acids (LCPUFA), have also been shown to improve vascular health (Lee, et al., 2008). We therefore hypothesised that these nutrient combinations have the potential to slow AD progression, by simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms. A similar diet composition has been supplied in mild AD patients in two randomized controlled clinical trials, showing improvements in the delayed verbal recall task and better cognitive performance (Cummings, 2012, Scheltens, et al., 2010, Scheltens, et al., 2012). One of the goals of our studies was to confirm their efficacy by using advanced imaging biomarkers and provide evidence of the mechanisms by which these dietary nutrients influence the pathophysiology of AD.

In agreement with our hypothesis, we showed that these multi-nutrient diets were able to significantly enhance the cortical and thalamic brain perfusion in 12-month-old \( \text{APP}_\text{swe}/\text{PS1}_{\text{de9}} \) transgenic mice (chapter 6). In addition, the brain diffusion changes found in this strain, which have been correlated with loss of myelin, decreased axonal density and connectivity in white matter (Song, et al., 2004), (Chen, et al., 2011) and neuronal loss in gray matter (Sykova, et al., 2005), were diminished in the mice fed with the multi-nutrient diet. Overall, the data presented strongly suggest an effect of the diets in maintaining both vascular and neuronal health in the \( \text{APP}_\text{swe}/\text{PS1}_{\text{de9}} \) mice onto physiological levels, possibly by slowing down the neurodegenerative processes compared to the transgenic mice on control diet (Zerbi, et al., 2013). These effects may explain the behavior and cognitive improvements found in \( \text{APP}_\text{swe}/\text{PS1}_{\text{de9}} \) on the similar multi-nutrient diet composition (Hooijmans, et al., 2009, Wiesmann, et al., 2013).

Some promising effects of these multi-nutrient diets are also shown in mouse models for vascular risk factors in AD (chapter 5). In 12-month-old apoE4 and apoE-ko mice this combination of nutrients decreased anxiety-related behavior,
partly reduced total cholesterol levels, and increased the concentration of omega-3 fatty acids in the brain. Furthermore, we found that wildtype and apoE-ko mice fed this multi-nutrient diet showed locally increased cerebral blood volume and decreased hippocampal glutamate levels. Based on these results, we suggest that a specific dietary intervention is able to alleviate early pathological consequences of hypercholesterolemia and vascular risk factors for AD. Overall, the work presented in this thesis demonstrated the utility of advanced MR neuroimaging tools for detecting changes in brain structure and function with respect to dietary intervention. Furthermore, we also revealed novel important mechanisms by which these diets may affect AD onset and development; up till now, the beneficial effect of these diets was suggested to derive from an increased production of phospholipids to sustain synaptic genesis and repair processes (Wurtman, et al., 2009, Wurtman, et al., 2006). In our studies, we show that the strongest and most consistent effect of these diets involved the amelioration of cerebrovascular health. Based on previous experiments in our lab and from literature, we associated this effect with the presence of omega-3 fatty acids and the B-vitamins in these diets (Hooijmans, et al., 2009, Lecerf, 2009). In our last experiment (chapter 6), we proved that these two protective mechanisms on vascular and neuronal health (revealed by diffusion) are both enhanced by the diets, and may positively influence each other. Although decreased cerebral perfusion has been recognized as an early and important contributor to AD pathology and cognitive decline (Altman and Rutledge, 2010), we believe that this aspect is not sufficiently considered in human nutritional intervention studies. To ultimately prove the translational aspect of this research, we therefore encourage physicians to include perfusion (and diffusion) MRI analysis in future human clinical studies. Indeed, in all our experiment the diets were supplemented before the pathology is documented — for each mouse model — and throughout the life of the animals; it would be interesting for future studies to understand whether a short period of supplementation would have similar effects. In this way, one can prove if these multinutrient diets should better be recommended to normal subjects as preventive strategy against AD development, or also to patients as an effective treatment.
Concluding remarks: towards understanding the biological underpinnings of AD’s biomarkers.

The uncovering of biomarkers for AD has dramatically increased the opportunities for a more effective diagnosis of the pathology, and for targeting therapeutics to patients who require them and who can best benefit from them. In this thesis we demonstrated some potential translational applications of these biomarkers, which could be applied to human studies. There are, however, many obstacles to overcome in achieving this purpose. The effectiveness of translational research is seriously limited by many factors. Some of these challenges are scientific, requiring standardization of the protocols for acquisition and processing, and a detailed characterization of the pathological changes of the animal models used. Also, more knowledge and validation of the biological basis of changes in surrogate biomarkers is needed. One example is the interpretation of the diffusion tensor imaging alterations. Despite that the technique is now widely used as well in the clinic, little is known about the neuroanatomical implication of the changes in water diffusivity; in most of the cases, many different interpretations of the results are at one’s disposal. With the unique possibilities offered by animal research, we tried to clarify the meaning of diffusion changes in AD by comparing MR results with immunohistochemical stainings. In this way, we were able to successfully correlate the increased hippocampal diffusivity found in our APP/PS1 mice, which well-resemble the findings in patients, with neurodegenerative processes in the granulate layer of the dentate gyrus (chapter 3). This result represents a real novelty of the study, but further investigations are possible; for instance, the exact origin of diffusion defects in the white matter is still uncertain: highly hydrophobic amyloid-β deposits might cause constraints on water diffusion but these defects might also come from loss of myelin or alterations in the fiber microstructure. It would be very valuable to characterize the white matter pathology in a future study with a multi-modal imaging approach; for example, by combining DT-MRI imaging with multi-photon microscopy, we could aim to test the hypothesis that changes in white matter water diffusion detectable with MRI represent and correlate with microstructural axonal disorganization and myelin loss (Farrar, et al., 2011). Resting-state fMRI is another increasingly used neuroimaging tool to measure brain function. The growing interest generated on this topic is reflected by the marked rise of publications in recent years.
Nevertheless, these measures ironically suffer from a lack of insight into their biological meaning. Trying to fill this gap, we assessed in this thesis resting-state functional connectivity (FC) in aging wildtype, apoE-ε4 and apoE-ko mice. Thanks to the advantages offered by animal research with respect to human studies, we were able to relate those changes with results from perfusion, diffusion and synaptic density measurements. We foresee that this study represents just the beginning of a new era in preclinical research, in which the use of multi modality approaches will expand the knowledge about how and why FC changes occur.
Appendices

List of abbreviations

References

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Curriculum vitae

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Donders Graduate School for Cognitive Neuroscience
### List of abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid-β</td>
</tr>
<tr>
<td>aca</td>
<td>anterior commissure</td>
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<tr>
<td>ACg</td>
<td>anterior cingulate gyrus</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>apoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>AβPP</td>
<td>amyloid-β precursor protein</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>bcc</td>
<td>body of corpus callosum</td>
</tr>
<tr>
<td>BOLD</td>
<td>blood oxygen level dependent</td>
</tr>
<tr>
<td>CA1</td>
<td>cornu ammonis 1</td>
</tr>
<tr>
<td>CA3</td>
<td>cornu ammonis 3</td>
</tr>
<tr>
<td>CAA</td>
<td>cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CATCH</td>
<td>critically attained threshold of cerebral hypoperfusion</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>CBV</td>
<td>cerebral blood volume</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO</td>
<td>control diet</td>
</tr>
<tr>
<td>cp</td>
<td>cerebral peduncle</td>
</tr>
<tr>
<td>CRLB</td>
<td>Cramér-Rao lower bounds</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>DEU</td>
<td>multi-nutrient diet enriched with docosahexaenoic acid, eicosapentaenoic acid, and uridine monophosphate</td>
</tr>
<tr>
<td>DT-MRI</td>
<td>diffusion tensor magnetic resonance imaging</td>
</tr>
<tr>
<td>ec</td>
<td>external capsule</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>echo planar imaging</td>
</tr>
<tr>
<td>FA</td>
<td>fractional anisotropy</td>
</tr>
<tr>
<td>FAs</td>
<td>fatty acids</td>
</tr>
<tr>
<td>FAD</td>
<td>familial Alzheimer’s disease</td>
</tr>
<tr>
<td>FAIR</td>
<td>flow-sensitive alternating inversion recovery</td>
</tr>
<tr>
<td>FC*</td>
<td>functional connectivity ( *chapter 4 )</td>
</tr>
<tr>
<td>FC</td>
<td>Fortasyn® Connect multi-nutrient diet enriched with docosahexaenoic acid, eicosapentaenoic acid, uridine monophosphate, phospholipids, choline, folic acid, vitamins B6, B12, C, E and selenium</td>
</tr>
<tr>
<td>fi</td>
<td>fimbria of the hippocampus</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>fo</td>
<td>fornix</td>
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<tr>
<td>FOV</td>
<td>field of view</td>
</tr>
<tr>
<td>gcc</td>
<td>genu of corpus callosum</td>
</tr>
<tr>
<td>GE</td>
<td>Gradient Echo</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
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<td>-------------------------------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>GLUT-1</td>
<td>glucose transporter type 1</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IML</td>
<td>inner molecular layer (of the dentate gyrus)</td>
</tr>
<tr>
<td>$\lambda_1$</td>
<td>axial diffusivity</td>
</tr>
<tr>
<td>lc-PUFAs</td>
<td>long-chain polyunsaturated fatty acids</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>$ml$</td>
<td>myo-Inositol</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>(1H) MRS</td>
<td>proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MD</td>
<td>mean diffusivity</td>
</tr>
<tr>
<td>MUFA</td>
<td>mono unsaturated fatty acids</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>n3</td>
<td>omega-3 fatty acids</td>
</tr>
<tr>
<td>n6</td>
<td>omega-6 fatty acids</td>
</tr>
<tr>
<td>NFTs</td>
<td>neurofibrillary tangles</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>OF</td>
<td>open field</td>
</tr>
<tr>
<td>OML</td>
<td>outer molecular layer (of the dentate gyrus)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PLA</td>
<td>prelimbic area</td>
</tr>
<tr>
<td>PS1</td>
<td>presenilin 1</td>
</tr>
<tr>
<td>PS2</td>
<td>presenilin 2</td>
</tr>
<tr>
<td>PUFA</td>
<td>poly unsaturated fatty acids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>rMWM</td>
<td>reverse Morris water maze</td>
</tr>
<tr>
<td>RD</td>
<td>radial diffusivity</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>rsfMRI</td>
<td>resting-state functional magnetic resonance imaging</td>
</tr>
<tr>
<td>SAD</td>
<td>sporadic Alzheimer’s disease</td>
</tr>
<tr>
<td>scc</td>
<td>splenium of corpus callosum</td>
</tr>
<tr>
<td>SE</td>
<td>Spin Echo</td>
</tr>
<tr>
<td>SFA</td>
<td>saturated fatty acids</td>
</tr>
<tr>
<td>SIPBs</td>
<td>synaptophysin-immunoreactive presynaptic boutons</td>
</tr>
<tr>
<td>SL</td>
<td>stratum lucidum (of the CA3 area)</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>SR</td>
<td>stratum radiatum (of the CA1 area)</td>
</tr>
<tr>
<td>STEAM</td>
<td>stimulated echo acquisition mode</td>
</tr>
<tr>
<td>TE</td>
<td>echo time</td>
</tr>
<tr>
<td>TI</td>
<td>inversion time</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine monophosphate</td>
</tr>
<tr>
<td>USPIO</td>
<td>ultra small particles of iron oxide</td>
</tr>
<tr>
<td>VAPOR</td>
<td>variable pulse power and optimized relaxation delays.</td>
</tr>
<tr>
<td>VBA</td>
<td>voxel-based analysis</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Abbreviations
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Acknowledgements

“If our brains were simple enough for us to understand them, we’d be so simple that we couldn’t.”
- Ian Stewart, The Collapse of Chaos: Discovering Simplicity in a Complex World

The first time I read this phrase, from Ian Stewart, I was fascinated by the message it carried. Indeed, studying the human brain is a difficult task, as it is probably the most complex system in our body and, perhaps, in life. But surprisingly, we have to deeply acknowledge its complexity, as we are all made of it.

There is, however, a second message in this sentence, hidden between the lines. Something less evident, but nonetheless crucial that caught my attention in a second moment.

If you read it more carefully, you will notice that everything in this sentence is written in plural form. “We”. “Our brains”. “For us”. “We’d be”. What is the reason for that? Is it perhaps just an elegant form to express an opinion, hiding in this way the author’s selfishness? Or there is something more?

I like to believe that the phrase’s construct has been chosen for a specific reason. Like in the big construction sites, with many people cooperating on the same project, the study of brain functions must be conducted in collaboration with many other researchers, including people not working on this specific field. You need to listen and to learn, to talk, to think, to discuss, to present your ideas and to attend other’s presentations. And you need a bright environment to grow your interests and get inspired.

My first acknowledges therefore go to my supervisor and my promoters, dr. Amanda Kiliaan, Prod. Dr. Arend Heerschap and Prof. Dr. Stan Gielen, who literally created the conditions and made possible all the work described in this thesis.
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Arend, you have been a mentor for me, someone who always motivated me to improved my precision and my scientific rigorousness. You have always been able to give the right suggestion and feedback, and I highly esteem how you were able to guide the department of Radiology, with a fresh view on every problem.

Stan, as the dean of the faculty of Science, you could only guide me from a certain distance, nonetheless I am grateful to your firm support to our research group demonstrated in these years.

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Curriculum vitae

Valerio was born on June 24th in 1983 in Borgomanero, Italy. After his bachelor degree in biomedical engineering (Politecnico of Milano, IT) he started working as clinical researcher in the cardio-thoracic surgery department at the San Raffaele Hospital (Milano, IT). There, he developed his passion and attitude for research, and he accomplished his master degree in biomedical engineering three years later, with an experimental thesis on the effects of surgical ablation of atrial fibrillation.

Looking forward a career in science, he applied for a PhD position at the Radboud University Nijmegen Medical Centre, the Netherlands, under the supervision of professor Amanda Kiliaan and professor Arend Heerschap. The objective of the PhD project, which is presented in this thesis, was to evaluate the therapeutic and interventional effects of lipid-based nutrition on cerebral vasculature and pathology in Alzheimer’s disease (AD).

His main interest and focus was to develop novel methodology with MR imaging and spectroscopy to evaluate biomarkers for AD progress in transgenic mouse models for AD and vascular disorders. As responsible for the MRI work in the research group, he collaborated on several different projects in the field of neuroscience. Up till now he has developed, both at high-field (7T) and ultra-high-field MR systems (11.7T), several methodologies for in vivo brain imaging, such as: susceptibility weighted-imaging for microvascular blood volume calculation; ASL-FAIR for cerebral blood flow quantification; $^1$H and $^{31}$P MR spectroscopy; high-resolution diffusion tensor imaging and resting-state fMRI.

He has supervised several excellent master students with their internship projects and theses, and he presented his research at several (inter)national conferences in oral/poster sessions, also as invited speaker and moderator. In the past year he was also actively involved in organizing the 2013 conference for the Benelux society of Magnetic Resonance in Medicine (http://www.beneluxismrm.org/).
List of publications


Donders Graduate School for Cognitive Neuroscience Series

Appendices


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