VASCULAR RISK FACTORS AND ALZHEIMER’S DISEASE
THERAPEUTIC APPROACHES IN MOUSE MODELS

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Vascular risk factors and Alzheimer’s disease

Therapeutic approaches in mouse models

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General introduction

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General introduction

The term dementia comprises several symptoms, for example a progressive loss of memory and behavioral changes, which together interfere with independent performance of tasks of daily life [1]. As a result of increasing life expectancy, dementia is developing into one of the major public health problems in our aging society. This is driven mostly by the increasing prevalence of Alzheimer’s disease (AD) with increasing age. AD and vascular dementia (VaD) are the number one and two disorders in terms of prevalence, and together they are responsible for most cases of dementia [2, 3]. These disorders are preceded by a stage in which the individual evidences cognitive decline but is still able to maintain independent functioning. In AD, this stage is referred to as mild cognitive impairment (MCI) due to AD, whereas in VaD this prodromal stage is termed vascular cognitive impairment (VCI). In this introduction the term AD will mostly reflect the continuum of MCI due to AD and dementia due to AD, and the term VaD will reflect the continuum of VCI and VaD.

Historically, AD and VaD have been considered as separate entities, and this separation remains driven by clinical classification criteria. Therefore, the inclusion of AD discussing vascular aspects of dementia may seem confusing. However, there is considerable overlap between these disorders, and the underlying interactions between VaD and AD will be explained and summarized in this introduction. Furthermore, two major risk factors for VaD and AD, hypertension and stroke, will be further discussed and investigated in this thesis.

Studies involving LC-n3-FA supplementation and manipulation of the renin-angiotensin system (RAS) have shown that these novel therapeutic approaches have the potential to lower the effect of hypertension and stroke. Therefore, these new preventive strategies against two major risk factors for cognitive impairment and dementia will be discussed as well in this chapter and later in this thesis.

Vascular dementia (VaD), classification and etiology

5-20% of dementia cases in the population are based on VaD,[4] which is a common disorder in the elderly but also prevalent among younger adults [5]. The concept of VaD consists of two main elements: the presence of a dementia syndrome and an underlying vascular cause [6]. To characterize VaD, criteria of State of California Alzheimer’s Disease Diagnostic and Treatment Centers and the National Institute for Neurological Disorders and Stroke-Association Internationale pour la Recherche et l’Enseignement en Neurosciences (NINDS-AIREN) are commonly used [4, 6, 7]. Dementia caused by ischemic or hemorrhagic cerebrovascular disease (CVD) or by ischemic-hypoxic brain lesions of cardiovascular origin is also
included in VaD [5, 8]. Among recurrent or first-ever stroke patients post-stroke dementia is a frequent sequel, ranging from 6 to 31.8% [9-11].

**Definitions of VaD and VCI**

The dementia stage of VaD can also be understood as the most severe form of vascular cognitive impairment (VCI) [4, 12]. VCI is a syndrome characterized by the presence of clinical stroke or vascular brain injury and cognitive impairment affecting more than one cognitive domain [13]. The VCI-VaD continuum can be divided into familial and sporadic forms [13]. The most frequent subtype of familial VaD, caused by genetic mutations, is the “cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy” (CADASIL) [13] caused by mutations in the Notch 3 gene [14]. Sporadic VaD has three major subtypes: multi infarct dementia, strategic infarct dementia, and subcortical vascular encephalopathy (synonymous with Binswanger’s disease) [13]. O’Brien et al. have published an alternative classification of the VaD subtypes [5, 15]: multi-infarct dementia (cortical VaD); small-vessel dementia (subcortical VaD); strategic infarct dementia; hypoperfusion dementia; hemorrhagic dementia; AD with CVD; and the familial variant of vascular dementia, CADASIL.

**Stroke and VaD**

Many stroke patients show a gradual but continuous deterioration following a single stroke lesion [16]. This deterioration is characterized clinically by cognitive and behavioral dysfunction. Stroke research has traditionally focused on motor impairment (e.g. limb paresis), where a number of patients show partial recovery indicating the brain’s capacity for repair or compensation after injury [17]. However, this research has paid little attention to cognitive and behavioral deficits induced by stroke. After stroke, recovery from these deficits is often absent, and, as indicated, in many patients stroke leads to progressive deterioration even in the absence of new stroke lesions. Novel research indicates that stroke-induced lesions to brain networks are responsible for this absence of recovery or even for progressive disease, leading to an increased mortality rate [18]. However, it is still not fully understood how stroke, cognitive decline and dementia are interconnected. Stroke may predispose older adults to developing VaD.

**AD, definition and etiology**

In 1906 Alois Alzheimer, mentioned already arteriosclerotic changes in cerebral blood vessels of the post mortem brain of his 55-year old patient Auguste D(eter) besides the neuropathological hallmarks amyloid plaques and neurofibrillary tangles [19, 20]. The production of Aβ peptides is increased in familial forms of AD and is thought to be the primary driving force in non-familial (sporadic) AD
pathogenesis [21]. This amyloid cascade hypothesis, is still the dominant theory for the pathogenesis of AD, but remains under debate as other researchers casted doubt that the Aβ plaques and the NFTs are really the main cause of the neurodegeneration in AD [22]. Experimental results showed that the density of senile Aβ plaques can be the same in patients affected by AD and non-affected patients [23, 24]. Recently, the focus of the research on amyloid-beta has shifted towards the oligomerization of Aβ since several studies showed that these oligomers and fibrils are in fact the toxic forms of Aβ-peptides [25].

Cerebral amyloid angiopathy and AD
The accumulation of Aβ in the walls of arteries and arterioles in the leptomeninges and cerebral cortex is called cerebral amyloid angiopathy (CAA) [26]. CAA has been linked to haemorrhages (microbleeds), most clearly shown in a mouse model for CAA [27]. Because CAA is found both in sporadic AD patients and also in cognitively normal individuals without prodromal AD, [28, 29] the exact relationship between AD and CAA remains uncertain.

Risk factors for VaD
The assumption has been made that risk factors for VCI and VaD would be the same as those for stroke [30]. The risk factors for stroke can be divided into three major classes, nonmodifiable (e.g. age, sex, genetic factors, etc); modifiable (e.g. hypertension, diabetes, hyperlipidemia, atrial fibrillation, smoking, obesity, etc); and potentially modifiable (e.g. alcohol abuse, infection) [31].

Hypertension has been shown to be the most common modifiable risk factor for stroke worldwide [32, 33]. Large-scale, placebo-controlled clinical trials have shown an association between hypertension and stroke [34, 35] and a linear relationship between blood pressure and stroke mortality has been revealed [36]. More specifically, a rise of only 1 mm Hg in systolic blood pressure of treated hypertensive patients increased stroke deaths by 2% [36]. A community-based prospective cohort study revealed that incremental increases in blood pressure were linked to an increase in microinfarcts in initially non-demented persons (65-80 years of age), but not in the older age group [37].

A history of stroke leads to a twofold increase of the risk of dementia in the population older than 65 years [38, 39] and this effect was also confirmed in animal studies [40]. Combining confirmed AD pathology and cerebral infarcts after autopsy with the test results of cognitive function revealed that AD patients with cerebral infarcts showed more cognitive impairment than patients without cerebral lesions [41, 42]. In the population-based Rotterdam Scan Study, 1015 participants underwent neuropsychological testing and cerebral MRI and were monitored for dementia during the study period [43]. In this study, silent brain
infarcts doubled the risk of dementia [43]. Furthermore, in subjects without dementia, presence of these infarcts increased the chances of a decline in global cognitive function [43]. Thus, an increased risk of incident stroke is associated with cognitive decline and dementia [44].

Even individuals who are stroke- and dementia-free, but have a higher risk to develop stroke, have more cognitive deficits than individuals with lower stroke risk [45]. As already mentioned for VaD, Stroke may also predispose older adults to developing AD. The mechanisms behind cognitive decline after the occurrence of a stroke could help us to develop new treatments to prevent the onset of dementia. Furthermore, new preventative treatments for stroke are needed to counteract both stroke and dementia. One possibility to reduce stroke would be to reduce the modifiable risk factors as lifestyle, hypertension, diabetes, hyperlipidemia, atrial fibrillation, smoking, obesity, etc..

Risk factors for AD
Several vascular risk factors for the development of AD have been demonstrated, e.g. hypertension, diabetes mellitus, atherosclerosis, atrial fibrillation, coronary artery disease, smoking, obesity, carrying apolipoprotein E ε4 allele (apoE4) and metabolic syndrome [46, 47]. Many studies have shown the association between increased blood pressure in mid-life and cognitive decline or AD in late-life, [48, 49] although conflicting studies are reported as well. The apolipoprotein E ε4 allele (apoE4) represents a strong genetic risk for AD as it is associated with increased cardiovascular risk factors [12, 50]. A recent review on apoE-related biomarker profiles in the early phase of AD further elucidates this relatively novel concept of the ε4 to be considered more as a vulnerability factor rather than a pathogenic factor [51]. Moreover, apoE4 carriers are clearly more susceptible to vascular brain damage (eg. stroke, brain haemorrhage; [52-54] and they display aberrant functional connectivity [55]. Additionally, changes in brain diffusivity, as a biomarker for white and gray matter integrity, have been reported in human -ε4 carriers [56-59]. These structural modifications may be linked to the isoform-specific role of apoE in synaptic development, dendrite formation and axonal guidance, which in some extent may be impaired in apoE4 carriers [60]. However, among all vascular risk factors hypertension seems to be the most powerful risk factor for AD [61]. Furthermore, recent studies demonstrated that in the elderly a history of stroke can double the AD prevalence [62]. The combination of the latter results demonstrates the impact of these two risk factors for AD. The risk factors for AD are almost the same as for VaD. Therefore, in the next paragraph the overlap and interactions of VaD and AD, but also between their risk factors are discussed.
Overlap and interactions of VaD and AD, and their risk factors

In the first instance, it may seem difficult to see the overlap between VaD and AD, as these entities are strictly separated in their clinical criteria. As a first illustration of why VaD and AD can no longer be strictly separated in this way, Biessels et al. have shown in their systematic review that diabetes as a risk factor may directly influence both vascular and neurodegenerative pathology [63]. Biessels et al. suggested from mechanistic studies that vascular disease and alterations in glucose, insulin, and amyloid metabolism were connected and may underlie the pathophysiology of both AD and VaD.

In line with this, several studies indeed indicate that Metabolic Syndrome (MetS) is a risk factor for lower cognitive function and dementia [64] and this association seems to be age-dependent [65-67]. MetS is characterized by the co-occurrence of several metabolic risk factors for cardiovascular disease (CVD), stroke, and/or type 2 diabetes mellitus (T2DM) [68, 69] and its increasing prevalence will drive the twin global epidemics for CVD and T2DM in the coming years [69]. The current epidemic of MetS in middle-age and the increasing prevalence of dementia with age, particularly AD, converge in findings that MetS occurring in middle-age is associated with an increased risk of cognitive decline and dementia later in life [70]. MCI can be considered as an intermediate stage of cognitive impairment wherein cognitive changes found in normal aging often proceed into those changes typically observed in dementia [71]. Indeed, one study described that MetS related risk for progression from MCI to dementia was increased significantly in a 3.5 year follow-up [72]. Other studies detected an association between MetS and AD [73, 74], neurodegenerative diseases [75], and VaD [76, 77]. Several factors of MetS have also been linked to cognitive impairment and dementia [78]. Obesity for instance, is recognized as a modifiable risk factor for dementia [79, 80] and also hypertension, especially mid-life hypertension, and both diabetes and prediabetes, increase the risk of developing dementia [81, 82]. Possible explanations for these correlations might be found in an impaired vasculature and/or altered CBF due to MetS, which in turn leads to neuronal damage [70]. To maintain the structural and functional integrity of the brain, a well-regulated and continuous cerebral blood supply is crucial. For this reason it is not surprising that alterations in the cerebral vasculature, as seen in MetS, have a profound impact on cognitive function [83]. The structural and functional alterations in the blood vessels due to MetS can affect the brain by accelerating cerebral small vessel disease that may result in cerebral microbleeds, white matter lesions (WML), and brain atrophy [84]. Among MetS components, obesity and hypertriglyceridemia are associated with smaller brain volume, whereas hypertension is associated with an increased occurrence of white matter hyperintensities (WMH) and infarcts [85]. Unfortunately, the components of MetS have often been considered
in an independent manner, as if the syndrome consists of a sum of different disorders with neuronal damage as the end stage where all components’ effects assemble [86]. However, this view will be reductive to give an explanation for the global metabolic effects on cognitive decline and therefore it has been suggested to consider the metabolic alterations as a continuum leading to various degrees of cognitive disorders [86, 87].

There is now more and more awareness that vascular risk factors play a key role in the pathogenesis of AD [88]. In aged subjects a relation between vascular risk factors and AD has been found [89, 90]. In addition, other epidemiological and clinical studies identified that AD and VaD share common risk factors such as hypertension, diabetes mellitus, hyperlipidemia, and arrhythmia [91-96]. This overlap in major risk factors for these clinically and pathologically different conditions (VaD and AD) may seem confusing when it comes to understanding pathogenesis. A simple practical consequence however is, for example, that hypertension is a major risk factor for cognitive decline and dementia in the elderly, regardless of whether this is due to stroke, VaD or AD, or combinations of these disorders. Another overlap between VaD and AD is found in the neurovascular unit. The neurovascular unit is the collective term for neurons, glia, and perivascular and vascular cells [12]. This unit is responsible for the strong increase in cerebral blood flow following cognitive activation. In AD and VCI-VaD patients the neurovascular unit is disrupted, and this could lead to insufficient perfusion during cognitive activation, contributing to further neuronal dysfunction [97-101].

In addition, alterations in cerebral microvascular structure are related to AD and VCI [102, 103]. In animal models, hypertension, aging, and diabetes, the major risk factors for AD and VCI interfere with endothelium-dependent responses in the microcirculation and in the functional hemodynamic response of the brain [12, 98, 104, 105]. Furthermore, hypertension promotes atherosclerosis in cerebral arteries [99] and induces lipohyalinosis, affecting the blood supply for the white matter [99]. These changes can result in lacunar infarction or brain hemorrhage [99]. Notably, studies have shown that vascular lesions lead to a decrease in the threshold for the clinical manifestation of AD [12]. In demented and non-demented Japanese-American men Petrovitch et al. showed that cerebrovascular lesions increase dementia frequency in patients with low neuritic plaque frequency [106]. From this finding, they concluded that the preservation on late-life cognitive function is dependent on the prevention of cerebrovascular lesions [106]. To support the idea that vascular lesions or CVD lower the threshold for dementia due to AD and α-synucleinopathies, Toledo et al. demonstrated that CVD is commonly found in aged subjects with dementia, while it is even more common in AD patients, especially in younger patients [107]. Furthermore, Toledo
et al. found that the presence of CVD increases the risk of dementia in patients with α-synucleinopathies and also in those being affected by AD [107]. These latter studies clearly show the relation between cerebrovascular impairments and dementia, especially AD and VaD.

**Hypertension – an overlapping risk factor for VaD and AD**

**Hypertension and AD**

One of the most common cardiovascular risk factors, arterial hypertension, has been shown to increase the risk of both AD and VaD [49, 108-115]. Skoog et al. demonstrated a relationship between hypertension and amyloid plaques, neurofibrillary tangles, and brain atrophy.[114] Furthermore, Skoog and Gustafson showed that increased blood pressure appeared decades before the onset of AD, followed by a decrease in blood pressure years before the start of AD [114]. This phenomenon of hypertension followed by a gradual reduction in blood pressure may be caused by difficulties in maintaining blood pressure homeostasis due to a damaged central nervous system [114, 116]. Studies that link blood pressure with dementia may be complicated by this non-stationary course of blood pressure in the trajectory of dementia.

Long-standing hypertension promotes atherosclerosis and vascular remodeling including increases in wall thickness. Arterial stiffness and severe atherosclerosis can lead to an increase in pulse pressure (the difference between systolic and diastolic blood pressure) [117]. In a community-based study, increased pulse pressure correlated with a higher risk of AD in older adults [117]. In the Rotterdam study the presence of atherosclerotic plaques or wall thickening has been associated with dementia and its two major subtypes AD and VaD [109]. Based on that study, arterial stiffening has been suggested to be a key player in the pathogenesis of dementia [118]. One hypothesis to explain how hypertension is a risk factor for dementia, which follows from these studies, is that hypertension could lead to atherosclerosis and arterial stiffness which in turn promotes the development of dementia (figure 1).

Disentangling the possible causal relationships between hypertension, cerebrovascular disease and Alzheimer in human studies is complex, if not impossible, because vascular risk factors like hypertension may take years or decades to lead to marked cerebrovascular and cognitive symptoms, and because Alzheimer pathology is thought to be present years to decades before clinical symptoms appear. Therefore, animal models are needed to elucidate these mechanisms and translate them to preventive or therapeutic interventions. Poulet developed an animal model to resemble hypertension-related ‘Alzheimer-like pathology’ [119, 120]. These mice were subjected to high blood pressure, and this resulted in accumulation of amyloid aggregates [119, 120]. Hypertension
was induced via a coarctation of the aortic arch between the two carotid arteries, causing changes in the CBF, leakage of the blood-brain barrier and neurodegenerative changes [119, 120]. However, there may also be a reverse association between AD and hypertension. To investigate the impact of Aβ on blood pressure, in spontaneously hypotensive Sprague-Dawley rats (mean arterial blood pressure < 100 mmHg) the intra-arterial infusion of Aβ increased the mean arterial blood pressure compared to vehicle distilled water infusion [121]. This finding suggests that Aβ may be able to induce hypertension, possibly through direct systemic vascular effects, without parenchymal Aβ deposition and before dementia onset [121]. Taken together, animal studies suggest that Aβ may be responsible for high blood pressure as well as for cerebrovascular impairment. Furthermore, also neurodegeneration is due to energetic deficiency, and can be explained by Aβ-induced cerebrovascular impairment, by e.g. impairing glucose transport in hippocampal and cortical neurons.[122] Hypothetically, these blood pressure and vascular effects of Aβ, which predate cognitive effects, may interact with common vascular disease (e.g. essential hypertension), potentially further elevating blood pressure levels, and synergistically induce cerebrovascular lesions.

**Hypertension and VaD**

Whereas the link between hypertension and VaD, through the well-established relationship between hypertension and stroke, may seem self-evident, it is often overlooked that in many cases of VaD there is no clear history of stroke. To be more precise, cortical stroke, which is often symptomatic and therefore more easily recognized, is not the most prevalent cause of VaD. VaD is most often caused by lacunar infarcts (e.g. multi-infarct dementia) and/or severe white matter disease, both related to small vessel disease, and these are frequently clinically unrecognized as acute stroke. As an illustration of this often clinically silent course, lacunar infarcts and white-matter disease are frequently found in studies in elderly subjects without known cognitive disorders [42, 123]. Depending on the location of these vascular lesions, their extent, and the patient’s cognitive reserve, these silent lesions can however have sufficient impact to cause VCI or VaD (figure 1).

**Can stroke cause VaD and AD?**

To strengthen the overlap and the connections between AD and VaD, accumulation of amyloid precursor protein and Aβ 1-42, hallmarks of AD, has been demonstrated in patients with multi-infarct dementia, which is the most prevalent form of VaD [124-126]. Also, animal studies using models of cerebral ischemia indicated a relationship between the amyloid precursor protein and
General introduction

Chapter 1

cerebral ischemia [124, 125, 127]. A highly sensitive fluorescent RT-PCR assay revealed a significant increase in the peripheral blood expression of amyloid precursor protein mRNA levels among patients who suffered from recent stroke [128]. A correlation between the density of cortical microinfarcts (CMIs) and the degree of cerebral amyloid angiopathy (CAA) was found in a postmortem analysis of human brains [129]. Although CAA may occur unrelated to AD, this example serves to explain that AD patients with CAA may present with stroke and cerebrovascular comorbidity.

Endothelin-induced ischemia mimicking small lacunar infarcts in the APP23 AD mouse model mice increased AD-like pathology and inflammatory markers of AD in the cortex and hippocampus of these transgenic mice [40]. In another murine study Garcia-Alloza et al. demonstrated that stroke accelerates amyloid deposition via interference with amyloid clearance pathways [130]. Garcia-Alloza et al. examined this association by using a transgenic AD mouse model (APP/PS1) subjected to microstrokes in the middle cerebral artery territory, an experimental stroke model using Rose bengal dye [130]. A fast increase in amyloid plaque burden and CAA was measured in the region surrounding the infarction [130]. As discussed in that paper, these changes were transient—and this may explain why these authors did not find amyloid plaques in post-infarcct brain tissue in humans in earlier work.

An association between cerebral hypoperfusion, caused by CAA, [131, 132] and CMIs was demonstrated by Okamoto et al. [129]. Chronic cerebral hypoperfusion due to bilateral common carotid artery stenosis in a CAA mouse model showed that the deposition of Aβ in leptomeningeal vessels was accelerated in combination with the development of microinfarcts [129]. Notably, in a rat model of AD and cerebral ischemia the accumulation of amyloid increased the infarct size, neuroinflammation and also cognitive deficits in these rats [133].

**Hemodynamic changes in VaD and AD**

A meta-analysis of transcranial Doppler studies has shown that both AD and VaD patients have evident changes in cerebrovascular hemodynamics (mostly reduced CBF and increased cerebrovascular resistance), albeit much more pronounced in VaD patients [134]. A study in transgenic mice overexpressing APP has demonstrated the impact of Aβ on cerebrovascular regulation [135]. Using quantitative autoradiography, resting CBF was shown to be reduced in the cerebral cortex and in the hippocampus [135]. These APP overexpressing mice also showed a disturbance in cerebrovascular autoregulation, as they were incapable to maintain a stable CBF during moderate hypotension or hypertension [136]. Whether this observation can be translated to human AD remains uncertain [137].
Figure 1 Proposed connections between risk factors, dementia and possible therapies affecting the major vascular risk factors for Alzheimer’s disease (AD) and vascular dementia (VaD), hypertension and stroke. Hypertension and stroke are major risk factors for dementia in particular AD and VaD, while hypertension itself is also a risk factor for stroke. Despite all research and drug development efforts, no curative pharmacological therapies are available for dementia and also no definitive treatments are attainable for VaD. In human and animal studies long-chain omega-3 polyunsaturated fatty acids (LC-n3-FA) and the manipulation of factors involved in the renin-angiotensin system (RAS) have shown to be beneficial to lower the risk of developing dementia like AD and VaD.

Preventive strategies – long-chain omega-3 polyunsaturated fatty acids and Renin-angiotensin system
Despite all research and drug development efforts, no curative pharmacological therapy are available for dementia and also no definitive treatments are attainable for VaD [5]. Therefore, in order to reduce the huge burden of disease, development of preventive strategies is urgent. Considering that vascular disorders contribute importantly to dementia as described above, such preventive strategies could consist of pharmaceutical interventions aimed at vascular dysfunctions (for example, treatment of hypertension and hyperlipidemia). In addition, current studies focus more and more on lifestyle. As already mentioned above, there is growing awareness that lifestyle components, e.g. diet and exercise, can influence modifiable risk factors such as hypertension, type 2 diabetes, and obesity. These modifiable risk factors affect the vascular system, thereby influencing the risk for
dementia, including AD [49, 138-141]. Modifying these risk factors via a change in lifestyle may potentially delay the onset of dementia and lead to a decrease in prevalence and public health burden of dementia [44, 141]. Aarsland et al. demonstrated in a systematic review that physical exercise may prevent the development of VaD [142]. Ravaglia et al. found in a population-based cohort study that physical activity is associated with a lower risk of VaD but not of AD [143]. In the review of Dichgans et al. the lifestyle risk factors for VaD, AD, Dementia (unspecified) and cognitive impairment are nicely summarized [144]. Based on epidemiological studies, they show that VaD and AD share common lifestyle risk factors such as smoking, decreased physical activity and obesity [144]. Furthermore, they also state from the existing epidemiological studies that insufficient evidence for diet as a potential risk factor for VaD exists, while concerning AD an improved dietary behavior was associated with a lower risk of cognitive decline [144].

Of the many important lifestyle factors, in this review we focus on (the supplementation of) long-chain omega-3 polyunsaturated fatty acids (LC-n3-FA) and its impact on cognition and the development of dementia. In addition, we also concentrate on the impact of these LC-n3-FA on risk factors for VaD and AD as hypertension and stroke to show that diet could have an impact on the development of both VaD and AD.

In a randomized, double-blind, placebo-controlled trial van de Rest et al. no effect of supplementation with eicosapentaenoic acid (EPA) combined with docosahexaenoic acid (DHA) for 26 weeks on mental well-being in independently living older individuals could be detected [145]. In another randomized, double-blind, placebo-controlled clinical trial Freund-Levi et al. the administration of LC-n3-FA in patients with mild to moderate AD did not slow down cognitive decline [146]. However, in a small subgroup of patients with very mild AD this administration led to beneficial effects [146]. A twenty-four week supplementation with 900 mg/d DHA led to an improved learning and memory function in age-related cognitive decline [147].

Many of these clinical trials had a relatively short duration of supplementation of LC-n3-FA or focused only on moderate or advanced AD patients. A systematic review with a meta-analysis in animal models of AD focusing on the effects of long-term LC-n3-FA supplementation on cognitive impairment, amyloid-β pathology, and neuronal loss revealed reduced Aβ burden, improved cognitive function, and decreased neuronal loss [148]. A very recent, double-blind randomized interventional study has shown that the intake of LC-n3-FA in healthy older adults significantly increased executive functions [149]. Furthermore, Witte et al. demonstrated that this intake had also beneficial effects on microstructural integrity and gray matter volume in frontal, temporal, parietal, and limbic areas, and on carotid intima media thickness and diastolic blood pressure [149].
Long-chain omega-3 polyunsaturated fatty acids and blood pressure
As already mentioned in the previous paragraph, Witte et al. were able to show that already in healthy older adults the LC-n3-FA supplementation led to a significant decrease in diastolic blood pressure [149]. In addition, a meta-analysis of 31 placebo-controlled trials in 1356 subjects revealed a dose-response effect of LC-n3-FA leading to a decrease of blood pressure [150]. This beneficial blood pressure lowering effect was even most prominent in hypertensive subjects and in patients with atherosclerosis or hypercholesterolemia [150]. This is in accordance with another study showing a reduction in blood pressure in untreated hypertensive patients with daily administration of LC-n3-FA [151]. However, the use of LC-n3-FA as antihypertensive treatment in humans needs to be analyzed in long-term studies. Long-term administration of DHA inhibits the development of hypertension in stroke-prone spontaneously hypertensive rats (SHRSP), a model for hypertension and stroke [152]. Notably, DHA also prolonged the life span of SHRSP [152]. But not only DHA may lower blood pressure. A meta-analysis of 25 randomized controlled trials revealed that an increased intake of dietary fibers also reduces blood pressure in hypertensive patients [153]. Reducing dietary salt intake may also be beneficial, as high salt intake increased the mortality rate, raised blood pressure and increased the number of cerebral aneurysms in SHRSP [154]. Also in humans, evidence supports the idea that reducing dietary salt intake can reduce hypertension-related disease [155]

Long-chain omega-3 polyunsaturated fatty acids and stroke
As mentioned above, fish consumption is recommended to reduce the risk of cardiovascular diseases [156]. Therefore it is likely to reduce the risk of developing stroke as well. A meta-analysis performed by He et al. revealed that already a very low fish consumption protects against the incidence of ischemic stroke [157]. De Goede et al. demonstrated a relationship between higher EPA-DHA and a lower stroke risk for women, while for men, these associations were not statistically significant [158]. Intraperitoneal pretreatment with DHA helped to reduce brain infarctions in Sprague-Dawley rats [159]. Moreover, Ozen et al. demonstrated a protective effect against cerebral ischemia in rats fed with a standard diet plus LC-n3-FA - including EPA and DHA - : these rats had a reduced number of apoptotic neurons in the prefrontal cortex [160]. This effect of DHA may be mediated by neuroprotectin 1. DHA is the precursor of neuroprotectin 1 (NPD1) and aspirin activates the synthesis of aspirin-triggered NPD1 (AT-NPD1) [161]. After the occlusion of the middle cerebral artery in Sprague-Dawley rats, inducing an experimental stroke, the administration of synthetic AT-NPD1 attenuated cerebral ischemic injury [161]. In women, intake of LC-n3-FA was associated with a lowered risk of total stroke, while dietary cholesterol was positively associated with risk of total stroke and cerebral infarction [162]. Increased intake of Mediterranean-style
diet was associated with a lowered risk of ischemic stroke, myocardial infarction, and vascular death [163]. The reduction in cardiovascular morbidity was most prominent for stroke, and was attributed to high intake of olive oil or nuts. But not only this diet originating around the Mediterranean Sea seems to have an influence on stroke incidence. A population based case-control study performed in southern Sweden demonstrated that stroke risk decreased with fat fish intake and especially in women the consumption of lean fish increased the stroke risk [164]. Additionally, also a deficient intake of alpha-linolenic acid, the plant-derived LC-n3-FA, may be a risk factor for the development of stroke [165]. This is in line with a murine study on rapeseed oil-enriched diets (rapeseed oil is a rich source of alpha-linolenic acid). Following MCA occlusion, the rapeseed oil fed groups demonstrated a decreased mortality rate, lowered levels of lipid peroxidation, and a reduced infarct size [166]. A meta-analysis by Arab et al. showed that individuals consuming daily three cups of either green or black tea had a 21% lower risk of stroke than those with a daily consumption of less than one cup of tea [167]. In summary, in human and animal studies LC-n3-FA have demonstrated to show beneficial effects on stroke and also on hypertension lowering the risk for dementia, see tables 1 and 2 and also figure 1.

Manipulation of the renin-angiotensin system – another possible therapy

The renin-angiotensin system is not only important in the cardiovascular system but also in the central nervous system. Angiotensin II binds to two main receptors, type 1 (AT$_1$) and type 2 (AT$_2$). AT$_2$ receptors are found in cerebral regions involved in control and learning of motor activity [168].

In Aβ injected mice the cognitive impairment was ameliorated by perindopril, a centrally active angiotensin-converting enzyme (ACE) inhibitor [169]. In these mice perindopril inhibited the cerebral, but not the peripheral ACE activity demonstrating a beneficial effect on AD as well as on hypertension [169]. In this animal study neither imidapril nor enalapril were able to reverse the cognitive impairment in spontaneous alteration and object recognition tests of the AD model mice [169]. In the SMART-MR study, patients treated with ARBs had less decline in CBF than patients treated with other hypertensive drugs (e.g. β-blockers, diuretics, calcium channel blockers, or ACE inhibitors) [170]. In line with this human study, in young AD transgenic mice models (APP23 mouse) treatment with the ARB olmesartan decreased oxidative stress in cerebral microvessels [171]. In the same study Takeda et al. used an acute mouse model induced by intracerebroventricular administration of Aβ 1-40, where pretreatment with a low dose of olmesartan completely prevented Aβ-induced vascular dysregulation and also partially reduced the impairment of hippocampal synaptic plasticity [171]. This preventive effect on cognitive decline by treatment with ARBs in AD was also demonstrated by another animal study performed by Tsukuda et al., using
intracerebroventricular injection of Aβ 1-40 in male ddY mice [172]. In these mice the ARB telmisartan decreased the cerebral Aβ 1-40 concentration and enhanced cerebral blood flow [172]. Furthermore, pretreatment with this ARB reduced the cognitive effects of Aβ 1-40 towards control level [172]. Using a similar model, Jing et al. demonstrated that the direct stimulation of the AT\textsubscript{2} receptor by a newly generated AT\textsubscript{2} receptor agonist, Compound 21 (C21), prevented cognitive decline [173]. In an observational study on cognitive function and systolic blood pressure reduction (OSCAR), in more than 60000 hypertensive patients, the specific use of the ARB eprosartan led to a reduction of blood pressure and also improved the cognitive function, revealing a positive correlation between cognitive decline or dementia and blood pressure levels [174].

ARBs do not only have the potential to reduce cognitive decline in animal and human studies (tables 1+2). In line with the described beneficial effect of ARBs on dementia, the Systolic Hypertension in the Elderly Program (SHEP) and the Systolic Hypertension in Europe (Syst-Eur) study demonstrated that antihypertensive treatment lowered the risk of developing stroke [34, 35]. In addition to the effect of an ARB in hypertension, an animal study showed a beneficial effect of ARBs on stroke [175]. After a MCA occlusion in apolipoprotein E-deficient mice – an atherosclerosis mouse model – treated with a cholesterol-high diet, the administration of the ARB telmisartan did not significantly decrease blood pressure, but decreased the ischemic area and also the atherosclerotic formation in the proximal aorta, and led to an improved cerebral blood flow in the penumbra of these treated mice [175]. In patients from the Kyoto heart study, who had coronary artery disease, the treatment with the ARB valsartan lowered the prevalence of stroke compared to the non-treated subjects [176]. Antihypertensive treatment with the ARB candesartan in elderly patients with isolated systolic hypertension, the relative risk of developing stroke was significantly reduced in comparison with other antihypertensive treatment, despite little difference in blood pressure reduction [177].

The potential mechanisms that could explain the link between hypertension and the development of AD need further investigation. Most human studies are epidemiological studies that show cross-sectional or longitudinal associations between hypertension and AD, but mechanistic studies or interventional studies are sparse (table 2). In animal studies, the focus of research has been mainly on the effect of low dosed blood pressure-lowering medication on Aβ accumulation and cognition. These preclinical studies indicate the possible potential for antihypertensive drugs against AD pathology and cognitive decline in AD patients [178]. Indeed, in a systematic review Shah et al. mentioned the need of large randomized clinical trials in order to explore the connection between blood pressure-lowering medications and dementia in humans [179].
### Table 1 Animal studies: Summary of intervention trials (long-chain omega-3 polyunsaturated fatty acids, LC-n3-FA, and the manipulation of factors involved in the renin-angiotensin system, RAS).

<table>
<thead>
<tr>
<th>Study</th>
<th>Specie</th>
<th>Model</th>
<th>Sex</th>
<th>Supplement+Dose</th>
<th>Route of administration</th>
<th>Start supplementation</th>
<th>Duration of supplementation</th>
<th>Results</th>
</tr>
</thead>
</table>
| [161] | Rat    | focal cerebral U/R | M    | 1. 333 µg/kg of AT-NPD1 in SS 2. 333 µg/kg of AT-NPD1 in Me 3. Vehicle | Intravenously | ? | 3h prior to ischemia | - AT-NPD1 reduces brain infarction size + brain edema  
- Improved tissue matrix  
- White matter protection |
| [152] | Rat    | SHRSP | ?    | 1. 1% DHA 2. 5% DHA 3. Control diet | Diet | 6 wk of age | 14 wk | - DHA has antihypertensive action  
- DHA ↑ acetylcholine levels  
- DHA ↑ life span |
| [166] | Mouse  | focal cerebral U/R | M    | 1. 5 % RSO 2. 10 % RSO 3. 20% RSO 4. 5% palm oil | Diet | ? | for 4 or 6 wk prior to ischemia | - Groups 2+3: decreased mortality rate, lowered levels of lipid peroxidation, and a reduced infarct size |
| [160] | Rat    | focal cerebral U/R | M    | 1. Control 2. Marinap capsules (EPA 38%, DHA 12%) | Gavage | 8-12 wk of age | 2 wk prior to ischemia | - ↓ in apoptotic neurons in prefrontal cortex in rat group given marinap capsules |
| [159] | Rat    | focal cerebral U/R | M    | 1. 100 nmol/kg 2. 500 nmol/kg 3. Vehicle | Intraperitoneally | ? | 1. Once 1 h prior to ischemia  
2. Once 3 d prior to ischemia  
3. Daily for 6 w prior to ischemia | - In all DHA injected animal groups: reduced infarct volume |
| [175] | Mouse  | ApoEKO + HCD + focal cerebral U/R | M    | 1. Telmisartan (0.3 mg/kg/d) | Osmotic minipump | 14 wk of age | for 2 wk prior to ischemia | - Telmisartan ↓ ischemic brain area, neurological deficit, reduction of cerebral blood flow in penumbra |
| [179] | Mouse  | Aβ 1-42 injection | M    | 1. C21 (1.0 µg/kg/d) 2. C21 (10.0 µg/kg/d) 3. Vehicle | Intraperitoneally | 10-12 wk of age | for 2 wk prior to MWM | - C21 prevented cognitive decline in this AD mouse model |
| [171] | Mouse  | APP23 mice | M    | 1. Omesartan (1.0 mg/kg/d) 2. Vehicle | Orally | 8 wk of age | Daily for 4-5 wk | - ARB olmesartan decreased oxidative stress in cerebral microvessels |
| [171] | Mouse  | Aβ 1-42 injection | M    | 1. Omesartan (0.5 mg/kg/d) 2. Omesartan (1.0 mg/kg/d) 3. Vehicle | Orally | 7-8 wk of age | for 4 wk prior to Aβ injection | - Omesartan completely prevented Aβ-induced vascular dysregulation and ↓ impairment of hippocampal synaptic plasticity |
| [172] | Mouse  | Aβ 1-42 injection | M    | 1. Telmisartan (0.35 mg/kg/d) 2. Telmisartan (0.35 mg/kg/d) + GW9662 3. Vehicle | Orally | 8 wk of age | for 4 wk prior to Aβ injection | - ↓ of Aβ 1-42 concentration by telmisartan  
- Telmisartan ↑ CBF  
- Telmisartan improved Aβ-induced cognitive decline |
| [178] | Mouse  | Tg2576 AD mouse model | M    | 1. Valsartan (10.0mg/kg/d) 2. Valsartan (40.0mg/kg/d) 3. Vehicle | Orally | 6 months of age | for 5 months | - Valsartan ↓ AD-type neuropathology  
- Valsartan ↓ development of Aβ-mediated cognitive deterioration |
| [169] | Mouse  | Aβ 1-42 injection | M    | 1. Perindopril (0.1, 0.3 or 1 mg/kg/d) 2. Imidapril (0.3, 1 or 3 mg/kg/d) 3. Enalapril (1, 3 or 10 mg/kg/d) 4. Vehicle | Orally | 5-6 wk of age | After Aβ 1-42-injection daily for five consecutive days | - Perindopril ameliorated the cognitive impairment in the AD model mice through inhibition of brain ACE activity |

**Used abbreviations:** DHA=docosahexaenoic acid, EPA=eicospentaenoic acid, AT-NPD1=aspirin-triggered NPD1, ME=Methyl ester, SS=Sodium salt, RSO=Rapeseed oil, ACE=angiotensin-converting enzyme, ARB=angiotensin receptor blocker, CBF=cerebral blood flow
Table 2 Human studies: Summary of intervention trials (long-chain omega-3 polyunsaturated fatty acids, LC-n3-FA, and the manipulation of factors involved in the renin-angiotensin system, RAS).

<table>
<thead>
<tr>
<th>Study</th>
<th>Age (y)</th>
<th>Subjects</th>
<th>Sex</th>
<th>N</th>
<th>Supplement + Dose / Intake</th>
<th>Route of administration</th>
<th>Duration of supplementation</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[165]</td>
<td>20-65</td>
<td>Healthy subjects</td>
<td>M+F</td>
<td>20069</td>
<td>- Habitual diet was assessed with a validated 178-item food frequency questionnaire</td>
<td>Orally</td>
<td>-</td>
<td>- Higher ALA intake ↓ risk of stroke</td>
</tr>
<tr>
<td>[158]</td>
<td>20-65</td>
<td>Healthy subjects</td>
<td>M+F</td>
<td>20069</td>
<td>- Intake of EPA-DHA was registered</td>
<td>Orally</td>
<td>-</td>
<td>- Women: Higher EPA-DHA and fish intake ↓ stroke risk</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[146]</td>
<td>74 ± 9</td>
<td>Patients with mild to moderate AD</td>
<td>M+F</td>
<td>204</td>
<td>1.17 g/d DHA 2.06 g/d EPA 3. Placebo</td>
<td>Orally</td>
<td>6 months</td>
<td>- No delay in rate of cognitive decline (MMSE/ cognitive portion of ADAS)</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>[163]</td>
<td>69 ± 1</td>
<td>Healthy subjects</td>
<td>M+F</td>
<td>2568</td>
<td>- Mediterranean-style diet registered by questionnaire</td>
<td>Orally</td>
<td>-</td>
<td>- Mediterranean-style diet ↓ ischemic stroke, myocardial infarction, and vascular death</td>
</tr>
<tr>
<td>[162]</td>
<td>49-83</td>
<td>Healthy subjects</td>
<td>F</td>
<td>34670</td>
<td>- Intake of LC-n3-FA was registered  - Intake of cholesterol was registered</td>
<td>Orally</td>
<td>-</td>
<td>- LC-n3-FA ↓ risk of stroke - Cholesterol ↑ risk of stroke</td>
</tr>
<tr>
<td>[164]</td>
<td>43-85</td>
<td>Healthy stroke patients</td>
<td>M+F</td>
<td>5191</td>
<td>- Fish intake was reported retrospectively</td>
<td>Orally</td>
<td>-</td>
<td>- Fat fish ↓ risk of ischemic stroke - Women: Lean fish ↑ risk of stroke</td>
</tr>
<tr>
<td>[145]</td>
<td>≥65</td>
<td>Nondepressed, older individuals</td>
<td>M+F</td>
<td>302</td>
<td>1.18 g/d EPA-DHA 2.04 g/d EPA-DHA 3. Placebo</td>
<td>Orally</td>
<td>26 wk</td>
<td>- Plasma concentrations of EPA-DHA ↑ in both test groups - No effect on mental wellbeing</td>
</tr>
<tr>
<td>[149]</td>
<td>50-75</td>
<td>Healthy subjects</td>
<td>F</td>
<td>30</td>
<td>1. (132 g EPA+ 0.88g DHA + 15mg vitamin E)/ d 2. Placebo</td>
<td>Orally</td>
<td>26 wk</td>
<td>- ↑ executive functions - Positive effects on white matter microstructure, gray matter volume and vascular markers - ↓ in diastolic blood pressure</td>
</tr>
<tr>
<td>[147]</td>
<td>≥65</td>
<td>Healthy subjects</td>
<td>M+F</td>
<td>485</td>
<td>1. 0.9 g/d DHA 2. Placebo</td>
<td>Orally</td>
<td>24 wk</td>
<td>- ↑ learning and memory function in ARCD</td>
</tr>
</tbody>
</table>

Used abbreviations: DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, MMSE=mini-mental state examination, ARCD=Age-related cognitive decline, ALA=alpha-linolenic acid, pCBF=parenchymal cerebral blood flow, ARB=angiotensin receptor blocker, CAD=coronary artery disease
Conclusions

Many studies showed that AD, VaD and stroke share hypertension as a common risk factor, while stroke in itself is also a risk factor for the development of AD or VaD (figure 1). Hypertension combined with aging decreases CBF results in changes in cerebrovascular structure and function [43, 44] leading to white matter changes, dysfunction of the blood-brain barrier, and to cognitive decline. Also MetS and its features induce structural and functional alterations in the cerebral vasculature, including resistance, stiffening, and remodeling. This seems to affect the brain in multiple ways. For instance, changes in the cerebral microcirculation can affect larger vessels and CBF. Changes in cerebral microcirculation also contribute to the development of cerebral small vessel disease possibly leading to WML, changes in gray matter microstructure, cerebral microbleeds, and brain cell (neuronal) atrophy. The affected brain tissue integrity might be a consequence of brain perfusion alterations, cerebral autoregulation disturbances, vascular reactivity abnormalities, or an altered production and secretion of peripheral adipokines being able to cross the BBB and to exert a wide range of effects on brain functioning. Eventually, all these factors together may lead to an increased risk of developing MCI, dementia, and stroke.

Notably, an increase in Aβ (regardless of the underlying cause) could lead to changes in cerebrovascular structure and function, and, if combined with for vascular disease could represent a vicious cycle of aggravated cerebrovascular disease, increasing Aβ pathology which in turn enhances vascular disease. As detailed, Aβ may increase blood pressure, decrease the amount of vascular endothelial cells, impair vascular function and decrease CBF, resulting in further neurodegeneration. Thus, elevated Aβ levels due to any cause could explain the association between hypertension, cerebrovascular disease and AD. Studying the causal relationships between hypertension, cerebrovascular disease and AD in humans is complex because of the long latency between pathological changes and clinical symptoms, which may span decades both in vascular disease and AD. Thus, properly designed animal studies that can be validly translated are needed to enlighten the underlying mechanisms and to translate them to preventive or therapeutic interventions. In murine studies, induced hypertension led to an increased accumulation of Aβ, neuroinflammation, changed CBF and disturbed BBB. Moreover, animal models for stroke showed also an increased AD-like pathology like enhanced amyloid deposition, neuroinflammation and cognitive deficits. If we consider hypertension and stroke as major risk factors for dementia, both for VaD and AD, there are two possible therapies for dementia. On the one hand, research has revealed that the supplementation with LC-n3-FA reduces
the risk of developing hypertension and stroke, thereby also lowering the risk of developing dementia (figure 1, tables 1+2). On the other hand, the manipulation of factors involved in the RAS like angiotensin II receptor blockers or ACE inhibitors showed beneficial effects in animal and human studies (figure 1, tables 1+2). As a sequel, future research needs to focus more on the role of other (both medical and lifestyle) treatment strategies to lower the risk factors for dementia. Such research could elucidate the importance of reducing the rate of hypertension and stroke. Until now, studies on stroke have concentrated on motor impairment. Therefore, more research is needed aiming at the cognitive and behavioral deficits induced by stroke. This would also help to understand the connection between stroke, cognitive decline and dementia and to elucidate the differences and overlap between VaD and AD.

**Thesis overview**

The main aims of this thesis are:

- To elucidate the underlying pathological processes of major vascular risk factors hypertension, apoE4, and stroke during very early development of neurodegenerative processes in AD using several mice models.
- To investigate whether vascular risk factors have the potential to accelerate the course of neurodegenerative processes in AD.
- To investigate the possible capacity of antihypertensives and specific multi-nutrient diets to serve as preventive or treatment against AD-like symptoms and vascular risk factors for AD.

In chapter 2 we elucidated the underlying vascular origin of neurodegenerative processes in AD in a 16-18 months old double transgenic AβPP\_swe/PS1\_dE9 (AβPP/PS1) mouse model for AD. Therefore, we investigated the relation between systolic blood pressure (SBP) cerebral blood flow (CBF) and vasoreactivity with brain structure and function in these aged AβPP/PS1 mice. These AβPP/PS1 mice are a murine model for familial very early-onset AD overexpressing Aβ. In familial forms of AD the production of Aβ peptides is also increased and is thought to be the primary driving force in non-familial (sporadic) AD pathogenesis [21]. Already the early stages of AD are characterized by the accumulation of Aβ affecting specific brain regions like the forebrain and medial temporal lobe structures like hippocampus, amygdala, and entorhinal cortex [23, 180, 181]. Almost all insoluble Aβ is accumulated within the neuritic plaques and cerebral vessel walls [182]. Using a multi-modal approach, including advanced MR neuroimaging tools, we investigated the relation between vascular parameters (systolic blood pressure
(SBP), cerebrovascular density, cerebral perfusion and vasoreactivity), brain tissue microstructure, neuroinflammation, neurogenesis, postsynaptic density, levels of fatty acids and sterols, and functional and structural connectivity, in relation to cognitive and behavioral alterations in this mouse model for the very early phase of AD.

The study presented in chapter 3 is directed on the impact of hypertension, the major vascular risk factor for AD, and its link with AD. The relation of hypertension with all (other) key markers of AD such as presence of Aβ plaques, neurofibrillary tangles, and brain atrophy, has been demonstrated [114]. Elevated angiotensin II (AngII) is an important cause of essential hypertension. AngII blocking agents can thus provide potential candidates to reduce AD risk factors in hypertensive patients. In this study, we studied the effect of 2 months AngII-infusion induced hypertension on SBP and CBF in 10 months-old wild-type (WT) C57bl/6j and AβPP/PS1 mice, and treatment with two different antihypertensives, eprosartan mesylate (EM) or hydrochlorothiazide, after 1 month of induced-hypertension. SBP was monitored each month via tail cuff plethysmography. CBF was measured with MR by flow-sensitive alternating inversion recovery. This study helps to gather more insight in the actions of hypertension and antihypertensives on brain processes and the vascular system, which may help to support the development of effective tailor-made blood pressure-lowering treatments for AD patients.

Since the research described in the previous chapter investigated the effect of AngII-induced hypertension on SBP and CBF in WT and AβPP/PS1 mice, chapter 4 reveals more understanding into the link between midlife hypertension, decreased cerebral hemodynamics and connectivity, and concomitant cognition in an AD mouse model. In this study we investigated if AngII-induced hypertension is able to induce AD pathology in WT mice, and if this AngII-induced hypertension could aggravate the AD pathology in the AβPP/PS1 mice. Another aim of this study was to study the effects of EM, an AngII receptor blocker, on cerebrovascular, metabolic, connectivity and cognitive changes in AD and WT mice. To determine the impact of hypertension and antihypertensive on Aβ, vascular density, and neuroinflammation immunohistochemical stainings were performed for Aβ, glucose transporter type 1 (GLUT-1), and ionized calcium-binding adapter molecule 1 (IBA-1).

Using the same AD-like transgenic mouse model as in the two latter chapters, in chapter 5 we aimed to study the efficacy of a specific multi-nutrient combination dietary approach against AD progression. Due to a lack of effective pharmacological interventions against AD, recent research has shifted towards the use of dietary interventions for the treatment and prevention of AD. Therefore, we investigated the effects of the specific nutrient combination Fortasyn, containing the
dietary precursors and cofactors for membrane synthesis, viz. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), UMP, choline, phospholipids, folic acid, vitamins B6, B12, C, E, and selenium, on spatial learning and memory in 11-month-old male AβPP/PS1 mice and WT mice. To this end, we analyzed the different search strategies to find the hidden platform in the Morris water maze using a parameter-based algorithm to assess the qualitative aspects of learning [183-187]. Our study examined whether Fortasyn may improve spatial learning of transgenic mice by facilitating the use of hippocampus-dependent search strategies.

Representing another strong genetic risk for sporadic AD [188], the apoE4 allele is associated with increased cardiovascular risk factors. Moreover, apoE4 carriers are clearly more susceptible to vascular brain damage (e.g., stroke, brain haemorrhage; [52-54] and they display aberrant functional connectivity [55]. ApoE4 has been related with increased toxicity, and loss of neuroprotective function in the pathogenesis of Alzheimer disease, dependent or independent from Aβ accumulation [29]. In chapter 6 another murine transgenic mouse model resembling this major vascular risk factor for AD, the ApoE4 mouse, was used to test if this aforementioned Fortasyn diet has the potential to reduce the occurrence of vulnerabilities for AD by simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms. To test this hypothesis, we evaluated the effect of the Fortasyn diet on cerebral and plasma levels of fatty acids and sterols, CBF, gray and white matter integrity, functional connectivity (FC) and post-synaptic density during aging in 12- and 18-month-old apoE4 mice.

Being another major risk factor for AD, ischemic strokes have been the target of many drug trials. Until now, thrombolysis and thrombectomy are the only used treatment options for ischemic stroke, but only a minority of patients benefit from these therapies due to the narrow therapeutic window and complexity of administration. Occlusion of the middle cerebral artery (MCAo) is among the most common causes of ischemic stroke in humans. Cerebral ischemia leads to brain lesions existing of an irreversibly injured core and an ischemic boundary zone, the penumbra, containing damaged but potentially salvageable tissue. As described in chapter 7, we applied transient occlusion (30 min) of the middle cerebral artery (tMCAo) in WT C57BL/6j mice in this cross-institutional study to investigate the neurorestorative efficacy of a dietary treatment (Fortasyn) as therapeutic approach counteracting neuroinflammation and impairment of cerebral (structural+functional) connectivity, CBF, and motor function. Male adult C57BL/6j mice were subjected to right tMCAo using the intraluminal filament model. At several time points after tMCAo, behavioral tests, and MRI and PET scanning were conducted to identify the impact of the multicomponent diet on
the elicited neuroinflammatory response, loss of cerebral connectivity, and the resulting impairment of motor function after experimental stroke. This study also integrates several multi-modal techniques revealing underlying pathological mechanisms of stroke revealing that multimodal neuroimaging combined with behavioral analysis is an excellent approach to assess brain function and motor function recovery after stroke. All study findings presented in thesis are summarized and are discussed in chapter 8 including suggestions for future studies.
Hypertension, cerebrovascular impairment, and cognitive decline in aged AβPP/PS1 mice

Abstract

Cardiovascular risk factors, especially hypertension, are also major risk factors for Alzheimer’s disease (AD). To elucidate the underlying vascular origin of neurodegenerative processes in AD, we investigated the relation between systolic blood pressure (SBP) cerebral blood flow (CBF) and vasoreactivity with brain structure and function in a 16-18 months old double transgenic AβPP<sub>swe</sub>/PS1<sub>de9</sub> (AβPP/PS1) mouse model for AD. These aging AβPP/PS1 mice showed an increased SBP linked to a declined regional CBF. Furthermore, using advanced MRI techniques, decline of functional and structural connectivity was revealed in the AD-like mice coupled to impaired cognition, increased locomotor activity, and anxiety-related behavior. Post mortem analyses demonstrated also increased neuroinflammation, and both decreased synaptogenesis and neurogenesis in the AβPP/PS1 mice. Additionally, deviant levels of fatty acids and sterols were present in the brain tissue of the AβPP/PS1 mice indicating maladapted brain fatty acid metabolism. Our findings suggest a link between increased SBP, decreased cerebral hemodynamics and connectivity in an AD mouse model during aging, leading to behavioral and cognitive impairments. As these results mirror the complex clinical symptomatology in the prodromal phase of AD, we suggest that this AD-like murine model could be used to investigate prevention and treatment strategies for early AD patients. Moreover, this study helps to develop more efficient therapies and diagnostics for this very early stage of AD.
Introduction

Dementia has become a public health problem in the aging world population [189, 190]. Alzheimer’s disease (AD) is responsible for most dementia cases followed by vascular dementia (VaD) [2, 3]. AD is characterized by brain atrophy and a gradual cognitive decline caused by neuronal death and loss of synapses in brain regions involved in learning and memory processes (e.g. temporal and frontal lobes) [182, 191]. Already the early stages of AD are characterized by the accumulation of Aβ affecting specific brain regions like the forebrain and medial temporal lobe structures like hippocampus, amygdala and entorhinal cortex [23, 180, 181]. Almost all insoluble Aβ is accumulated within the neuritic plaques and cerebral vessel walls [182]. Epidemiological and clinical studies revealed that AD and VaD share common vascular related risk factors such as hypertension, diabetes, hyperlipidemia, cerebrovascular disease, and arrhythmia [91-93, 95, 96, 192, 193]. Furthermore, it has been shown that vascular risk factors can influence the development of AD pathology (the vascular hypothesis) [194]. A recent study showed that hypertension affects the expression of tau as well as that of Aβ in an AD mouse model [195]. Further evidence supporting this link between hypertension and AD comes from clinical studies indicating that antihypertensives may reduce the development of AD [196, 197]. Other clinical studies suggested that resting cerebral blood flow (CBF) is decreased among hypertensive patients as opposed to that of normotensive subjects [198-202]. In elderly patients a decreased CBF has been shown to increase hippocampal and amygdalar atrophy [203, 204]. Therefore, an impaired or diminished cerebral perfusion may play a role in the development of AD via decreased delivery of oxygen in ischemia-sensitive brain regions like the hippocampus, inducing neurodegeneration and subsequent cognitive decline [205]. Notably, maladapted cerebral hemodynamics could lead to alteration in structural and functional connectivity in the brain represented by white matter lesions/hyperintensities. Impaired functional connectivity is also found in AD patients [206-208] and additionally, a link between the incidence of white matter lesions and the severity of the underlying AD pathology has been reported [209-211]. Many studies have shown the relationship between increased blood pressure in mid-life and cognitive decline or AD in late-life [48, 49] indicating the relevance of proper blood pressure maintenance. Therefore, to elucidate the underlying vascular origin of neurodegenerative processes in AD, we investigate the relation between vascular parameters (systolic blood pressure (SBP), cerebrovascular density, cerebral perfusion and vasoreactivity), functional and structural connectivity, and postmortem markers for neuroinflammation, neurogenesis, postsynaptic density, and levels of fatty acids and sterols and, in relation to cognition in the AβPP<sub>swe</sub>/PS1<sub>dE9</sub> (AβPP/PS1)
mouse model for AD. To develop and evaluate potential therapeutic targets for early stages in AD it is necessary to understand underlying (neurovascular) pathological processes in AD by studying well characterized early stage AD animal models.

Methods

Animals
The AβPP_{swe}/PS1_{dE9} (AβPP/PS1) founder mice were originally obtained from John Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) [212, 213] and a colony was first established at the University of Kuopio, Finland and thereafter a colony was bred at the Central Animal Facility at Radboud university medical center, The Netherlands. In short, mice were created by co-injection of chimeric mouse/human AβPP{swe} (mouse AβPP695 harbouring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transfected genes co-integrate and co-segregate as a single locus [213]. This line was originally maintained on a hybrid background by backcrossing to C3HeJ×C57BL/6J F1 mice (so-called pseudo F2 stage). For the present work, the breeder mice were backcrossed to C57BL/6J for fourteen generations to obtain mice for the current study. Before the actual experiments, animals were housed socially with a maximum of six animals per cage, with room temperature at 21°C, and 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 medical center, Nijmegen, The Netherlands, and the Animal Experiment Committee (called the Dierexperimentencommissie or DEC, RU-DEC 2011-058) of the Radboud university, Nijmegen, The Netherlands. The reporting of the animal experiments conforms with the ARRIVE guidelines [214].

For the present experiment, we used a total of 17 16-month-old males mice (ten WT littermates, and seven AβPP/PS1 mice); at eighteen months of age, all animals completed the experiments and were euthanized. All behavioural and MRI experiments were performed in the Preclinical Imaging Centre (PRIME) of the Radboud university medical center between 8 a.m. and 6 p.m..

Study design, randomization, blinding, and sample size
This was a single-institution, randomized, and double-blind controlled study (blinded for investigators and outcomes assessor) conducted at the preclinical imaging center (PRIME) of the Radboudumc (Nijmegen, the Netherlands). Per
experimental subgroup, the selection of animals was randomized. All animals completed the study, no animals were replaced. The sample size of minimal 6 mice per experimental group was chosen based on formal calculation of power as described in the approved protocols (RU-DEC 2011-058; WP: 110149) and using results from our previous study [40]. At 16, 17, and 18 months of age systolic blood pressure measurements were performed. At 17 months of age (WT: 16.8±0.03; AβPP/PS1: 16.6±0.04) all animals underwent behavioural testing in the open field and Morris water maze (MWM) and subsequently MRI measurements at 18 months of age (WT: 18.0±0.02; AβPP/PS1: 17.9±0.05). No animals were excluded from analysis. Post mortem immunohistochemical and biochemical procedures were performed on all brains.

**Tail-cuff plethysmography**

Mice were trained for two consecutive days in the warmed tail-cuff device (IITC Life Scientific Instruments, Woodland Hills, CA) to accustom them to the procedure. Starting from sixteen months of age and being repeated at seventeen and eighteen month of age, SBP was measured for two consecutive days monthly in trained, conscious and preheated mice using computerized tail-cuff plethysmography (IITC Life Scientific Instruments, Woodland Hills, CA), as previously described [41-44].

**Behavioral analyses**

The results of the behavioral analyses are placed in the supplementary material.

**Morris water maze**

The Morris water maze (MWM) is used to test spatial learning and memory in rodents. In short, at 17 months of age mice were placed in a circular pool, filled with opaque water, and were trained to find a submerged platform in the northeast (NE) quadrant of the pool by using distant visual cues.

*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 (s) to find the hidden platform was scored. Starting positions were south (S), north (N), east (E) and west (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):* At the start of the fifth day, mice performed a single probe trial (starting position: S), in which the platform was removed from the swimming pool. Mice were allowed to swim for 120 s and the time spent swimming and searching in the NE quadrant (where the platform had been located) was recorded.
Open field
Locomotion and explorative behavior were evaluated for 30 minutes in the open field at 17 months of age, as previously described [40, 45, 46]. Using EthoVision XT10.1 (Noldus, Wageningen, The Netherlands), locomotion was automatically recorded. The floor of the arena was divided into center, periphery, and corners. The frequency of entering these zones was measured automatically. In addition, exploration was manually scored (walking, sitting, wall leaning, jumping, rearing, grooming) and analyzed as described previously [47, 48].

MRI protocol
Following the behavioral examinations, at 18 months of age 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 operating on the Paravision 5.1 software platform (Bruker, Karlsruhe, Germany). We used a circular polarised volume resonator for radiofrequency transmission and an actively decoupled mouse brain quadrature surface coil with integrated combiner and preamplifier for receive (Bruker BioSpin). For the imaging procedure, the animals were anesthetized with isoflurane (3.5% for fast induction and 1.8% for maintenance) in a 2:1 oxygen and N$_2$O mixture (normal gas condition) or in a 3:0 oxygen and N$_2$O mixture (vasoconstriction), and placed in a stereotactic holder to prevent unwanted movement during the scanning. Body temperature was monitored with a rectal temperature probe and maintained at 37°C with heated airflow. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). First gradient echo T2*-weighted images covering the entire mouse brain were acquired in three directions for anatomical reference using previously described acquisition parameters [49].

Cerebral blood flow
MR perfusion data were measured with flow-sensitive alternating inversion recovery (FAIR) MRI techniques; from a series of echo planar imaging (EPI)-images in three different regions of interest (ROI) were evaluated [50, 51] in the cerebral cortex (all cortical regions above corpus callosum), hippocampus, and thalamus according to the Franklin - Paxinos atlas [52]. Twelve images with increasing inversion times (TIs; 40–3000 ms) were obtained for the T1 calculations, accounting to a total scan time of 12 min. Inversion recovery data from the imaging slice were acquired after selective inversion interleaved with nonselective inversion. To evaluate vasoreactivity, a 2:1 oxygen and N2O mixture (normal gas condition) and a 3:0 oxygen and N2O mixture (vasoconstriction) were used. To calculate regional CBF we used the protocol that is described in [49].
**Diffusion tensor imaging**

Diffusion of water was imaged as described previously [40, 45, 53]. In short, 22 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/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.

The diffusion tensor was estimated for every voxel using the PATCH algorithm [54]; mean water diffusivity (MD) and fractional anisotropy (FA) were derived from the tensor estimation following a protocol as described elsewhere [45]. MD and FA values were measured in several white matter (WM) and grey matter (GM) areas, manually selected based on an anatomical atlas [52].

**Resting state fMRI**

Subsequently after the acquisition of the anatomical reference images, resting state fMRI (rsfMRI) datasets were acquired using a single-shot spin-echo sequence with echo-planar readout (SE-EPI) sequence. Six hundred repetitions with a repetition time (TR) of 1.8 s and echo time of 16.9 ms were recorded for a total acquisition time of 18 min. 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). Mean and maximum displacement across the six degrees of freedom (along the x-, y-, and z-axes and on three rotation parameters pitch, roll, and yaw) were measured in each mouse. The mean SE-EPI images for each mouse were then used to generate a study-specific template through linear affine and nonlinear diffeomorphic transformation (ANTs. v1.9; http://picl.upenn.edu/ANTS/). Visual inspection of the normalised dataset was performed to screen for possible normalization biases. On the template, 12 areas were selected in left and right hemisphere. The selected regions were based on previous work concerning functional connectivity in mice [55], and included: left and right dorsal hippocampus, left and right ventral hippocampus, left and right auditory cortex, left and right motor cortex, left and right somatosensory cortex, and left and right visual cortex. All cortical ROIs were selected 1–2 voxels away from the edge of the cortex, to minimise the impact of susceptibility artefacts, which are more prominent in areas close to tissue interfaces (e.g., near the skull or near the ear canals). In-plane spatial smoothing (0.4 × 0.4 mm), linear detrending, and temporal high-pass filtering (cut-off at 0.01 Hz) were applied to compensate for small across-mouse misregistration and temporal low-frequency noise. FC group comparison between ROIs were calculated from the BOLD time series.
using total and partial correlation analyses implemented in FSLNets (FSLNets v0.3; www.fmrib.ox.ac.uk/fsl). Pearson’s correlation values were Fisher transformed to Z-scores for group comparisons and statistical analysis.

Post mortem brain tissue preparation

Directly following the MR measurements at 18 months of age, anaesthetized mice were sacrificed by transcardial perfusion with 0.1M phosphate buffered saline (PBS) at room temperature. The perfused brains were cut mid-sagittally and the right hemispheres were snap frozen in liquid nitrogen and stored at -80°C, before further biochemical processing. The left hemispheres were immersion fixed 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.

Immunohistochemistry

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.

For every staining, one complete series with 240 µm distance between the sections was used. Immunohistochemistry was performed using standard free-floating labeling procedures, as described previously, and was carried out on a shaker table at room temperature [40].

Postsynaptic density (PSD) was visualized with anti-PSD-95 antibody (1:2000; Abcam, catalog #ab18258, RRID:AB_444362) using one subseries of brain sections per animal. Donkey anti-rabbit biotin 1:1500 (Jackson ImmunoResearch, West Grove, PA, USA) was used as secondary antibody.

To visualize immature neurons we used an anti-doublecortin (DCX) antibody (1:4000; polyclonal goat anti-doublecortin (C18): sc-8066, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) with one subseries of brain sections per animal. Being a microtubule-associated protein, Doublecortin is exclusively found in somata and processes of migrating and differentiating neurons [56, 57]. Donkey-goat biotin 1:1500 (Jackson ImmunoResearch, West Grove, PA, USA) was used as secondary antibody.

To visualize and quantify the amount of macrophages and microglia, an anti-ionized calcium binding adapter molecule 1 (IBA-1) antibody (1:1500; polyclonal goat anti-IBA1 (C18); Abcam Inc., Cambridge, UK) was used with one subseries of brain sections per animal. Donkey-goat biotin 1:1500 (Jackson ImmunoResearch, West Grove, PA, USA) was used as secondary antibody.

The amount of glucose transporter type 1 (GLUT-1) was visualized using an anti-GLUT-1 antibody (1:2000; rabbit anti GLUT-1 transporter, Chemicon AB 1340, Chemicon International, Inc., Temecula, CA, USA). Donkey-rabbit biotin 1:1500
(Jackson ImmunoResearch, West Grove, PA, USA) was used as secondary antibody. Results of all immunohistochemical stainings for PSD-95, DCX, IBA-1, and GLUT-1 can be found in the supplementary material.

Quantification – PSD-95
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 [52] and quantified in five regions of the hippocampus: the inner molecular layer (IML), outer molecular layer (OML), cornu 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, and Bethesda, Maryland, USA). The contrast was manually enhanced, following the same procedure for all digitized images, and the amount of tissue stained was measured with a threshold-based approach.

Quantification – DCX, IBA-1 & GLUT-1
Brain sections between bregma -1.46 and -2.30 [52] were preselected for quantification. Quantification was done at a 5x magnification using an Axio Imager (A2, Zeiss Germany). ImageJ (National Institute of Health, Bethesda, MD, USA) was used to analyze the regions of interest: Cortex (IBA-1 & GLUT-1), hippocampus (DCX, IBA-1 & GLUT-1).

Biochemical analyses
Serum and brain sterol levels were measured by gas-chromatography-mass-spectrometry-selected-ion-monitoring (GC-MS-SIM) as described in detail previously [58]. The cerebellum of the right hemisphere was homogenized and sterols were extracted overnight by chloroform/methanol trimethylsilylation prior to GC-MS-SIM analysis [58]. Brain fatty acid analyses were performed with a part of the brain homogenate (olfactory bulb and part of frontal cortex), as described previously [45].

Statistics
For the statistical analysis, IBM SPSS 22 software (IBM Corporation, New York, NY, USA) was used. Multivariate ANOVA (Repeated measures ANOVA for the MWM and SBP data) with Bonferroni corrections was conducted with between-group-factors genotype to analyze possible differences in all the other parameters. Statistical significance was set at p ≤ 0.05, while a tendency was set at 0.05 < p < 0.08. All data are expressed as mean ± SEM.
Results

Body Weight
18-month-old AβPP/PS1 mice had the same body weight as their WT littermates before both MR scan sessions (Figure 1A, F(1,15)=2.7, p<0.124).

Systolic blood pressure
Using tail-cuff plethysmography to measure systolic blood pressure (SBP), AβPP/PS1 mice demonstrated an increased SBP compared to their WT littermates from 16 to 18 months of age (Figure 1B, F(1,15)=5.6, p<0.032). Both AβPP/PS1 and WT mice exhibited a decrease in SBP over time from 16 to 18 months of age (Figure 1B, F(2,30)=8.1, p<0.002).

MR measurements
Cerebral blood flow and vasoreactivity
To study the effect of genotype differences on cerebrovascular health, CBF (Figure 2A) and vasoreactivity (Figure 2B) were measured with FAIR ASL using normal (200 \( O_2 \): 100 \( N_2O \) mL/min) and vasoconstrictive (300 \( O_2 \): 0 \( N_2O \) mL/min) gas conditions in three regions of interest (ROI): cortex, hippocampus and thalamus. Under normal gas condition a decreased CBF was observed in the cortex (F(1,14)=4.2, p<0.060) and thalamus (F(1,14)=10.5, p<0.007) of AβPP/PS1 mice compared to...
their WT littermates. In addition, under vasoconstrictive condition our AD model mice had again a lower thalamic CBF \((F(1,14)=5.1, p<0.040)\) than the WT mice. Both AβPP/PS1 and WT mice showed an intact vasoreactivity in the cortex (AβPP/PS1, \(F(1,6)=6.2, p<0.048\); WT, \(F(1,8)=13.7, p<0.006\)) and thalamus (AβPP/PS1, \(F(1,6)=13.2, p<0.011\); WT, \(F(1,8)=35.4, p<0.001\)). In contrast, in the hippocampus only WT mice (\(F(1,8)=13.8, p<0.006\)) revealed an intact vasoreactivity, while AβPP/PS1 mice (\(F(1,6)=1.6, p<0.253\)) had an impaired vasoreactivity indicating an incapability to adapt to the vasoconstrictive gas condition.

**Diffusion tensor imaging**
Quantitative assessment of the diffusion tensor indices fractional anisotropy (FA); and mean diffusivity (MD) was performed for ROIs drawn in several white and gray matter regions to assess effects of AD-like pathology in 18-month-old AβPP/PS1 and WT mice (Figure 2C+D). AβPP/PS1 mice showed impaired white matter integrity as indicated by a decrease in FA in the visual cortex, not being present in their WT littermates (Figure 2C, \(F(1,9)=24.5, p<0.001\)). No genotype effect was present for the MD (Figure 2D).

**Resting state fMRI**
To analyze the impact of AD-like pathology on the functional connectivity (FC) patterns at 18 months of age, rsfMRI data were statistically analyzed based on total correlation (Figure 3A) and partial correlation (Figure 3B).

Total correlation analyses
AβPP/PS1 mice showed no differences in FC, as analyzed with total correlations compared to their WT littermates (Figure 3A).

Partial correlation analyses
In comparison with the total correlation analysis, partial correlation analysis accentuates the direct connectivity between two ROI, while regressing the temporal BOLD signal from all other ROI. Resulting connectivity was thresholded at \(|Z| > 1.0\). For the partial correlations we found significant genotype effects at 18 months of age (Figure 3B). AβPP/PS1 mice showed a disturbed FC between several brain regions. While revealing an increased FC between the left motor cortex to left auditory cortex (\(F(1,12)=5.5, p<0.037\)), AβPP/PS1 mice compared to their WT littermates demonstrated a decreased FC between left auditory cortex to left ventral hippocampus (\(F(1,12)=5.7, p<0.034\)) and left somatosensory cortex to left visual cortex (\(F(1,12)=6.3, p<0.028\)).
Figure 2 Genotype effects on the cortical, hippocampal and thalamic cerebral blood flow (A, CBF) and vasoreactivity (B) using normal (200 O\textsubscript{2} : 100 N\textsubscript{2}O mL/min) and vasoconstrictive (300 O\textsubscript{2} : 0 N\textsubscript{2}O mL/min) gas conditions in 18-month-old A\betaPP/PS1 and wild-type (WT) mice. Quantitative assessment of diffusion tensor derived indices was performed for several ROI (AUC= Auditory cortex, CC=Corpus callosum, F=Fornix, HC=Hippocampus, MC=Motor cortex, OT=Optic tract, SSC=Somatosensory cortex, VC=Visual cortex) drawn in white and gray matter to assess effects of AD-like pathology in 18-Month-old A\betaPP/PS1 and WT mice on fractional anisotropy (C) and mean diffusivity (D). (A) CBF was measured with flow-sensitive alternating inversion recovery MRI technique; from a series of echo planar imaging images. Under normal gas condition, A\betaPP/PS1 mice had a lower cortical (p<0.060) and thalamic (p<0.007) CBF than their WT littermates. Under vasoconstrictive gas conditions, A\betaPP/PS1 mice showed again a lower thalamic CBF (p<0.040) than their WT littermates. (B) Both A\betaPP/PS1 and WT mice showed an intact vasoreactivity in the cortex (A\betaPP/PS1, p<0.048; WT, p<0.006) and thalamus (A\betaPP/PS1, p<0.011; WT, p<0.001). In contrast, in the hippocampus only WT mice (p<0.006) revealed an intact vasoreactivity, while A\betaPP/PS1 mice (p<0.253) had an impaired vasoreactivity indicating an incapability to adapt to the vasoconstrictive gas condition. (C) A\betaPP/PS1 mice had a lower FA in the VC than WT mice indicating an impaired white matter integrity (p<0.001). (D) No genotype effect was found for the MD in all measured ROI.
Figure 3 Resting-state functional connectivity (FC) based on total (A) and partial (B) correlation analyses of 12 ROI in the brain of 18-Month-old AβPP/PS1 and WT mice. (A) For the overall correlations no significant genotype effects were detected in the dorsal (DHC) and ventral hippocampus (VHC), and auditory (AUC), motor (MC), somatosensory (SSC), and visual cortices (VC). (B) AβPP/PS1 mice showed a disturbed FC between several brain regions. Between the left motor cortex to left auditory cortex (p<0.037) AβPP/PS1 mice had a higher FC than their WT littermates, while showing a decreased FC between left auditory cortex to left ventral hippocampus (p<0.034) and left somatosensory cortex to left visual cortex (p<0.028).

**Fatty acids in brain tissue**

Fatty acid content was determined in frontal cortex (Supplementary Table 1). No genotype effects were found for the brain fatty acids palmitic acid, stearic acid, saturated fatty acid, oleic acid, and mono-unsaturated fatty acid. However, AβPP/PS1 mice showed increased arachidonic acid (F(1,15)=6.8, p<0.020), increased total omega-6 (F(1,15)=4.1, p<0.061), a decreased DHA (F(1,15)=9.6, p<0.008), a decreased overall omega-3 (F(1,15)=10.6, p<0.006), and a resulting decreased omega-3/omega-6 ratio (F(1,15)=8.6, p<0.011).

**Sterol levels**

Sterol levels were determined in the blood serum (Supplementary Table 2) and in the cerebellum of the brain (Supplementary Table 3).
Blood serum
No genotype effects were found in levels of cholestanol, lathosterol, campesterol, campestanol, stigmasterol, sitosterol, sitostanol, avenasterol, brassicasterol, lanosterol, desmosterol, dihydro-lanosterol, 24OH-cholesterol, 7αOH-cholesterol, 27OH-cholesterol, and global cholesterol.

Cerebellum
In brain tissue of 18-month-old AβPP/PS1 mice, levels of dihydro-lanosterol (F(1,15)=5.8, p<0.030) were higher than in the brain tissue of their WT littermates. No genotype effects were revealed for the sterol levels of cholestanol, lathosterol, campesterol, stigmasterin, sitosterol, lanosterol, desmosterol, 24OH-cholesterol, 27OH-cholesterol, and global cholesterol.

Table 1. Summary of all significant results.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood pressure</strong></td>
<td>↑ in AD mice</td>
</tr>
<tr>
<td><strong>CBF</strong></td>
<td>↓ in cortex of AD mice, ↓ in thalamus of AD mice</td>
</tr>
<tr>
<td><strong>Vasoconstriction</strong></td>
<td>↓ in thalamus of AD mice</td>
</tr>
<tr>
<td><strong>Vasoreactivity</strong></td>
<td>↓ in hippocampus of AD mice</td>
</tr>
<tr>
<td><strong>DTI</strong></td>
<td>↓ in visual cortex of AD mice</td>
</tr>
<tr>
<td><strong>Mean diffusivity</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Total correlations</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Partial correlations</strong></td>
<td>↑ between left motor cortex to left auditory cortex in AD mice, ↓ between left ventral hippocampus to left auditory cortex in AD mice, ↓ between left visual cortex to left somatosensory cortex in AD mice</td>
</tr>
<tr>
<td><strong>Acquisition</strong></td>
<td>↓ spatial learning in AD mice</td>
</tr>
<tr>
<td><strong>Distance</strong></td>
<td>↑ in AD mice</td>
</tr>
<tr>
<td><strong>Velocity</strong></td>
<td>↑ in AD mice</td>
</tr>
<tr>
<td><strong>Platform crossings</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Distance</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Velocity</strong></td>
<td></td>
</tr>
</tbody>
</table>
| **Open field** | (
| **Activity parameters** | ↑ Walking in AD mice, ↓ Sitting in AD mice, ↑ Leaning in AD mice |
| **Stationary time** | |
| **Time spent in center/ corners/ periphery** | ↑ time spent in corners than in center in AD+WT mice, ↑ time spent in periphery than in center in AD+WT mice, ↑ time spent in corners than in periphery in AD mice |
| **Velocity** | ↑ in AD mice |
| **HIC** | |
| **GLUT-1** | ↑ in AD mice |
| **IBA-1** | ↑ neuroinflammation in cortex of AD mouse |
| **DCX** | ↓ neurogenesis in AD mouse |
| **PSD-95** | ↓ in CA1 and OML in AD mouse |
| **Fatty acids** | ↑ ARA in AD mice, ↑ D-6 in AD mice, ↓ DHA in AD mice, ↓ D-3 in AD mice, ↓ ratio of D-3/D-6 in AD mice |
| **Sterol levels** | |
| **Blood** | |
| **Brain** | ↑ dihydro-lanosterol in AD mice |

Used abbreviations: AD (Alzheimer's disease), SBP (systolic blood pressure), CBF (cerebral blood flow), DTI (diffusion tensor imaging), FA (fractional anisotropy), MD (mean diffusivity), rsfMRI (resting state functional MRI), MWM (Morris water maze), GLUT-1 (glucose transporter-1), IBA-1 (ionized calcium-binding adapter molecule 1), DCX (doublecortin), PSD-95 (postsynaptic density 95), CA1 (cornus ammonis 1), OML (outer molecular layer of the dentate gyrus), ARA (arachidonic acid), DHA (docosahexaenoic acid).
Discussion

Hypertension is the most common cardiovascular risk factor [36, 59-65], and associated with all (other) key markers of AD such as presence of amyloid-β (Aβ) plaques, neurofibrillary tangles, and brain atrophy [64]. The increasing prevalence of hypertension due to the aging world population and growing prevalence of obesity could also increase the number of AD patients, since midlife hypertension almost doubles the risk of developing AD in later life [36, 66]. In agreement with this, the AβPP/PS1 AD-like model mice used in the present study, demonstrated an increased systolic blood pressure (SBP) and concomitant decreased regional CBF in the cortical and thalamic areas, decreased vasoreactivity of the circulation of the hippocampus, and impaired cognition. The WT and AβPP/PS1 mice showed a decrease in SBP over time, while the AβPP/PS1 mice always demonstrated a higher SBP than their WT littermates. This is in line with the AGES-Reykjavik study which has shown that midlife hypertension combined with a lower late-life BP is associated with a lowered total brain and gray matter volume [67]. Long-standing hypertension stimulates atherosclerosis and vascular remodeling leading to increases in wall thickness. Arterial stiffness and severe atherosclerosis can lead to an increase in pulse pressure [68]. An increased pulse pressure is correlated with a higher risk of AD in older adults [68]. In the Rotterdam study the presence of atherosclerotic plaques or wall thickening has been associated with dementia and its two major subtypes AD and vascular dementia [66]. In accordance to previous research, this AD-like mouse model suffers from early-impaired cerebrovascular autoregulation and CBF, but also shows abnormalities in the cortical microvasculature [69-72]. These results support the significant reduction in regional and global CBF as identified in studies on MCI and AD patients [73]. A lowered CBF is a common feature in the early phase of AD, which may possibly be caused by an accumulation of Aβ in the vessel walls or in close vicinity of the blood vessels [27, 74, 75]. Furthermore, the impaired vasoreactivity in our transgenic mice is also a clinical hallmark of AD [76]. Aβ has shown to directly enhance the vasoconstriction of the cerebral arteries and to stimulate selected constrictor responses, resulting in a reduced CBF [74]. In addition, deposition of Aβ in the cerebral microvessels promotes vascular pathology and dysfunction [77-79], as embodied by the impaired hippocampal vasoreactivity in the brains of the AβPP/PS1 mice. Hypertension may contribute to this remodeling through increase in smooth muscle tone and damage of endothelial cell function resulting in arterial wall thickening and microvascular rarefaction [80]. In line with our research, Toth et al. demonstrated that in aging mice hypertension could induce an impaired cerebrovascular autoregulation [81], cerebromicrovascular injury and neuroinflammation. In the present study we investigated brain diffusivity
with DTI as an imaging biomarker for white and gray matter integrity. FA is a
marker of the degree of myelination and fiber density of white matter, while
MD characterizes an inverse measure of the membrane density and is sensitive
to cellularity, edema, and necrosis in gray matter (GM) [82-84]. In addition to
an impaired cerebrovascular function, a reduced FA value of the visual cortex
was found in our AD model mice indicating a subtle change in microstructural
integrity. This impaired microstructural integrity could imply axonal degeneration
or demyelination [84] in the visual cortex. Nevertheless, no genotype effects were
found for MD, being considered to be more informative for gray matter regions
like the visual cortex. In our previous research using DTI in 12-month-old WT and
AβPP/PS1 mice as well, fiber tract volume reduction, loss of axonal neurofilaments,
and myelin breakdown in axonal bundles of several white matter regions (i.e.
body of the corpus callosum) were detected in AβPP/PS1 mice compared to WT
[85]. In this recent study minor genotype effect on FA and no genotype effects
on MD were found. One reason for these missing genotype effects could be that
already in normal aging significant white matter deterioration occurs due to
myelin degeneration [86]. Therefore, myelin degeneration in our 18 months WT
mice may mask partly the significant changes regarding structural connectivity
in APP/PS1 mice. Nevertheless, in line with the loss of structural connectivity,
a disturbed FC pattern in cortical and hippocampal brain regions was revealed
in the 18-month-old AβPP/PS1 mice. In support, a mouse model for cerebral
amyloidosis demonstrated a compromised FC affecting the sensory motor cortex
already in pre-plaque stage [87]. Our latter results are in accordance with clinical
studies, in which a decreased FC was also demonstrated in AD patients [29-31].
A limiting factor of the acquired FC results is that the rsfMRI measurements are
acquired in isoflurane anesthetized mice. Fortunately, using rsfMRI a general
structure of the functional networks transcending levels of consciousness was
detected in both preclinical and clinical studies [88-90]. Notably, under isoflurane
anaesthesia a bilateral cortical connectivity in several cortical regions was measured
[91, 92]. Nevertheless, also contradictory results were detected in isoflurane
anesthetized mice showing not the same level of bilateral connectivity [55, 93].
However, in our previous and recent studies using isoflurane as anaesthesia, we
confirmed the presence of networks in several well defined cortical and subcortical
brain regions in two different murine models for vascular risk factors for AD [94,
95]. In our previous work we confirmed that both FC and CBF are dependent on
isoflurane concentrations, and both FC and CBF decline with concentrations of
isoflurane >2.2%, but do not further decline below a concentration of 2.2% [90].
Therefore, using the low-dose isoflurane (~1.7%) in this recent experiment will
preserve the resting-state networks and will not interfere with the outcome of
this study, as all animals were kept under the same low isoflurane concentration.
The combination of impaired cerebral hemodynamics, disturbed structural and functional connectivity, may underlie the impaired spatial learning capability found in the MWM. Here, the AβPP/PS1 mice needed more time to learn the position of the hidden platform. Moreover, these transgenic mice demonstrated a larger swim distance and higher swim velocity indicating an increased usage of non-spatial search strategies instead of hippocampus-dependent search strategies to find the hidden platform in the MWM [96, 97]. The behavior of the APP/PS1 mouse has been well-characterized. Mirroring the cognitive impairment being present in early AD patients, this cognitive deficit is a well-known feature of this AD mouse model. While at 4 and 7 months of age no learning deficit could be determined in AβPP/PS1 mice [98, 99], these AD model mice tend to exhibit an impaired memory capacity at 8 months of age in our earlier studies [48] and a significantly impaired spatial memory at 12 months of age, as demonstrated in studies from Lalonde et al. [100]. In fear-conditioning tests Kilgore et al. showed at already six months of age an impaired contextual memory in the APP/PS1 mouse model [101]. However, Webster et al. performed recently a comprehensive behavioral analysis of a knock-in AβPP/PS1 mouse model [102] using four different age groups (7, 11, 15, and 24 months) in which cognitive deficits in spatial reference memory (radial arm water maze) appeared from 11 months of age and becoming apparent as the disease progressed and recognition memory (novel object recognition) was demonstrated just from 15 months of age [102]. In line with previous results from our lab on younger (8-/12-/15-month-old) AβPP/PS1 mice [40, 48], also our 18-month-old AβPP/PS1 mice exhibited an increased locomotor activity and anxiety-related behavior in the open field. Being linked to elevated anxiety levels, this heightened activity has been observed in many transgenic AD model mice [103-105]. This hyperactivity and anxiety-related behavior resembles restlessness and anxiety-related symptoms found in up to 71% of AD patients [106, 107]. The increased anxiety of our mice could reveal a possible pathological mechanism for the elevation in SBP. This phenotype of anxiety is an expression of a stressful chronic condition. Therefore, it is well known that mental stress is able per se to provoke an increase in blood pressure in mice [108, 109]. The influence of the overexpression of AβPP and PS1 on cognition is well-characterized in several AD mouse models, while the non-cognitive behavior has not been considered as systematically [110]. Pugh et al. demonstrated reduced spontaneous motor activity, disinhibition, heightened frequency and duration of feeding bouts, decreased body weight and, by 10 months, increased activity over a 24h period in both female and male AβPP/PS1 mice [110]. In addition, male mice also expressed a heightened aggression relative to WT controls [110]. In the postmortem part of the study we revealed a decreased postsynaptic density and less DCX+ cells in the hippocampus of AβPP/PS1 mice.
indicating respectively a lowered synaptogenesis and neurogenesis, which may be the cause of the profound cognitive impairment found in the MWM in this AD-like transgenic mouse model. Notably, in our earlier studies, AβPP/PS1 mice showed decreased neurogenesis combined with a lowered hippocampal CBF already at 12-month of age [40, 49]. Moreover, in these younger 12-month-old AβPP/PS1 mice Zerbi et al. also detected impaired structural connectivity in the corpus callosum, fimbria, and cortex measured also via DTI [49]. This decrease in hippocampal CBF was no longer observed in the 18-month-old AβPP/PS1 mice granting a possible pathological timeframe starting with an impaired hippocampal CBF provoking a decreased synapto- and neurogenesis resulting in an impaired cognition. In another study, Ermini et al. showed as well a decreased neurogenesis in younger (8 months of age) AβPP/PS1 and (5 months of age) AβPP23 mice [111]. In many transgenic mouse models of AD an increased neuroinflammation has been observed reporting modulation of cytokine levels, activation of microglia and in some cases an activation of the complement system [112]. In the early pre-plaque stages of transgenic AD mice and, to a much lesser extent, in old WT mice highly activated microglia have been found [113-117]. In addition, Aβ-plaques in AD are engulfed by activated microglia [117]. To measure neuroinflammation, we immunohistochemically stained the mouse brains with ionized calcium-binding adapter molecule 1 (IBA-1) being a marker for activated microglia [118-120]. Here, in our AD model mice more IBA+ cells were detected in cortical regions compared to their WT littermates. In accordance to the results of Minogue et al., in this mouse model after 14 months of age an age-associated dysregulation of microglial activation is coupled with an enhanced blood-brain barrier permeability [114]. Furthermore, Meadowcroft et al. demonstrated activated microglial cells surrounding Aβ plaques in the brain of AβPP/PS1 mice. In accordance to our previous study, 18-month-old AβPP/PS1 mice did not show any differences in hippocampal capillary density measured via immunohistochemical staining for GLUT-1 [121]. Notably, these mice exhibited the highest level of Aβ in combination with hippocampal atrophy [121]. While 8-month-old AβPP/PS1 mice demonstrated also an increased amount of Aβ deposition in the dentate gyrus, but did not show any differences in hippocampal atrophy [121]. Besides all other AD-like pathological changes in this AD mouse model, we also detected an increase in cerebral omega-6 fatty (n6) acid content (especially arachidonic acid, ARA) combined with a decrease in cerebral omega-3 (n3) fatty acid content (in particular docosahexaenoic acid, DHA) resulting in a pronounced decreased n3/n6-ratio, which is thought to elevate the risk for AD [122, 123]. In accordance to our results, Dutch drug-naïve patients with mild AD (Mini-Mental State Examination (MMSE) = 25.0) showed a decreased relative content of n3 fatty acids (including DHA) and an increased relative content of ARA in erythrocyte membranes compared to a group of Dutch healthy controls.
In accordance, ARA is a mediator of inflammatory pathways, and its metabolites are involved in the production of Aβ and in the pathogenesis of AD [125]. In our previous research using the same transgenic mouse model, a multi-nutrient diet was able to induce a pronounced shift in n3/n6 ratio in favor of the n3 fatty acids as compared to the control diet [40]. Here, the reduction of the relative n6 content was mainly caused by a decrease in ARA, while the higher n3 content was mainly caused by an increase in DHA. Fabelo et al. revealed that AβPP/PS1 mice show a more rapid lipid raft aging compared to WT, which is related to an increased saturation of phospholipids (higher saturated fatty acids content and decreased content of the long chain unsaturated fatty acids ARA and DHA) and increased sphingomyelin levels rather than to alterations in cholesterol [126]. Moreover, Fabelo et al. demonstrated that both levels of ARA and DHA were gradually lowered with age in AβPP/PS1 mice, with its maximal reduction of approximately 50% at 14 months of age [126]. In our recent study the aged 18-month-old AβPP/PS1 mice demonstrated a pronounced decreased n3/n6-ratio in the frontal cortices due to a decrease in DHA and increased ARA. This contradictory data on ARA levels may be explained by age and the brain area measured, as Fabelo et al. analyzed homogenized cortex of a total hemisphere. Notably, Perez et al. revealed that both male and female 6-month-old AβPP/PS1 mice show neither a change in ARA nor a decrease in DHA brain fatty acid content [127] indicating the pathological role of aging in disturbing cerebral membrane composition. Notably, a novel finding is the increased level of dihydro-lanosterol in our AD-model mice, being an intermediate in the cholesterol synthesis. Nevertheless, no genotype effects on cholesterol levels in blood and brain tissue were found. Decreased brain cholesterol levels were measured in our previous research using 15-month-old AβPP/PS1 mice compared to their WT littermates [48]. In addition, mice fed a high cholesterol-containing typical western diet showed a significant decreased regional cerebral blood volume compared to mice fed a standard control chow diet [48]. Notably, Park et al. showed an increased Aβ synthesis and senile Aβ plaque deposition only in female AD model (Tg2576) mice when using lovastatin (a blood-brain barrier crossing inhibitor of the cholesterol biosynthesis pathway) [128]. Dysregulation of the cerebral cholesterol homeostasis has been increasingly associated with chronic neurodegenerative disorders, including AD, Huntington’s disease, and Parkinson’s disease [129]. Furthermore, the E4 isoform of apolipoprotein E, being a cholesterol-carrying protein, is linked to an increased risk of developing AD [129]. In conclusion, in this study we detected an increased SBP, impairments in cerebral hemodynamics resulting in an impaired cognition and structural and functional connectivity, increased locomotor activity, and anxiety-related behavior in an AD-like mouse model. In accordance, already in 4.5 months old AβPP/PS1 mice Cifuentes et al. indicated that hypertension is able to accelerate the development
of Alzheimer disease-related structural and functional alterations, partially through cerebral vasculature impairment and reduced nitric oxide production [130]. Future research should also consider gender differences in these AβPP/PS1 mice. In detail, male mice develop Aβ plaques more rapidly than female mice, being saturated at nine months of age, while in female mice plaque accumulation does not reach its maximum in female mice until around 12 months of age [131]. Notably, using both male and female 4-, 12-, and 17-month-old AβPP/PS1 mice Wang et al. demonstrated that female AβPP/PS1 mice have a higher hippocampal Aβ40 and Aβ42 levels than male AβPP/PS1 mice at 4 months of age Aβ and female AβPP/PS1 mice had a heavier Aβ burden and higher plaque number compared to male mice of the same age, both at 12 and at 17 months of age [132]. Our results indicate that vascular impairment plays an important role in the very early stage of AD but whether it is a causative factor or just a contributor aggravating the disease progress, remains to be elucidated. Thus, future therapeutic approaches should focus on (cerebro)vascular impairment as a promising strategy for the prevention of AD. The present AD mouse model showing hypertension and cerebral circulation impairment could serve as translational tool for the development of treatments to inhibit neurodegenerative diseases like AD already in the prodromal phase of the disease.

Supplementary material

Results

Behavioural tests
Open field (OF)
We used the OF to measure the effect of the AD-like pathology on explorative and anxiety-related behavior. In the open field, locomotor activity (walk distance & walk velocity) and active exploration parameters (walking, sitting, wall leaning, rearing) and grooming were scored for 30 minutes. AβPP/PS1 mice seemed to be more active in the OF than their WT littermates. AβPP/PS1 mice walked more (Supplementary figure 1C, F(1,15)=4.0, p<0.063) and sat less (Supplementary figure 1C, F(1,15)=3.6, p<0.078) than their WT littermates. This resulted in a larger distance walked (Supplementary figure 1D, F(1,15)=3.5, p<0.080) and increased walk velocity (Supplementary figure 1F, F(1,15)=3.7, p<0.074) in our AD model mice than in their non-transgenic controls. AβPP/PS1 mice exhibited more explorative behaviour against the walls of the open field like wall leaning (Supplementary figure 1C, F(1,15)=5.5, p<0.033) than their WT littermates. Both AβPP/PS1 and WT mice spent more time in both corners (Supplementary figure 1E, F(1,30)=220.6, p<0.001) and periphery (Supplementary figure 1E, F(1,30)=285.0, p<0.001) than
in the center of the open field. Especially, the AβPP/PS1 mice (Supplementary figure 1E, F(1,12)=5.4, p<0.039) stayed longer in the corners than in the periphery indicating an increased anxiety in these transgenic mice.

Supplementary figure 1  Explorative and anxiety-related behavior, and locomotor activity (C-F) was measured in the open field (OF) in 17-month-old AβPP/PS1 and wild-type (WT) mice. (A) In the OF AβPP/PS1 mice walked more (p<0.063) and sat less (p<0.078) than their WT littermates. AβPP/PS1 mice exhibited more wall leaning (p<0.033) than their WT littermates. (B+D) An increased walk distance (p<0.080) and walk velocity (p<0.074) was found in our AD model mice. (C) Both AβPP/PS1 and WT mice spent more time in both corners (p<0.001) and periphery (p<0.001) than in the center of the open field. Notably, only AβPP/PS1 mice (p<0.039) stayed longer in the corners than in the periphery indicating an increased anxiety in these transgenic mice.

Morris water maze (MWM)
We used the MWM to investigate the impact of the AD-like pathology on spatial learning abilities. During the acquisition phase AβPP/PS1 mice demonstrated a longer latency time to reach the hidden platform than their WT littermates (Supplementary figure 2A, F(1,15)=8.6, p<0.011). Moreover, these AD mice swam a larger distance than their WT littermates to find the hidden platform.
during acquisition (*Supplementary figure 2B*, F(1,15)=5.5, p<0.033), while only at acquisition days 2 and 3 the AD model mice swam faster than their WT littermates (*Supplementary figure 2C*, Day 2: F(1,15)=3.6, p<0.078; Day 3: F(1,15)=7.1, p<0.018).

During the probe phase no significant genotype differences were found in the mean number of platform area crossings (*Supplementary figure 2F*, F(1,15)=1.1, p<0.310), the swim distance (*Supplementary figure 2D*, F(1,15)=0.2, p<0.677) and the swim velocity (*Supplementary figure 2E*, F(1,15)=0.2, p<0.684).

**Morris water maze**

**Acquisition**

**Probe**

**D**

**E**

**F**

*Supplementary figure 2* Morris water maze learning and memory in 18-month-old AβPP/PS1 and wild-type (WT) mice. (A) Determining the latency to find a hidden platform in the North-East (NE) quadrant in a 4-day acquisition phase, spatial learning was measured. (A) During all four acquisition days, AβPP/PS1 mice reached the hidden platform slower than their WT littersmates (p<0.011). (B) AβPP/PS1 mice showed a larger swim distance during all acquisition days compared to their WT littermates (p<0.033). (C) During the second (p<0.078) and third (p<0.018) acquisition day, all AβPP/PS1 mice swam faster than their WT littermates. (D+E+F) Spatial memory was tested in the probe phase measuring the frequency of crossing the former platform location. During the probe phase no significant genotype differences were found in the frequency crossing the platform area, the swim distance and swim velocity.
Immunohistochemical procedures

**PSD-95**
Postsynaptic density (PSD) was stained with a polyclonal antibody against PSD-95 reflecting synaptic function. PSD was measured in the visual (V1) and somatosensory cortex (SSC), and in several hippocampal subregions: cornu ammonis (CA)2, stratum lucidum (SL) of CA3, stratum radiatum (SR) of CA1, outer molecular layer (OML) of the dentate gyrus (DG), and inner molecular layer (IML) of DG (Supplementary figure 3A+B). In the V1, SSC, CA2, SL of CA3, and IML of DG no genotype effects were found. In the SR of the CA1 (F(1,14)=5.1, p<0.041) and OML of the DG (F(1,14)=16.5, p<0.002), AβPP/PS1 mice had less PSD95+area than their WT littermates revealing a decreased PSD in these transgenic mice.

**DCX**
Immature neurons were visualized in all mice with a polyclonal antibody against doublecortin (DCX). As a measure for neurogenesis, DCX+ cells were counted in the subgranular zone of the dentate gyrus (Supplementary figure 3C+D). In the hippocampus of AβPP/PS1 mice less DCX+ neurons were counted than in the hippocampus of their WT littermates (F(1,15)=7.3, p<0.017) indicating a decreased neurogenesis in these AD model mice.

**IBA-1**
Brain sections of all mice were immunohistochemically stained against ionized calcium-binding adapter molecule 1 (IBA-1). IBA-1 is a marker for active and resting microglia, but also a marker for phagocytes in general (monocytes and macrophages). Here, we measured the relative area of the total section area being stained for IBA-1 (for results see the supplementary material) and the number of IBA-1+ cells in the cortex, hippocampus, and thalamus (Supplementary figure 3E-G). Only for the number of IBA-1+ cells in the cortex a genotype effect was found. In detail, AβPP/PS1 mice had more IBA-1+cells in the cortex (Supplementary figure 3F, F(1,15)=6.5, p<0.023) than their WT littermates revealing an increased inflammation in these transgenic mice. No results were detected for the relative area of the total section area being stained for IBA-1 (Supplementary figure 3G).

**GLUT-1**
All brains were processed for immunohistochemical staining with glucose transporter-1 (GLUT-1, Supplementary figure 3H-J) antibody as well. In order to reveal the changes in total amount of GLUT-1, we measured the relative area of the total section area being stained for GLUT-1 in the cortex, hippocampus, and thalamus (Supplementary figure 3J). Furthermore, we also measured vascular
density represented by the number of GLUT-1+ blood vessels (Supplementary figure 3I). For both amount of GLUT-1 and vascular density, no genotype effects were found.

Supplementary figure 3 Immunohistochemical stainings for Postsynaptic Density-95 Protein (PSD-95, A+B), for doublecortin (DCX, C+D), for ionized calcium-binding adapter molecule 1 (IBA-1, E-G), and for glucose transporter-1 (GLUT-1, H-J) performed on brains of 18-Month-old AβPP/PS1 and wild-type (WT) mice. (A+B) PSD-95 was measured in the visual (V1, representative photo + magnified photo A, scale bar = 10µm) and somatosensory cortex (SSC), and in several hippocampal subregions: cornu ammonis (CA)2, stratum lucidum (SL) of CA3, stratum radiatum (SR) of CA1, outer molecular layer (OML) of the dentate gyrus (DG), and inner molecular layer (IML) of DG. In the SR of the CA1 (p<0.041) and OML of the DG (p<0.002), AβPP/PS1 mice had less PSD95+-area than their WT littermates revealing a decreased PSD in these transgenic mice. (C+D) In the hippocampus (DG, representative photo + magnified photo C, scale bar = 50µm), AβPP/PS1 mice had less DCX+ neurons than their WT littermates (p<0.017) indicating a decreased neurogenesis in these AD model mice. (E-G) IBA-1 is specifically expressed in activated microglia. Here, we measured the number of IBA-1+ cells (F) and the relative area of the total section area being stained for IBA-1 (G) in the cortex, hippocampus, and thalamus (representative photo + magnified photo E, scale bar = 200µm). AβPP/PS1 mice had more IBA-1+ cells in the cortex (p<0.023) than their WT littermates revealing an increased inflammation in these transgenic mice. In contrast, for the relative area of the total section area being stained for IBA-1 no genotype effects were detected. (H-J) We
measured the vascular density via the number of GLUT-1+ blood vessels (I) and the total amount of GLUT-1 being stained for GLUT-1 (J) in the cortex, hippocampus, and thalamus (representative photo + magnified photo C, scale bar = 200µm). For both, amount of GLUT-1 and vascular density no genotype effects were found.

Table 1 Relative brain fatty acid content represented in average ± SEM for each experimental group. (Used abbreviations: PA=Palmitic acid; SA=Stearic acid; SFA=Saturated fatty acid; OA=Oleic acid; MUFA=Mono-unsaturated fatty acid; AA=Arachidonic acid; Ω-6=Omega-6; DHA=Docosahexaenoic acid; Ω-3=Omega-3; Ω3/6=Omega 3/6); # P = 0.05 - 0.08, * P ≤ 0.05, ** P ≤ 0.05

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<th>SFA</th>
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<th>MUFA</th>
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<td>14.3±0.2 #</td>
<td>16.6±0.3 **</td>
<td>17.3±0.3 **</td>
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Table 2 Plasma sterol levels represented in average ± SEM for each experimental group.

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<th>Campestatin</th>
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<th>Brassicasterol</th>
<th>Dihydro-sterol</th>
<th>7αOH-Cholesterol</th>
<th>7βOH-Cholesterol</th>
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Table 3 Brain sterol levels represented in average ± SEM for each experimental group; * P ≤ 0.05

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Hypertension impairs cerebral blood flow in a mouse model for Alzheimer’s disease

Chapter 3 Hypertension impairs cerebral blood flow in a mouse model for Alzheimer’s disease

Abstract

Hypertension, a risk factor for Alzheimer’s disease (AD), is a treatable condition, which offers possibilities for prevention of AD. Elevated angiotensin II (AngII) is an important cause of essential hypertension. AngII has deleterious effects on endothelial function and cerebral blood flow (CBF), which may contribute to AD. AngII blocking agents can thus provide potential candidates to reduce AD risk factors in hypertensive patients. We studied the effect of 2 months induced hypertension (AngII-infusion via osmotic micropumps) on systolic blood pressure (SBP) and CBF in 10 months-old wild-type (WT) C57bl/6j and AβPP_{swd}/PS1_{de9} (AβPP/PS1) mice, and treatment with two different antihypertensives, 1) eprosartan mesylate (EM, 0.35mg/kg) or 2) hydrochlorotiazide (HCT, 7.5mg/kg), after 1 month of induced-hypertension. SBP was monitored twice each month via tail cuff plethysmography. CBF was measured with MR by flow-sensitive alternating inversion recovery. Chronic AngII-infusion induced an increase in SBP in both AβPP/PS1 and WT mice accompanied by a decrease in hippocampal and thalamic CBF only in the AβPP/PS1 mice. An additional difference between the AβPP/PS1 mice and WT mice was that SBP was much higher in AβPP/PS1 mice in both hypertensive and normotensive conditions. Moreover, both antihypertensives were less effective in reducing AngII-induced hypertension to normal levels in AβPP/PS1 mice, while being effective in WT mice. It can be concluded that AngII-induced elevated SBP results in impaired CBF and a decreased response to blood pressure lowering treatment in a transgenic model of AD. Our findings suggest a relation between midlife hypertension and decreased CBF in an AD mouse model, similar to the relation which has been found in AD patients. This translational mouse model could be used to investigate possible prevention and treatment strategies for AD.
Introduction

Dementia has become a global public health problem as a result of the rapidly aging world population [189, 276]. Alzheimer’s disease (AD) is responsible for most dementia cases, followed by vascular dementia as second most cause (VaD) [2, 3]. In epidemiological and clinical studies it has been identified that AD and VaD share common risk factors like hypertension, diabetes mellitus, hyperlipidemia, cerebrovascular disease, and arrhythmia [91-96, 277]. Hypertension is the most common cardiovascular risk factor [49, 108-115], and its relation with all (other) key markers of AD such as presence of amyloid-β (Aβ) plaques, neurofibrillary tangles, and brain atrophy, has been demonstrated [114].

Further evidence supporting this link between hypertension and AD comes from preclinical and clinical studies in which it is argued that antihypertensives may reduce the development of AD [196, 197]. Angiotensin receptor blockers (ARBs) [278, 279] and the calcium antagonist nitrendipine [280] have been associated specifically with a reduced risk of AD. In the last decade many studies with contradictory results have been performed.

To illustrate this point: no significant effect of active treatment on the incidence of dementia [281, 282] was found compared to placebo as a result of two randomized, placebo-controlled studies. These two studies were conducted by ‘Study on Cognition and Prognosis in the Elderly’ (abbreviated to SCOPE; candesartan/ hydrochlorothiazide vs placebo) and ‘Systolic Hypertension in the Elderly Program’ (SHEP; chlorthalidone/ atenolol/ reserpine vs placebo). Contrarily to the example above, two other randomized, placebo-controlled studies named ‘Systolic Hypertension in Europe’ (Syst-Eur) and ‘Perindopril Protection Against Recurrent Stroke Study’ (PROGRESS), showed beneficial effects on the incidence of dementia and cognitive decline [280, 283, 284]. The Syst-Eur study revealed that active treatment with nitrendipine, enalapril, and/or hydrochlorothiazide decreased the incidence of dementia [280, 283]. The PROGRESS study demonstrated a reduced cognitive decline, caused by treatment with angiotensin-converting enzyme inhibitors (ACEIs), resulting in a decrease in the incidence of stroke-related dementia. The use of diuretics did not have any impact [284].

It remains uncertain, however, which hypertension mechanisms increase the risk for AD and, on the other hand, which mechanisms of antihypertensive agents reduce the risk for AD. Elucidation of these mechanisms would provide much needed insight in the pathophysiology of AD and would boost the development of effective preventive strategies.
Long-standing hypertension promotes atherosclerosis, cerebrovascular remodeling, and increased cerebral vessel wall thickening. In a community-based study, increased wall thickness and associated increased pulse pressure were correlated with a higher risk of AD [117] and, moreover, in the Rotterdam study it was published that atherosclerotic plaques and vessel wall thickening were associated with dementia, including AD [109]. These findings suggest that cerebrovascular changes could be an important link between hypertension and AD.

Angiotensin II (AngII), a component of the renin-angiotensin system (RAS), is a highly plausible candidate for this mechanistic link between hypertension and AD [285], due to its involvement in BP regulation [286]. AngII causes vasoconstriction and increases BP by means of binding to AT1 and AT2 receptors [225, 286, 287]. When present during mid-life, chronically elevated levels of AngII are an important cause of hypertension which induce cerebral vascular dysfunction by promoting vascular remodeling and inflammation [286, 288]. This causes malfunctioning of the neurovascular unit [289, 290] and promotes the expression of AβPP and Aβ production [290]. Decreased cerebral blood flow (CBF) is usually present in the early phase of AD, possibly caused by an accumulation of Aβ in the vessel walls or in close vicinity of the bloodvessels [204, 247, 248]. Preclinical data show that cerebrovascular dysfunction is partly caused by AngII, and not merely by an increased BP [217]. Recent clinical trials and studies in animal models suggest that manipulation of the renin-angiotensin-system (RAS) could reduce the pathologies of AD and VaD [169, 170, 172, 174].

We hypothesize that AngII affects the neurovascular function by impairing the cerebral blood flow (CBF) and also decreasing the response to antihypertensive treatment in a transgenic model of AD. To investigate this hypothesis, we designed a translational animal model to investigate the possible link between AngII and AD. We used wild-type littermate mice and an AD mouse model to investigate the hypothesis that AngII can initiate AD related cerebrovascular changes. To differentiate between the effect of AngII and elevated systolic blood pressure (SBP) *per se*, we also investigated the intervention by using two different blood pressure lowering agents: 1) diuretic hydrochlorothiazide (HCT) and 2) AngII receptor antagonist eprosartan mesylate (EM). With this study we aimed at increasing knowledge about antihypertensive actions on the cardio- and cerebrovascular system in AD, possibly supporting the development of effective, tailor-made blood pressure lowering treatments for AD patients.
Materials and Methods

Animals
The AβPP<sub>swe</sub>/PS1<sub>dE9</sub> (AβPP/PS1) founder mice were originally obtained from John Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) [213, 291]. A colony was first established at the University of Kuopio, Finland, and, thereafter, a colony was bred at the Central Animal Facility of the Radboud University Medical Centre, The Netherlands. Mice were created by co-injection of chimeric mouse/human AβPPswe (mouse AβPP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transfected genes cointegrate and co-segregate as a single locus [213]. This line was originally maintained on a hybrid background by backcrossing to C3HeJ×C57BL/6J F1 mice (so-called pseudo F2 stage). Breeder mice were backcrossed to C57BL/6J for 14 generations to obtain mice for the current study at the Central Animal Facility of the Radboud university medical center, Nijmegen, The Netherlands. Before experiments were carried out, animals were socially housed with a maximum of six animals per cage, at a room temperature of 21°C, with an artificial 12:12h light source: dark cycle (lights on at 7 a.m.). After the micro-osmotic pump implantation and throughout the experiments, the mice were housed separately (see below) to maintain a correct supplementation of drinking water or water mixed with medication (see below). Food and water were available ad libitum. Experiments were performed in compliance with Dutch federal regulations for animal protection and were approved by the Veterinary Authority of the Radboud University Medical Centre, Nijmegen, The Netherlands.

For the experiment, we used a total of 67 ten-months-old male mice (34 WT littermates, and 33 AβPP/PS1 mice). After completing the experiments for this study, all animals were euthanized, at twelve months of age.

Study overview
The AβPP/PS1 and WT mice were divided in two main groups: the first group received induced hypertension (AngII infusion delivered by subcutaneously implanted micro-osmotic pumps) and the second group consisted of controls and was given saline infusion. Table 1 provides an overview of the study and number of animals per group. After one month, both animal groups on AngII or saline infusion were further divided into 3 subgroups: treatment with EM (angiotensin receptor type 1 blocker, ARB), HCT (diuretic), and standard drinking water as control. SBP was measured in all mice at the age of eleven months. MRI measurements were performed at mice at the age of twelve months.
Table 1 Summary of the study overview: both AβPP/PS1 and wild-type (WT) mice were divided into two main groups as soon as they reached the age of ten months: the first group was infused with angiotensin II (AngII), the second group with saline (Sal). This was applied by using implanted micro-osmotic pumps, containing a dose that was sufficient for 4 weeks. After one month (when mice were aged 11 months), a replacement micro-osmotic pump was implanted in all mice. Mice were then given eprosartan mesylate (EM), hydrochlorothiazide (HCT) simultaneously, or just plain water (CTR). Between the ages of 11 and 12 months, systolic blood pressure (SBP) was measured using computerized tail-cuff plethysmography. MRI measurements were performed on mice aged 12 months.

<table>
<thead>
<tr>
<th>Months of age</th>
<th>Techniques</th>
<th>WT (N=34)</th>
<th>AβPP/PS1 (N=33)</th>
</tr>
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<tr>
<td>10-11</td>
<td>1st &amp; 2nd micro-osmotic pump implantation</td>
<td>Sal N=19</td>
<td>AngII N=15</td>
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<td></td>
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<td></td>
<td>Sal N=17</td>
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<tr>
<td>11-12</td>
<td>Supplementation with EM/HCT</td>
<td>CTR N=6</td>
<td>EM N=8</td>
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<tr>
<td>11</td>
<td>SBP measurements</td>
<td>All groups N=19</td>
<td>All groups N=15</td>
</tr>
<tr>
<td>12</td>
<td>CBF measurements</td>
<td>All groups N=19</td>
<td>All groups N=15</td>
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Implantation of Minipumps & Tail-cuff Plethysmography
Mice at 10 months of age received AngII (Sigma-Aldrich, Missouri, USA) or sterile saline (Sigma-Aldrich, Missouri, USA) for a period of 28 days via a newly implanted micro-osmotic pump (ALZET, CA, USA, model 1004). This period was followed by the implantation of a replacement micro-osmotic pump which delivered the same solution as the first pump for another 28 days. Micro-osmotic pumps were either filled with AngII or sterile saline. The micro-osmotic pumps were implanted subcutaneously under 2% of isoflurane anesthesia. Concentrations and delivery rates of AngII (500 ng·kg⁻¹·min⁻¹) were adjusted for each single mouse weight to produce comparable levels of SBP elevation.

Mice were trained in the warmed tail-cuff plethysmography device (IITC Life Scientific Instruments, Woodland Hills, CA) for two consecutive days, in order to accustom them to the procedure. One week after the second micro-osmotic pump implantation, SBP was measured in trained, conscious and warmed mice. For this purpose, computerized tail-cuff plethysmography (IITC Life Scientific Instruments, Woodland Hills, CA), as previously described [216, 217, 292, 293], was used for two consecutive days.

Drug treatment
To avoid stress caused by repeated injections, drugs were administered orally via drinking water mimicking the human route of administration. Administration of these drugs was initiated immediately after the subcutaneous implantation of the second micro-osmotic pump.
To investigate the effects of AngII receptor antagonists, mice received a supplement of ARB, EM (Sigma-Aldrich, Missouri, USA), dissolved in drinking water for four weeks (0.35 mg·kg\(^{-1}\)·day\(^{-1}\)). To investigate diuretic effects, mice received HCT (Sigma-Aldrich, Missouri, USA), dissolved in drinking water for four weeks (7.5 mg·kg\(^{-1}\)·day\(^{-1}\)) (group-size: \(n = 4-8\)). EM was dissolved in a small amount of \(\text{C}_2\text{H}_6\text{O}\), while HCT was dissolved in \(\text{H}_2\text{O}\).

Plain drinking water was used as a control for each condition. All drinking solutions were freshly prepared twice a week and kept at room temperature to block precipitation from solution.

**MRI protocol**

MRI 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 and operating on Paravision 5.1 software platform (Bruker, Karlsruhe, Germany). We used a circular polarized volume resonator for signal transmission and an actively decoupled mouse brain quadrature surface coil with integrated combiner and preamplifier as receiver (Bruker BioSpin). As part of the imaging procedure, the mice were anaesthetized with isoflurane (3.5% for induction and 1.8% for maintenance) in a 2:1 oxygen and \(\text{N}_2\text{O}\) mixture and placed in a stereotactic holder to prevent unwanted movement during scanning. Body temperature was monitored with a rectal temperature probe and maintained at 37°C using heated airflow. Respiration of each animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). First gradient echo images were acquired using previously described image parameters [294]. MR perfusion data were acquired during resting conditions using established methods via flow-sensitive alternating inversion recovery (FAIR) technique [226, 295].

Regional perfusion was evaluated in the cerebral cortex (all cortical regions above the corpus callosum), hippocampus and thalamus, upon consultation of the mouse brain atlas of Franklin and Paxinos [296].

One representation of high-resolution voxel-wise analyzed CBF image is shown in Figure 2A. In order to calculate regional CBF, the same protocol was used as previously described [294].

**Statistics**

IBM SPSS Statistics 20 software was used (IBM Corporation, New York, NY, USA) for statistical analysis.

For the assessment of the effects of genotype, antihypertensive treatments, and interactions between genotype and treatment on SBP and cerebral perfusion,
SBP data for hypertensive and normotensive animals were analyzed separately. Univariate ANOVA was used for the SBP data and multivariate ANOVA for the CBF data (cortex, hippocampus and thalamus).

To unravel the impact of genotype, AngII-induced hypertension, and their interaction on SBP and CBF, SBP data were additionally analyzed separately for each single treatment (plain drinking water as control, EM and HCT) with univariate ANOVA for the SBP data and multivariate ANOVA for the CBF data (cortex, hippocampus and thalamus).

To demonstrate the meaning of AngII-induced hypertension, antihypertensive treatments and interrelations with SBP and CBF, SBP data were analyzed separately for transgenic and non-transgenic mice. Univariate ANOVA was used for the SBP data and multivariate ANOVA for the CBF data (cortex, hippocampus and thalamus).

Whenever statistical analysis revealed a significant effect in the antihypertensive treatments, we used the post-hoc Tukey’s HSD test to focus further on the separate groups. After overall analysis of SBP and CBF, data that showed significant interactions were divided into groups based on their specific interacting factors. Statistical significance was set at $p \leq 0.05$. All values used are expressed as mean ± SEM.

Results

Systolic blood pressure

AngII and systolic blood pressure

In both genotypes, infusion with AngII effectively increased SBP (AβPP/PS1: 56.7±6.6 mmHg; WT: 48.9±7.4 mmHg), in comparison to mice that did not receive these antihypertensive drugs but merely plain drinking water (Figure 1: WT, F(1,9)=43.5, p=0.001; AβPP/PS1, F(1,7)=74.9, p=0.001).

Antihypertensive treatment and systolic blood pressure

In wild type animals, EM and HCT treatment lowered SBP under both normotensive (saline) (Figure 1: F(2,11)=12.4, p=0.002) and hypertensive (AngII) conditions (Figure 1: F(2,14)=19.3, p=0.001). Post hoc tests revealed that both EM (Figure 1: Sal, p=0.001; AngII, p=0.0001) and HCT (Figure 1: Sal, p=0.017; AngII, p=0.001) decreased SBP in saline-infused and AngII-infused WT mice. In AngII-induced hypertension, SBP was reduced by EM and HCT to near-normal values in WT mice as well (Figure 1: EM, F(1,7)=4.8, p=0.064; HCT, F(1,9)=4.6, p=0.061). In contrast, EM and HCT lowered SBP only in hypertensive AβPP/PS1 mice only but not in normotensive AβPP/PS1 mice (Figure 1: F(2,11)=24.5, p=0.001). The post hoc
test showed here that EM (Figure 2C: AngII, p=0.000) and HCT (Figure 2C: AngII, p=0.000) lowered SBP in hypertensive AβPP/PS1 mice only. It is remarkable that EM and HTC decreased the AngII-induced hypertension less efficiently in AβPP/PS1 mice, EM and HCT, whereas it decreased AngII-induced hypertension to normotensive values in their WT littermates that were only offered plain drinking water (Figure 1: EM, F(1,8)=7.8, p=0.023; HCT, F(1,7)=8.0, p=0.025). In addition, AβPP/PS1 mice on EM demonstrated an increased SBP (12.5±4.6 mmHg) in comparison to their WT littermates (F(1,15)=7.4, p=0.016).

Overall, the hypertensive effect of AngII (significant increase in SBP; different in EM of 16.1±4.6 mmHg, a difference of HCT 20.6±6.0 mmHg) persisted in AβPP/PS1 and WT mice despite treatment with antihypertensives: EM (F(1,15)=12.8, p=0.003) and HCT (F(1,16)=12.0, p=0.003).

Genotype and blood pressure
In the normotensive group (saline condition), AβPP/PS1 mice had a higher SBP (a difference of 7.3±2.9 mmHg) than their WT littermates (Figure 1: F(1,22)=6.5, p=0.018). This overall genotype effect was also observed in the hypertension group (AngII-infusion). SBP in AβPP/PS1 mice (a difference of 11.6±5.2 mmHg) was higher than the SBP of their WT littermates (Figure 1: F(1,25)=5.1, p=0.034). In summary, sal-infused and AngII-infused AβPP/PS1 mice had a higher SBP than their WT littermates. Remarkably, AngII increased SBP in both transgenic and non-transgenic animals whereas both antihypertensive treatments (EM and HCT) lowered SBP in all experimental groups. However, these antihypertensives could not merely reduce AngII-induced increased SBP in AβPP/PS1 mice within the normal range. In addition, EM decreased SBP in a less efficient manner in AβPP/PS1 mice than in their WT littermates.
Figure 1 Effect of angiotensin II (AngII)-infusion on systolic blood pressure (SBP) measurements of AβPP/PS1 and wild-type (WT) mice treated with two antihypertensives, eprosartan mesylate (EM) and hydrochlorothiazide (HCT), versus water as control. Without antihypertensive drugs, SBP was higher in AngII-infused AβPP/PS1 (j vs g) and WT (d vs a) mice than in saline (sal)-infused animals. In all AngII-infused animals, EM and HCT significantly lowered the SBP (WT: e/f vs d; AβPP/PS1: k/l vs j) while only WT mice under sal-infusion (b/c vs a) also demonstrated a decreased SBP after both antihypertensive treatments. In AβPP/PS1 mice, EM and HCT (EM: k vs h; HCT: l vs i) were not able to restore SBP to normal values, whereas in the WT littermates only a non-significant difference (EM: e vs b; HCT: f vs c) was detectable.

Cerebral blood flow
Neither AngII-induced hypertension nor antihypertensive treatment with EM and HCT had an effect on CBF in the cortex of both genotypes (Figure 2B).
In contrast, AngII-induced hypertensive AβPP/PS1 mice had a lower hippocampal CBF than their AngII-induced hypertensive WT littermates (Figure 2C: F(1,24)=4.4, p=0.046), while in normotensive animals this genotype effect was not visible. Hypertensive AβPP/PS1 mice also showed a trend towards decreased CBF in the thalamus in comparison to their hypertensive WT littermates (Figure 2D: F(1,24)=4.0, p=0.057).
In all ROI, no effects of antihypertensive treatment on CBF were found in WT and neither in AβPP/PS1 mice (Figures 2B-D).
Hypertension impairs cerebral blood flow in a mouse model for Alzheimer’s disease

Chapter 3

Figure 2 Cerebral blood flow (CBF) measurements of AβPP/PS1 and wild-type (WT) mice. CBF was measured by means of the flow-sensitive alternating inversion recovery (FAIR) MRI technique; from a series of echo planar imaging (EPI)-images. One representation of high-resolution voxel-wise analyzed CBF image is shown (A). The figure shows the cerebral perfusion data of AβPP/PS1 and WT mice infused with either saline (sal) or angiotensin II (AngII) and treated with either eprosartan mesylate (EM) or hydrochlorothiazide (HCT) compared to water as control. The CBF was measured in three different regions of interest (ROI): Cortex (B), hippocampus (C) and thalamus (D). No effect of AngII infusion or treatment with EM and HCT could be observed in the cortex (B). In contrast to controls (sal), under AngII infusion AβPP/PS1 mice had a lower hippocampal CBF than their WT littermates (C). Treatment with EM or HCT, however, did not influence hippocampal CBF either (C). On AngII, thalamic CBF was lowered in transgenic mice compared to their WT littermates, no decrease was present with sal infusion (D). This effect was only a statistical trend, however. In addition, no treatment effect on cerebral perfusion could be demonstrated in the thalamus in WT and AβPP/PS1 mice (D).

Discussion

This study assessed the relation between hypertension, antihypertensive treatment and cerebral blood flow (CBF) in a mouse model for Alzheimer’s disease (AD) and their wild-type littermates (WT), which were used as controls. We confirmed in part our hypothesis that AngII in interaction with Aβ negatively affects CBF by demonstrating a reduction in CBF and a decreased adaptive response to blood pressure lowering treatments in the hippocampus of the transgenic mouse model of AD compared to WT mice.

Several studies [95, 297] have demonstrated that midlife elevated BP compared to late-life elevated BP is associated with cognitive impairment and AD [298]. In this study, we showed that middle-aged 10 to 12 months old male AβPP/PS1 mice had a higher SBP compared to their WT littermates, both under control conditions (saline) and under AngII-infusion. This finding in our studies shows the same link between midlife hypertension and AD in mice as can be found in humans. Paris et al. showed that soluble Aβ peptides can enhance vasoconstriction induced by endothelin-1 (ET-1), which is an endogenous vasoconstrictor [299]. In contrast, Danielyan et al. reported that there was no genotype effect in 7-months old female AβPP/PS1 mice and WT mice [300]. In support of our hypothesis that AD pathology could contribute to higher blood pressure in our AD mouse model,
however, is the observation in spontaneously hypotensive Sprague-Dawley rats (mean arterial BP < 100 mmHg). In these rats, the intra-arterial infusion of Aβ increased the mean arterial BP as opposed to those rats who were given a vehicle distilled water infusion [121]. This finding suggests that Aβ may be able to increase SBP, possibly through direct systemic vascular effects, without parenchymal Aβ deposition and before dementia onset [121]. In addition, besides the parenchymal Aβ accumulation, this AβPP/PS1 mouse model has an early impairment in cerebrovascular autoregulation and CBF, but also in abnormalities in the cortical microvasculature [242-245]. A recent study showed that hypertension affects the expression of tau as well as that of Aβ in an AD mouse model (57). One limitation of our study is that all the features found in typical AD cannot be retrieved in AβPP/PS1 mice. These mice are characterized by early amyloid deposition in the brain parenchyma but not by neurofibrillary tangles and hyperphosphorylated tau. With this study we could therefore only look at the interaction between AngII and one feature found in AD. Hawkes et al. showed that particular regions of the brain demonstrate different vascular effects of perivascular Aβ [301], which could explain our findings that only the hippocampal and thalamic regions, and not the cortical regions, demonstrated a lowered CBF in hypertensive AD mice. Furthermore, we assessed whether antihypertensive treatment is able to reduce the effects of AngII-induced hypertension in both WT and AβPP/PS1 mice. As shown before, AngII increased SBP in non-transgenic mice, and we have now also reported this effect in an AD mouse model. Both antihypertensives, i.e. EM and HCT, lowered the SBP. However, EM was less effective in lowering BP in AβPP/PS1 mice than in their WT littermates. It can be suggested that this is a possible consequence of the vasotoxic effects of Aβ deposition, which is present in the cerebral vessel walls [302-304]. AD is associated with Aβ depositions in the wall of cerebral vessels or in close vicinity of the vasculature in the brain and was found in AβPP/PS1 mice as well [302, 304-306]. A study supporting this hypothesis was performed by Wang et al. showing that Valsartan treatment reduced AD neuropathology and attenuated the development of Aβ-mediated cognitive deterioration in Tg2576 mice, expressing double mutated human AβPP[307]. In accordance with the study of Wang et al., Danielyan and co-workers reported that intranasally administered ARB losartan, at a dosage below the systemic antihypertensive dose, decreased Aβ plaque burden in AβPP/PS1 mice [300]. In both studies, ARBs were used at sub-threshold dosages, being not effective for hypertension treatment in humans; and the mice were treated at least for two months with the ARB. In our study, a clinical dosage of EM was supplemented to the mice for one month only. It is possible that EM can also directly target Aβ by inhibiting its accumulation. Wang et al. have shown that another ARB, Valsartan, was capable of attenuating oligomerization of Aβ peptides into high-molecular-weight
Hypertension impairs cerebral blood flow in a mouse model for Alzheimer's disease

Chapter 3

oligomeric peptides [307]. Due to increased Aβ plaque production in AβPP/PS1 mice, which was already known beforehand, EM molecules may be bound to Aβ peptides. As a consequence, less free EM molecules will be available to block AngII activity mediated by its receptor, and, therefore, less EM is able to bind to the AngII receptor blocking its antihypertensive effect. This mechanism may explain the genotype effect of EM in our study in which EM decreased SBP in AβPP/PS1 mice in a less effective manner than in their WT littermates. In this respect, Savaskan et al. have also shown that the staining intensity of the AngII type 1 receptor was increased in the parietal cortex of AD patients but not in their aged controls, suggesting an enhanced brain RAS system activity in the disease process [308]. Furthermore, our results correspond with the human SCOPE and ProFESS studies in which AngII receptor blockers, candesartan and telmisartan, were less effective [309, 310]. As already mentioned above, HCT is also less efficient in decreasing SBP in AβPP/PS1 mice than in WT littermates.

In this study we also found that AngII-infused AβPP/PS1 mice had a lower CBF in the hippocampus and thalamus than their AngII-infused control WT littermates. This may indicate that the combination of AngII-induced hypertension and presence of highly expressed Aβ in the AD mouse brain [291] has the potential to influence cerebral hemodynamics. No effect of AngII on CBF was found in the middle-aged healthy WT mice. Aβ may inhibit the neurogenic vascular control due to its toxicity to basal forebrain cholinergic neurons [311-313] and this blocking effect might be exacerbated by AngII. In addition, Meyer and colleagues already observed cerebrovascular changes in young transgenic mice overexpressing AβPP before the appearance of Aβ plaques [245]. In line with our results, other human studies suggested that resting CBF is decreased among hypertensive patients as opposed to that in normotensive subjects [198-202]. These results show that vascular diseases like hypertension may affect the development of AD already at middle-age.

The second aim of this study was to investigate whether CBF is affected by specific AngII-increasing blood pressure mechanisms or other pathways. Therefore, we used the ARB, EM, to block AngII-receptor-interaction and also a diuretic, HCT, to lower SBP without influencing the actions of AngII. In our study neither of the two antihypertensives, EM and HCT, were able to restore the decrease in hippocampal and thalamic CBF in transgenic animals. In addition, no other treatment effects on CBF were found in this study. This indicates that AngII plays a crucial role in cerebral hemodynamics. Kume et al. showed that Telmisartan, another ARB like EM, was able to increase regional CBF in hypertensive patients, as opposed to those treated with amlodipine [314]. Recently, Toth et al. found an impaired cerebrovascular autoregulation in 24-month-old hypertensive mice [254]. We revealed that, after comparing the effect in both AD mice and WT littermates, the
cerebral perfusion is only impaired in hypertensive AD mice. Furthermore, the chronic infusion of AngII increased SBP but lowered CBF in these 12-month-old AD mice. An increased Aβ burden could possibly already disturb BP and CBF systems in younger mice, while Toth et al. showed that hypertension in aging mice could also impair cerebrovascular autoregulation [254]. Furthermore, Iadecola et al. have shown that Aβ acts directly on cerebral arteries to enhance vasoconstriction and to stimulate selected constrictor responses, resulting in a reduced CBF [247]. In our study, infusion of the vasoconstrictor AngII in Aβ overexpressing transgenic mice also decreased thalamic and hippocampal CBF, showing an exacerbating effect of AngII-induced hypertension on cerebral hemodynamics in Aβ expressing AD mice brains. In contrast, in AngII-infused WT mice no effect on CBF could be observed. Other studies demonstrated that hypertension also triggers neuroinflammation prior to Aβ deposition and that chronic hypertension could lead to an impaired blood-brain barrier permeability with deposition of Aβ in brain tissue [315, 316]. In conclusion, hypertension triggers different pathways to impair cerebrovascular function, also possibly resulting in cognitive impairment [317]. We showed that chronic infusion of AngII increased BP in both transgenic and WT mice, but only decreased the subsequent cerebral perfusion only in the hippocampus of the AD-like transgenic mouse model. This result supports the Honolulu Asia Aging Study which showed that untreated midlife blood pressure increases the risk for developing hippocampal atrophy indicating the hippocampal vulnerability to hypertension [318]. In addition, antihypertensive treatments acted differently in our AD-like transgenic mouse model resulting in a less effective blood-lowering response compared to that in WT animals. More specifically, the antihypertensives, EM and HCT, were not able to restore BP in AD mice in the same way as in their WT littermates. A scheme summarizing the potential effect of hypertension on neurovascular coupling and CBF in non-Alzheimer and Alzheimer mice is given in figure 3. Future studies should therefore include CBF response to stimulation, which could demonstrate the possible dysfunctional CBF regulation (neurovascular coupling) in hypertensive AβPP/PS1 transgenic mice. Gathering more insight in the actions of antihypertensives on brain processes and the vascular system could support the development of more efficient treatments for AD. Future studies are thus needed to elucidate the specific link between impaired cerebral perfusion and hypertension, in relation to functional and structural connectivity and cognitive impairment in AD.
Hypertension impairs cerebral blood flow in a mouse model for Alzheimer’s disease

Chapter 3

Figure 3 Schematic representation of the potential effect of hypertension on neurovascular coupling and cerebral blood flow (CBF) in non-Alzheimer versus Alzheimer mice. In non-Alzheimer mice, hypertension did not affect CBF. This is possibly due to a neurovascular coupling that is still intact. Furthermore, both Alzheimer and non-Alzheimer mice in this study were on antihypertensives: an angiotensin receptor type 1 blocker and a diuretic; both antihypertensives were less effective in Alzheimer mice. Inducing hypertension in our amyloid β (Aβ) overexpressing Alzheimer-like mice showed a higher increase in systolic blood pressure than that in non-Alzheimer mice. This induced hypertension may further stimulate aggregation of Aβ, which will affect the neurovasculature directly and increase hypertension even further, resulting in cerebrovascular impairment and a decreased hippocampal CBF.
Angiotensin II, hypertension, and angiotensin II receptor antagonism: Roles in the behavioural and brain pathology of a mouse model of Alzheimer’s disease

Abstract

Elevated angiotensin II (AngII) causes hypertension and contributes to Alzheimer’s disease (AD) by affecting cerebral blood flow (CBF). AngII receptor blockers (ARB) may provide candidates to reduce (vascular) risk factors for AD. We studied effects of 2 months AngII-induced hypertension on systolic blood pressure (SBP), and treatment with the ARB, eprosartan mesylate (EM), after 1 month of induced-hypertension in wild-type (WT) C57bl/6j and AβPP<sup>swe</sup>/PS1<sub>de9</sub> (AβPP/PS1 / AD) mice. AβPP/PS1 showed higher SBP than WT. Subsequent EM-treatment restored this elevated SBP in all mice. Functional connectivity (FC) was decreased in AngII-infused AD and WT mice, and only 12 months AD mice showed impaired CBF. Only AngII-infused AD mice exhibited decreased spatial learning in the Morris water maze. Altogether, AngII-induced hypertension exacerbated AD-like pathological changes such as impairment of CBF, FC, and cognition only in AD-model mice, but it also induced decreased FC in WT mice. However, we could not detect hypertension-induced overexpression of Aβ nor increased neuroinflammation. Our findings suggest a link between midlife hypertension, decreased cerebral hemodynamics and connectivity in an AD mouse model. EM treatment restored and beneficially affected CBF and connectivity. This model could be used to investigate prevention/ treatment strategies in early AD.
Introduction

Dementia has become a public health problem in the aging world population [189, 190]. Alzheimer’s disease (AD) and vascular dementia (VaD) are the number one and two disorders, together responsible for most cases of dementia [2, 3]. Epidemiological and clinical studies revealed that AD and VaD share common vascular related risk factors such as hypertension, diabetes, hyperlipidemia, cerebrovascular disease, and arrhythmia [91-96, 192, 193]. Of these, hypertension is the most common cardiovascular risk factor for dementia. In coming years, the expected increase in the prevalence of hypertension due to the aging world population could also raise the number of AD patients, since midlife and late-life hypertension almost double the risk of developing AD in later life [239, 240]. Whether this association between hypertension and AD is causal—and if so, through which mechanism—remains unknown. Angiotensin II (AngII), a component of the renin-angiotensin system (RAS), is a candidate for the hypothesised mechanistic link between hypertension and AD [285]. AngII has a prominent role in blood pressure (BP) regulation [286], causing vasoconstriction and elevated BP by binding to the AngII-receptor type1 (AGTR1) and type2 (AGTR2) [286, 287] and is considered to be a key mediator of the clinical syndrome of essential hypertension [319], through chronically induced vasoconstriction, increased aldosterone secretion, increased sympathetic tone, and cardiac and vascular hypertrophy [320, 321]. How could AngII be linked mechanistically to AD? When initiated during mid-life, chronically elevated levels of AngII, in addition to causing hypertension, may induce cerebral vascular dysfunction by promoting vascular remodelling and inflammation [286, 288], causing malfunctioning of the neurovascular unit [289, 290] and promoting expression of amyloid-β precursor protein (AβPP) and amyloid-β (Aβ) production [290]. Preclinical data show that cerebrovascular dysfunction is partly caused by AngII directly, and not via elevated BP alone [217]. A decreased cerebral blood flow (CBF) is an important and consistent early feature of AD, possibly as result of the accumulation of Aβ in or close to blood vessels [204, 247, 248]. Another hallmark of AD is a reduced glucose metabolism particularly in the temporal and parietal cortex [322, 323]. Being selectively highly expressed in the capillary endothelium of the brain, glucose transporter type 1 (GLUT-1) is responsible for transfer of glucose across the blood brain barrier [324]. A decreased hippocampal and cortical amount of GLUT-1 have been revealed in the brains of AD patients [325-327]. Another important component of AD pathology is neuroinflammation, manifested by activation of microglia [328-330]. Ionised calcium-binding adapter molecule 1 (IBA-1) is a marker for active and resting microglia [236-238]. GLUT-1 and IBA-1 are used in this study as markers for changes in metabolism, vascular density, and neuroinflammation. Further support for this mechanistic link
Angiotensin II, hypertension, and angiotensin II receptor antagonism

between AngII and AD comes from recent clinical trials and studies in animal models showing that AD pathology can be reduced via RAS [169, 170, 172, 174]. Angiotensin receptor blockers (ARBs) specifically inhibit the activation of AGTR1 and thus the actions of AngII [170]. Preclinical and clinical studies indicate that antihypertensives may reduce the development of AD [196, 197]. Specifically, ARBs have been associated with a reduced risk of AD [278, 279]. Findings from the Observational Study on Cognitive function And SBP Reduction (OSCAR) suggest that Eprosartan (ARB) is associated with preservation or improvement of cognitive performance [331]. A case control analysis showed that patients on ARBs and ACE-Is had a respectively 53% and 24% reduced risk of AD, compared to patients on other anti-hypertensives [278].

If indeed AngII is a causal factor linking hypertension to AD, we hypothesise 3 potential mechanisms: 1) AngII initiates Aβ accumulation through increased production and/or reduced clearance, leading to AD pathology; 2) Aβ accumulation is initiated by other factors, but AngII contributes to AD by enhancing cerebrovascular toxicity and inflammatory potency of Aβ, and/or by contributing to enhanced Aβ production or reduced Aβ clearance; 3) AngII leads to neurovascular dysfunction causing cognitive decline independent of AD pathology, but adding to the cognitive effects of AD or even mimicking AD in absence of underlying AD pathology. To investigate these hypotheses, we developed a translational animal model to study the following mechanisms: 1) Can AngII induce AD related pathology in wild-type (WT) mice; 2) Can AngII exacerbate AD pathology in the AβPP/PS1 mouse model for AD; 3) Can AngII induce cerebrovascular, metabolic/inflammatory and/or functional connectivity changes that mimic AD in WT animals or that add to the effects of AD pathology in AβPP/PS1 mice. In addition, to uncover the effects of AngII on AD, we aim to investigate whether treatment with the AngII receptor antagonist eprosartan mesylate (EM) is able to prevent or reduce AngII induced changes. The overall aim of this study is to elucidate the mechanisms through which hypertension and antihypertensive treatment influence AD. Translational characteristics of this study are the exposure to AngII at 10 months to simulate midlife hypertension, inclusion of WT animals in addition to transgenic animals to better translate to sporadic AD, and the use of MRI techniques with protocols similar to those used in clinical studies. We use flow-sensitive alternating inversion recovery arterial spin labeling (FAIR-ASL) to measure changes in CBF and DTI to reveal pathological adaptations in microstructural integrity of the white and grey matter. Several studies have revealed decreases in CBF in AD compared to healthy controls such as prefrontal cortex [332], temporo-occipital and parieto-occipital association cortices [333], and hippocampus [334]. Mean diffusivity (MD) is a measure of DTI which increases in presence of tissue damage [335]. Previous studies demonstrated an increased
MD in frontal [336, 337] and temporal lobes [336-341], but also in intercerebral tracts, including the superior superior longitudinal fasciculus [342] and the corpus callosum [337].

**Materials and Methods**

*Animals*

The AβPP<sup>swe</sup>/PS1<sub>de3</sub> (AβPP/PS1) founder mice were originally obtained from John Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) [291, 343] and a colony is bred at the Central Animal Facility at Radboud university medical center, The Netherlands. This line was originally maintained on a hybrid background by backcrossing to C3HeJ×C57BL/6J F1 mice and for the present work, the breeder mice were backcrossed to C57BL/6J for fifteen generations to obtain mice for the current study. Before the actual experiments, animals were housed socially with a maximum of six animals per cage, with room temperature at 21°C, and artificial 12:12h light:dark cycle (lights on at 7 a.m.). Throughout the experiments, the mice were housed separately after the micro-osmotic pump implantation to control intake of drinking water or water mixed with medication. Food and water were available *ad libitum*.

We used fifty-one ten months old male mice (twenty-nine WT littermates, and twenty-two AβPP/PS1 mice); at twelve months of age, all animals completed the experiments and were euthanised. All experiments were performed between 8 a.m. and 6 p.m..

*Study design, randomization, and blinding*

This was a randomised and controlled assessor-double-blind study. The experiments were performed according to the Dutch federal regulations for animal protection and approved (WP130084 + RU-DEC 2013-001) by the Veterinary Authority of Radboud university medical center, Nijmegen, The Netherlands, and the Animal Experiment Committee of the Radboud university, Nijmegen, The Netherlands. The reporting of the animal experiments are according to ARRIVE guidelines [214]. Per experimental subgroup, the selection of animals was randomised. There was no increased mortality (n=0) in this study. For the sample size calculation and ranked experimental outcomes per research question, see the supplementary materials. The AβPP/PS1 and WT mice were split in two main groups: 1) induced hypertension (using AngII infusion delivered by subcutaneously implanted micro-osmotic pumps) and 2) controls (saline infusion, further called Sal-infused animals/ mice). Supplemental table 1 provides an overview of the study design and number of animals per group. No animals were excluded from analysis. After one month, both animal groups on AngII or saline infusion were further
divided into 2 subgroups: treatment with EM (ARB) and standard drinking water as control. At age ten and eleven months, SBP was measured. Morris water maze (MWM) was exerted at eleven months. MRI measurements were performed at the age of twelve months. Post mortem immunohistochemical and biochemical procedures were performed.

**Micropumps implantation & tail-cuff plethysmography**
The implantation of the micro-osmotic pumps (ALZET, CA, USA, model 1004) to deliver either AngII (500 ng·kg$^{-1}$·min$^{-1}$, Sigma-Aldrich, Missouri, USA) or sterile saline (Sigma-Aldrich, Missouri, USA) and the tail-cuff plethysmography (IITC Life Scientific Instruments, Woodland Hills, CA) were performed, as previously described [344].

**Drug treatment**
To investigate the effects of an AngII receptor antagonist, mice were supplemented with EM (Sigma-Aldrich, Missouri, USA) dissolved in drinking water for four weeks (0.35 mg·kg$^{-1}$·day$^{-1}$), like previously described [344].

**Morris water maze**
Spatial learning and memory were tested in the MWM (for more detailed information, see [345]).

**MRI protocol**
MRI measurements were performed, as previously described [346]. For the imaging procedure, the animals were anesthetised with isoflurane (3.5% for fast induction and 1.7% for maintenance) in a 2:1 oxygen and N$_2$O mixture (normal gas condition) or in a 3:0 oxygen and N$_2$O mixture (vasoconstriction) with an air flow of 300 mL/min, and placed in a stereotactic holder to prevent movement during the scanning. Body temperature was monitored with a rectal temperature probe and maintained at 37°C with heated airflow. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). First gradient echo T2*-weighted images covering the entire mouse brain were acquired in three directions for anatomical reference using previously described image parameters [294].

**Cerebral blood flow**
MR perfusion data were measured with flow-sensitive alternating inversion recovery (FAIR) MRI techniques; from a series of echo planar imaging (EPI)-images in three different regions of interest (ROI) were evaluated [225, 226] in the cerebral cortex (all cortical regions above corpus callosum), hippocampus, thalamus
according to the Franklin - Paxinos atlas [227]. Regional CBF was calculated as described earlier [294].

**Diffusion tensor imaging**

Diffusion of water was imaged as described previously [229, 347]. Mean water diffusivity (MD) and fractional anisotropy (FA) were derived from the tensor estimation following a protocol as described elsewhere [229]. MD and FA values were measured in several white matter (WM) and grey matter (GM) areas, manually selected based on an anatomical atlas [227].

**Resting state fMRI**

Subsequently after the acquisition of the anatomical reference images, resting state fMRI (rsfMRI) datasets were acquired using a single-shot spin-echo sequence combined with echo-planar imaging (SE-EPI) sequence in anesthetised mice, as published previously [346]. FC group comparison between ROIs (left and right dorsal hippocampus, left and right ventral hippocampus, left and right auditory cortex, left and right motor cortex, left and right somatosensory cortex, and left and right visual cortex) were calculated from the BOLD time series using total correlation analyses implemented in FSLNets (FSLNets v0.3; www.fmrib.ox.ac.uk/fsl), using previously published protocol [346]. Pearson’s correlation values were Fisher transformed to Z-scores for group comparisons and statistical analysis.

**Immunohistochemical procedures**

After scanning, the mice were sacrificed by transcardial perfusion using 0.1M phosphate-buffered saline (PBS). Details on tissue preparation and sectioning are described earlier [346].

A triple fluorescent staining for glucose transporter-1 (GLUT-1), ionised calcium-binding adapter molecule 1 (IBA-1), and amyloid-β (Aβ) was performed on free-floating brain sections on shaker tables at room temperature. Brain sections were first rinsed with 0.1 M PBS and pre-incubated in 0.5 ml 0.1 M PBS-BT for 30 minutes. After pre-incubation, primary antibodies (Aβ: Mouse anti-human-Aβ (1:10000; WO-2 antibody, mouse anti-human Aβ4-10, T Hartmann, Heidelberg, Germany), IBA-1: Goat anti-IBA-1 (1:750; Abcam, Cambridge, UK), GLUT-1: Rabbit anti-GLUT-1 (1:5000; Millipore, Billerica, MA, USA) were added for 16 hours. All sections were rinsed and the secondary antibodies (Aβ: Alexa647-conjugated donkey anti-mouse IgG, IBA-1: Cy3-conjugated donkey anti-goat IgG, GLUT-1: Alexa 488-conjugated donkey anti-Rabbit IgG; Jackson Immunoresearch Inc, Allentown, PA, USA) was added for 3 hours in the dark. After rinsing, mounting, and drying sections were enclosed with Fluorsave (Calbuichem, Canada).
**Quantification**

Brain sections between bregma -1.46 and -2.30 [227] were preselected for quantification. Quantification was done at a 5x magnification using a Axio Imager (A2, Zeiss Germany). ImageJ (National Institute of Health, Bethesda, MD, USA) was used to analyse the regions of interest (cortex, hippocampus and thalamus).

**Statistics**

For statistical analyses IBM SPSS Statistics 20 software was used (IBM Corporation, New York, NY, USA).

We first analysed data gathered under untreated conditions (no EM). To unravel the impact of AngII-induced hypertension on all parameters and the possible genotype by AngII-induced hypertension interactions, data were analysed separately with univariate/ multivariate ANOVA with Bonferroni correction.

To assess effects of treatment (EM), data were analysed separately for AngII-infused and Sal-infused animals with univariate/ multivariate ANOVA with Bonferroni correction for multiple comparisons. After overall analysis, data were split into the specific interacting factors, when significantly different.

The repeated measures ANOVA with Bonferroni correction was only used for the acquisition phase of the MWM (with the repeated measure: acquisition days/time). All values of the MWM acquisition are expressed as mean ± SEM for. All other results are shown in box plots with whiskers. The hinges of the boxes extend from the 25th to 75th percentiles of the data. The whiskers are drawn down to the 5th percentile and up to the 95th. For all statistical analyses there are significant comparisons between groups and treatments over all experimental outcome measures, and Type I error was consistently controlled and limited to \( p \leq 0.05 \) overall.

**Results**

Body weight was not influenced by genotype, AngII-infusion nor treatment with EM before (10-month), and during the experiment (11- and 12-month) (Figure 1A).

At 11 months of age AngII-infused mice, both AβPP/PS1 and WT mice drank daily more water than Sal-infused mice (Figure 1B, \( F(1,22)=7.5, p=0.012 \)).

**Systolic blood pressure**

* Aging by genotype interactions on the relationship between AngII and SBP

Repeated measures ANOVA revealed a genotype by AngII interaction on SBP (\( p=0.032 \)) over time (Figure 1C).

AngII effectively increased SBP (11 months: 44.6±4.8 mmHg; 12 months: 45.5±4.9 mmHg)...
mmHg) in all mice compared to littermates without AngII at 11 and 12 months of age (11 months, F(1,22)=85.0, p<0.001; 12 months, F(1,22)=85.6, p<0.001).
At 12 months of age all AβPP/PS1 mice had a higher SBP than corresponding WT (11.4±4.9 mmHg; F(1,22)=5.4, p=0.030).

AngII, antihypertensive treatment with EM, and SBP
At 12 months of age, EM treatment lowered SBP (Figure 1D; -42.5±6.5 mmHg; F(1,22)=42.8, p<0.001) only in AngII-infused animals, and not in Sal-infused animals).

Figure 1 Effect of aging, angiotensin II (AngII)-infusion and treatment with eprosartan mesylate (EM) on body weight (A), drinking behaviour (B), systolic blood pressure (C+D, SBP) of AβPP/PS1 and wild-type (WT) mice treated with eprosartan mesylate (EM) versus water as control. (A) No significant genotype, AngII or EM effects were observed on body weight. (B) AngII-infusion increased water intake both in untreated AβPP/PS1 and WT (p=0.012) compared to their corresponding control Sal-infused (saline) littermates. This effect was not influenced by genotype or treatment with EM in AngII-infused and Sal-infused animals. (C) SBP was measured for two consecutive days in trained, conscious and preheated mice using computerised tail-cuff plethysmography. During both experimental months, infusion with AngII effectively increased SBP (11-Months: 44.6±4.8 mmHg; 12-Months: 45.5±4.9 mmHg) in all mice compared to littermates without AngII (11-Months, p<0.001; 12-Months, p<0.001). At 12-months of age untreated (no EM) Sal-infused and AngII-infused AβPP/PS1 mice had a higher SBP than their corresponding WT mice (11.4±4.9 mmHg; p=0.030). (D) Only in AngII-infused animals, not in Sal-infused animals, treatment with EM lowered SBP (-42.5±6.5 mmHg; p<0.001).
Cerebral blood flow

One representative high-resolution voxel-wise analysed CBF image is shown (Figure 2A). At 12 months of age, CBF was measured in the cortex (Figure 2B), hippocampus (Figure 2C) and thalamus (Figure 2D). Furthermore, CBF was acquired in two standardised gas concentrations: normal (200 O₂ : 100 N₂ O mL/min) and vasoconstriction (300 O₂ : 0 N₂ O mL/min). For each ROI (cortex, hippocampus, thalamus) and each gas concentration (normal (200 O₂ : 100 N₂ O mL/min) and vasoconstriction (300 O₂ : 0 N₂ O mL/min)) data were analysed on the one hand to detect genotype effects on the relationship between AngII and CBF, and on the other hand to investigate the relationship between AngII, antihypertensive treatment with EM, and CBF.

Cortex
Normal gas condition
AβPP/PS1 mice had a lower cortical CBF than WT littermates (-36.7±13.2 mL/100 g/min; F(1,19)=7.7, p=0.012).

Gas condition inducing vasoconstriction
AβPP/PS1 mice had a decreased cortical CBF compared to corresponding WT littermates (-38.5±13.1 mL/100 g/min; F(1,19)=8.6, p=0.009).

Hippocampus
Normal gas condition
Treatment with EM increased hippocampal CBF both in AngII (44.5±10.9 mL/100 g/min; F(1,21)=16.5, p=0.001) and Sal-infused (25.5±9.0 mL/100 g/min; F(1,16)=8.0, p=0.012) animals compared with untreated (no EM) animals.

Gas condition inducing vasoconstriction
No significant differences were found.

Thalamus
Normal gas condition
No significant differences were found.

Gas condition inducing vasoconstriction
No significant differences were found.
Angiotensin II, hypertension, and angiotensin II receptor antagonism

Chapter 4

A

Normal

Vasoconstriction

B

Cortex

CBF (ml/100g/min)

WT  AβPP/PS1  WT  AβPP/PS1
Normal  Vasoconstriction

C

Hippocampus

CBF (ml/100g/min)

WT  AβPP/PS1  WT  AβPP/PS1
Normal  Vasoconstriction

D

Thalamus

CBF (ml/100g/min)

WT  AβPP/PS1  WT  AβPP/PS1
Normal  Vasoconstriction

Sal-Water

Sal-EM

AngII-Water

AngII-EM

* P < 0.05

** P < 0.001

*** P < 0.0001
Figure 2 Cerebral blood flow (CBF) measurements of the brains of 12-month-old saline-infused (Sal) and Angiotensin II-infused (AngII) AβPP/PS1 and wild-type (WT) mice, treated with eprosartan mesylate (EM) vs normal drinking water as control. CBF was measured with flow-sensitive alternating inversion recovery MRI technique; from a series of echo planar imaging images. One representative high-resolution voxel-wise analysed CBF image for each single animal group is shown (A). The figure shows the cerebral perfusion data of AβPP/PS1 and WT mice infused with either saline (sal) or angiotensin II (AngII) and treated with either eprosartan mesylate (EM) compared to water as control. The CBF was measured in three different regions of interest (ROI): Cortex (B), hippocampus (C) and thalamus (D). (B) Under normal gas concentration (normoxia=2:1 O₂ and N₂O mixture) all untreated (no EM) AβPP/PS1 mice exhibited a decreased cortical CBF compared to their corresponding WT littermates (p=0.012). Again, under gas concentration inducing vasoconstriction (3: O₂ and N₂O mixture), all untreated (no EM) AβPP/PS1 mice demonstrated a decreased cortical CBF compared to their corresponding WT littermates (p=0.009). (C) No significant results were found in the hippocampal CBF. (D) No significant results were found in the thalamic CBF.

**Diffusion tensor imaging**

In this study brain diffusivity determined with DTI is a measure for white and grey matter integrity. One DTI measure is fractional anisotropy (FA) being a marker for myelination and fiber density of white matter (WM) and MD represents an inverse measure for membrane density, and sensitive measure for cellularity, edema, and necrosis in grey matter [348-350]. Quantitative assessment of diffusion tensor derived indices was performed for ROIs drawn in white and gray matter regions (Figure 3A) to assess effects of AngII-induced hypertension and genotype in 12-Month old AβPP/PS1 and WT mice (Figure 3B and C). Moreover, in AngII-infused and Sal-infused mice the impact of antihypertensive treatment with EM on microstructural integrity in white and grey matter regions was measured.

**Fractional anisotropy**

Treatment with EM led to higher hippocampal FA in all AngII-infused animals (transgenic and WT) (F(1,18)=5.0, p=0.039), also in the optic tract (F(1,17)=12.9, p=0.002) compared to animals without EM.

**Mean diffusivity**

EM treatment decreased MD in the motor cortex of all Sal-infused mice (F(1,13)=8.0, p=0.014) compared to Sal-infused mice without EM. Only in AngII-infused animals a genotype by EM interaction was detected for MD in motor cortex (p=0.036) and optic tract (p=0.008).

In detail, not EM treated AngII-infused AβPP/PS1 mice showed lower MD in the optic tract compared to WT (F(1,8)=7.4, p=0.026).
However, on EM treatment the MD in the motor cortex of AngII-infused AβPP/PS1 mice was decreased compared to the non-treated mouse (F(1,9)=6.4, p=0.033). In AngII-infused EM treated WT a decreased MD in the optic tract was found compared to the non-treated mouse (F(1,8)=6.4, p=0.035).

Figure 3 Diffusion tensor imaging (DTI) measurements of the brains of 12-month-old saline-infused (Sal) and Angiotensin II-infused (AngII) AβPP/PS1 (Alzheimer’s disease, AD) and wild-type (WT) mice, treated with eprosartan mesylate (EM) vs normal drinking water as control. Quantitative assessment of diffusion tensor derived indices was performed for regions of interest (ROI; AUC= Auditory cortex, CC=Corpus callosum, F=Fornix, HC=Hippocampus, MC=Motor cortex, OT=Optic tract, SSC=Somatosensory cortex, VC=Visual cortex). (A) ROIs were drawn in white and gray matter to assess effects of AngII-infusion (induced hypertension) and antihypertensive treatment with EM in 12-Month old AβPP/PS1 and WT mice on fractional anisotropy (B) and mean diffusivity (C). (A) Under AngII-infusion, hippocampal FA (p=0.039) and FA in the optic tract (p=0.002) were higher if animals were treated with EM compared with AngII without EM. (C) In all Sal-infused mice EM treatment decreased MD in the motor cortex (p=0.014). AngII-infused AβPP/PS1 mice (no EM) had a lower MD in the optic tract than their WT littermates (p=0.026). However, on EM treatment MD in the motor cortex of AngII-infused AβPP/PS1 mice was decreased compared to their non-treated littermates (p=0.033). Under AngII-infusion, WT mice on EM had a decreased MD in the optic tract compared to their untreated littermates (no EM) (p=0.035).
**Resting state fMRI**

Resting state fMRI is based on the principle that during the resting state in functionally related brain regions spontaneous low-frequency fluctuations (SLFF) (<0.08 Hz) of the blood oxygen level dependent (BOLD) signals occur synchronously implying a possible neuronal functional connectivity (FC). Li et al. showed that AD patients have a different FC including alterations in the visual, sensory-motor, and visual networks [351]. Therefore, we also included these ROI (visual, motor, and somatosensory cortex) in our analysis. To compare FC patterns in 12 months old mice (Figure 4) rsfMRI data were analysed for genotype and AngII effects in untreated animals. In addition, in AngII-infused and Sal-infused mice the effect of antihypertensive treatment with EM on FC was investigated.

![Resting-state functional connectivity (FC) based on total correlation analyses of 12 ROI in the brain of 12-month-old Sal-infused (saline) and AngII-infused (Angiotensin II) AβPP/PS1 and wild-type (WT) mice, treated with eprosartan mesylate (EM) vs normal drinking water as control.](image)

In summary, for the overall correlations no significant genotype effects were detected in the dorsal and ventral hippocampus (hipp), and auditory, motor, somatosensory (SS) and visual cortex (ctx) of untreated animals (no EM). Notably, in all mice, AβPP/PS1 and WT mice, AngII-induced hypertension led to a decrease in FC between several brain regions, i.e. left dorsal hippocampus to right ventral hippocampus, left ventral hippocampus to left visual cortex, and right auditory ctx to right motor ctx. Investigating the effect of antihypertensive treatment with EM on FC in AngII-infused and Sal-infused animals revealed only negative effects of EM in normotensive animals and only positive effects on the FC in AngII-infused animals. A detailed description of the effects found in the total correlation analyses of the resting-state FC data is given in supplementary table 2.
Total correlation analyses

AβPP/PS1 showed no differences in FC compared to their WT littermates. Notably, in both AβPP/PS1 and WT mice, AngII-induced hypertension led to a decreased FC between several brain regions, see supplementary table 2. Investigating the effect of antihypertensive treatment with EM on FC in AngII-infused and Sal-infused animals revealed only negative effects of EM in Sal-infused animals and only positive effects on the FC in AngII-infused animals. More specifically, Sal-infused mice treated with EM had a lower FC between several brain regions than Sal-infused mice not treated with EM, see supplementary table 2. In contrast, AngII-infused mice on EM showed a higher FC than non EM treated AngII-infused mice between many brain regions, see supplementary table 2.

Morris water maze

All mice were trained in the water maze task to assess spatial learning capacity at 11 months of age (Figure 5). During task acquisition, the latency to reach the platform was assessed (Figure 5A+B+C). Spatial memory was measured with the probe trial one hour after the last regular trial on the last day of the MWM experiment using the frequency of crossing the NE quadrant containing the platform position (Figure 5D).

 Acquisition

A genotype by AngII interaction was found for the latency to reach the platform in the acquisition phase (Figure 5A, p=0.009). Sal-infused AβPP/PS1 (F(3,9)=4.2, p=0.041) and WT mice (F(3,24)=5.4, p=0.006), and also AngII-infused WT mice (F(3,18)=5.9, p=0.005) were able to learn to find the platform from acquisition day 1 to day 4, whereas AngII-infused AβPP/PS1 mice (F(3,15)=2.5, p=0.100) did not learn to find the platform from acquisition day 1 to day 4. Combining EM-treated with untreated mice revealed no effect or a genotype by antihypertensive treatment interaction (Figure 5B+C).

 Probe

We detected no effects of genotype, AngII-infusion nor antihypertensive treatment with EM (Figure 5D).
Figure 5 Analysed data of the Morris water maze (MWM) performed by 11-month-old Sal-infused (saline) and AngII-infused (Angiotensin II) AβPP/PS1 (Alzheimer’s disease, AD) and wild-type (WT) mice, treated with eprosartan mesylate (EM) vs normal drinking water as control. While during task acquisition, all mice were trained in the MWM to assess spatial learning capabilities during 11-Month of age (A+B+C), during the probe phase the ability to remember the former platform location in the pool area was measured using the frequency of crossing NE quadrant of the pool area containing the platform location was measured as probe trial performance (D). (A) Task acquisition performance revealed that in untreated mice (no EM) almost all animal groups, Sal-infused WT mice (p=0.006), Sal-infused AβPP/PS1 mice (p=0.041), and AngII-infused WT mice (p=0.005), were able to learn to find the platform from acquisition day 1 to day 4, except of the AngII-infused AβPP/PS1 mice. This latter experimental group could not learn to find the platform from acquisition day 1 to day 4 (p=0.100). (B+C) Combining EM-treated with untreated mice revealed no effect nor a genotype by antihypertensive treatment interaction. (D) During the probe trial of the MWM the ability to cross NE quadrant of the pool area containing the platform location was measured as probe trial performance indicating spatial memory capacity. No effect on spatial memory was found.

Immunohistochemical procedures
To analyse effects of genotype and AngII in untreated animals, AngII-infused and Sal-infused mice and also the effect of an antihypertensive treatment with EM on metabolism, vascular density, neuroinflammation and amount of Aβ, all brains were stained for glucose transporter-1 (GLUT-1), against ionised calcium-binding adapter molecule 1 (IBA-1), and WO-2 antibody (mouse anti-human Aβ4–10) (Figure 6A).

GLUT-1 staining
All brains were processed for immunohistochemical staining with GLUT-1 antibody. In order to reveal the changes in total amount of GLUT-1, we measured the relative area of the total section area being stained for GLUT-1 in the cortex, hippocampus, and thalamus (Figure 6B). While in the cortical and hippocampal regions no effect were detected, all AngII-infused animals displayed increased
GLUT-1 in the thalamus compared to their Sal-infused littermates (F(1,22)=3.4, p=0.039). Only in thalamic regions of all Sal-infused mice, EM-treatment increased amount of GLUT-1 (F(1,20)=5.3, p=0.032). We also measured vascular density via the number of GLUT-1+ blood vessels, but no significant effects were found (data not shown).

**IBA-1 staining**

The brains of all mice were immunohistochemically stained for IBA-1. IBA-1 is specifically expressed in activated microglia. The typical example of the combined picture of figure 6A demonstrates clearly the colocalisation of IBA-1 with Aβ. We measured relative area of the total section area being stained for IBA-1 in the cortex, hippocampus, and thalamus as measure for amount of activated microglia (Figure 6C). No significant results were found for the amount of activated microglia.

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**Figure 6 Distribution of glucose transporter-1 (GLUT-1), ionised calcium-binding adapter molecule 1 (IBA-1), and amyloid-β (Aβ) in the cortex, hippocampus, and thalamus of 12-month-old Sal-infused (saline) and AngII-infused (Angiotensin II) AβPP/PS1 (Alzheimer’s disease, AD) and wild-type (WT) mice, treated with eprosartan mesylate (EM) vs normal drinking water as control.** A representative example of vessel density (GLUT-1), neuroinflammation (IBA-1), Aβ staining, and an overlay of all three staining in an AβPP/PS1 mouse is shown in (A). (B) The brains of all mice underwent immunohistochemical staining with GLUT-1 antibody representing amount of GLUT-1. While in the cortical and hippocampal regions no effect were detected, all AngII-infused animals displayed an increased amount of GLUT-1 in the thalamus compared to their Sal-infused littermates (p=0.039). Only in thalamic regions of all Sal-infused mice, EM-treatment increased amount of GLUT-1 (p=0.032). (C) The brains of all mice were immunohistochemically stained with an antibody against IBA-1 being specifically expressed in activated microglia. Notably, Aβ and microglia are colocalised as demonstrated in the combined picture. No significant differences were found for IBA-1. (D) The brains of all AβPP/PS1 mice were immunohistochemically stained with WO-2/Aβ antibody (mouse anti-human Aβ4–10). No significant AngII- or antihypertensive treatment effects were found.
β-Amyloid staining

The brains of all AβPP/PS1 mice were immunohistochemically stained with WO-2 antibody. As previously shown, brains of WT mice showed no immunoreactivity with this antibody [352], while Aβ deposits in transgenic mice were intensively stained. We measured the relative area of the total section area covered with Aβ deposits in the cortex, hippocampus, and thalamus (Figure 6D). No significant results were found for the area covered with Aβ depositions in the cortex, hippocampus, and thalamus.

A summary of all experimental results is given in table 1.

Table 1. Summary of all significant results.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
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<tbody>
<tr>
<td>Body weight</td>
<td></td>
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<tr>
<td>Drinking behavior</td>
<td>↑ in AngII-infused mice</td>
</tr>
<tr>
<td>SBP</td>
<td></td>
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<tr>
<td>Aging</td>
<td>↑ in AngII-infused mice</td>
</tr>
<tr>
<td>12-Month</td>
<td>↑ in AngII-infused mice; ↑ in AD mice; ↓ in AngII-infused mice on EM</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>↑ in mice on EM</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
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<tr>
<td>FA</td>
<td></td>
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<tr>
<td>Cortical</td>
<td></td>
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<tr>
<td>Auditory Cortex</td>
<td></td>
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<tr>
<td>Corpus callosum</td>
<td></td>
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<tr>
<td>Fornix</td>
<td></td>
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<tr>
<td>Hippocampus</td>
<td>↑ in AngII-infused animals on EM</td>
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<tr>
<td>Motor cortex</td>
<td></td>
</tr>
<tr>
<td>Optic tract</td>
<td>↑ in AngII-infused animals on EM</td>
</tr>
<tr>
<td>DTI</td>
<td></td>
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<tr>
<td>Somatosensory cortex</td>
<td></td>
</tr>
<tr>
<td>Visual cortex</td>
<td></td>
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<tr>
<td>FA</td>
<td></td>
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<tr>
<td>Neocortex</td>
<td></td>
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<tr>
<td>Auditory Cortex</td>
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<td>Corpus callosum</td>
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<td>Fornix</td>
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<tr>
<td>Hippocampus</td>
<td></td>
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<tr>
<td>Motor cortex</td>
<td>↓ in Sal-infused mice on EM; ↓ in AngII-infused AD mice on EM</td>
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<tr>
<td>Optic tract</td>
<td>↓ in AngII-infused WT mice on EM</td>
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<tr>
<td>Somatosensory cortex</td>
<td></td>
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<tr>
<td>Visual cortex</td>
<td></td>
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<tr>
<td>rsfMRI</td>
<td></td>
</tr>
<tr>
<td>Total correlations</td>
<td>↓ in AngII-infused mice; ↓ in Sal-infused mice on EM; ↑ in AngII-infused mice on EM</td>
</tr>
<tr>
<td>MWM</td>
<td></td>
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<tr>
<td>Acquisition</td>
<td>Latency times</td>
</tr>
<tr>
<td>Probe</td>
<td># NE-quadrant</td>
</tr>
<tr>
<td>IHC</td>
<td></td>
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<tr>
<td>GLUT-1</td>
<td>↑ in thalamus of AngII-infused mice; ↑ in thalamus of mice on EM</td>
</tr>
<tr>
<td>IBA-1</td>
<td></td>
</tr>
<tr>
<td>Aβ</td>
<td></td>
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</table>

Used abbreviations: AngII (Angiotensin II), AD (Alzheimer’s disease), SBP (systolic blood pressure), EM (eprosartan mesylate), CBF (cerebral blood flow, DTI (diffusion tensor imaging), FA (fractional anisotropy), MD (mean diffusivity), Sal (saline), rsfMRI (resting state functional MRI), Total (total correlations), partial (partial correlations), MWM (Morris water maze), NE (North-East), GLUT-1 (glucose transporter-1), IBA-1 (ionised calcium-binding adapter molecule 1), Aβ (amyloid-β).
Discussion

This study assessed the potential relationships between angiotensin-II induced hypertension and AD related cerebrovascular, metabolic, connectivity and cognitive changes in a mouse model for AD and wild-type controls. A summary of all significant results can be found in table 1.

We found that AngII-induced hypertension did not induce a total AD pathology in WT mice. One explanation for these results could be the use of relatively young mice. In accordance, Toth et al. showed that aging impairs autoregulatory protection in the brain, and this aggravates potentially cerebromicrovascular injury and neuroinflammation [353].

However, AngII led to a reduced functional connectivity (FC) in these WT mice, see supplementary table 2. In clinical studies a decreased FC was found in AD patients [206-208]. In support, a preclinical study using a mouse model for cerebral amyloidosis showed a compromised FC resulting in functional impairments affecting the sensory motor cortex already in pre-plaque stage [258]. In the current study we showed that in a hypertension model a reduction in FC could be introduced via AngII-induced hypertension, in absence of classical AD pathology (amyloidosis). A limitation of the acquired FC results is that the rsfMRI measurements are performed in isoflurane anesthetised mice. Fortunately, in both preclinical and clinical studies using rsfMRI a general structure of the functional networks transcending levels of consciousness was detected [354-356]. Liang et al. and Zhou et al. have confirmed the presence of a bilateral cortical connectivity in several cortical regions also under isoflurane anaesthesia [357, 358]. Nevertheless, in isoflurane anesthetised mice Jonckers et al. and Guilfoyle et al. did not find the same level of bilateral connectivity [231, 359]. However, in our previous and recent studies using isoflurane as anaesthesia, we confirmed the presence of networks in several well defined cortical and subcortical brain regions in two different murine models for vascular risk factors for AD [346, 360]. In our previous work we confirmed that both FC and CBF are dependent on isoflurane concentrations, and both FC and CBF decline with concentrations of isoflurane >2.2%, but do not further decline below a concentration of 2.2% 90. Therefore, using the low- dose isoflurane (~1.7%) in this recent experiment will preserve the resting-state networks and will not interfere with the outcome of this study, as all animals were kept under the same low isoflurane concentration.

Second, we used an AD mouse model to study the influence of AngII-induced hypertension on AD pathology. As expected, in all mice (AD and WT), AngII increased SBP. This result is in line with other studies showing that AngII causes vasoconstriction and increased BP by binding to AT1 and AT2 receptors [225,
However, at 12 months of age, our AD mice had a higher SBP than their WT littermates, confirming recent findings [344]. Our results revealed a relationship between aging, AngII-induced hypertension and Alzheimer-like (Aβ-overexpression) pathology leading to an increased SBP. A possible explanation for this finding is that Aβ enhances the vasoconstrictive effect of AngII, or causes vasoconstriction independent of AngII. Niwa et al. have shown that Aβ acts directly on cerebral arteries to enhance vasoconstriction and to stimulate selected constrictor responses, resulting in a reduced CBF [247]. In accordance, we showed that our AD mice showed also an impaired CBF, similar to our earlier study in the same AD mouse model [294]. Studies in MCI and AD patients also identified a significant reduction in regional and global CBF [246]. Whether this reduction is due to reduced metabolic demand or due to vascular pathology is difficult to disentangle in human studies. Contrarily to our expectations we could not detect hypertension-induced overexpression of Aβ in the cortex, hippocampus nor thalamus in the brains of the AD-like mice, while these mice do show impaired cortical CBF. Sun et al. induced hypoxia, a direct consequence of hypoperfusion, in another Aβ overexpressing mouse model at eight months of age and revealed both an increased Aβ deposition and neuritic plaque formation [361], whereas in our study hypertension was induced by AngII-infusion for only two months starting from 10 months of age. A limitation of this study is the usage of a relatively high dosage (500 ng·kg−1·min−1) of AngII being infused via osmotic minipumps for two months starting from ten months of age. Using this hypertension model we wanted to simulate hypertension during mid-life via chronically elevated levels of AngII for only two months. Therefore, we needed a slightly higher dosage of AngII increasing SBP already after a short period of time to induce AngII-induced hypertension and the consequent pathological changes resembling the human situation. For example, the Honolulu Asia Aging Study revealed that untreated hypertension during midlife can increase the risk for late age dementia in men [95]. Dosages of 200 ng·kg−1·min−1 and 400 ng·kg−1·min−1 using the same slow pressor model have proven not to elevate SBP within the first six days after the implantation of the osmotic minipumps, while a dosage of 1,000 ng·kg−1·min−1 heightened SBP already at three days after the implantation [362]. However, Qi et al. also infused 6 weeks old mice via osmotic minipumps with even a higher dosage of AngII (1,500 ng·kg−1·min−1) for 7 days increasing SBP already at 4 days post operation [363]. Nevertheless, Edgley et al. have proven that intravenous infusions of AngII into the systemic circulation requires much lower dosages of AngII [364].

In summary, our AD mice exhibited an increased SBP and a decreased cortical CBF. In addition, AD mice on AngII showed a decreased FC like their WT littermates, but only in these AngII-infused AD mice did this lead to a lowered spatial learning
capacity. RsfMRI has developed into a method for the analysis of spontaneous neural activity through the blood-oxygen-level-dependent (BOLD) signal change [365] representing FC. Our findings indicating an impaired FC in AngII-infused mice are in line with a recent study that found reduced connectivity in cortex regions of older hypertensive AD patients relative to older non-hypertensive AD patients [366]. We found no cognitive effects of AngII induced hypertension in WT animals. Haley et al. demonstrated even in healthy young adults that a family history of hypertension was associated with subtle changes in visuospatial attention combined with a lowered task-related activation in several brain regions during this visuospatial working memory task [367]. This result is in accordance with a study revealing an impaired memory and learning in hypertensive rats [368]. Hypertensive patients had deficits in the white matter and functional connectivity in frontal and parietal lobes, associated with cognitive decline [369]. However only our AngII-infused AD model mice exhibited a decreased spatial learning capacity during the MWM, which could be due to the functional connective impairments combined with the loss of cortical CBF being only present in this experimental group.

We could not observe genotype or AngII effects on structural connectivity. In this study AngII-infusion was administrated only for two months starting from 10 months of age. This short duration of AngII-infusion could be one reason we found no changes in structural connectivity measured via DTI. Another explanation could be the increased amount of GLUT-1 measured in the thalamus of AngII-infused mice as a compensatory mechanism. GLUT-1 is responsible for the transfer of glucose across the blood brain barrier and being selectively expressed at high levels in the capillary endothelium of the brain [324]. GLUT-1 is a marker for vascular endothelial cells and cerebral metabolism. In several animal studies, myocardial ischemia increased the synthesis of GLUT-1 mRNA and protein levels in both ischemic and also nonischemic cardiac regions indicating also a potential rescue mechanism for myocardial ischemia [370, 371]. Notably, McCall et al. reported an overexpression of GLUT-1 in microvessels after the onset of global cerebral ischemia in the rat brain [372]. Moreover, Gerhart et al. and Urabe et al. also showed also an overexpression of GLUT-1 in the cerebral cortex in two ischemia and reperfusion animal models [373, 374]. However, no effects of genotype or AngII-infusion were found on vascular density.

In summary, regarding our second aim of this study, AD model mice had an increased SBP and an impaired CBF. Furthermore, AngII-induced hypertension exacerbated several AD-like pathological changes such as impaired CBF, impaired functional connectivity, and cognitive impairment. These results are in accordance with the recent study of Cifuentes et al. demonstrating that hypertension advances the development of AD-like structural and functional alterations, partially through
cerebral vasculature impairment and reduced nitric oxide production [375]. This result of Cifuentes et al. is in line with our results of the immunohistochemical data of IBA-1, a marker for activated microglia [236-238]. Here, no differences in amount of IBA-1+ cells nor immunohistochemically stained surface in the AngII-infused and AD model mice could be observed.

Another aim of this study was to investigate the effect of an ARB such as EM on possible (neuro)pathological changes induced by hypertension and to indicate a relationship between the AD-like pathology, AngII, and its receptors. In all AngII-infused experimental mice, treatment with the angiotensin-receptor blocker EM restored SBP, in line with previous work [376]. Treatment of hypertension with EM enhanced hippocampal CBF. This beneficial effect was shown in all mice, and was not restricted to transgenic animals only, indicating that either blood pressure lowering or blocking effects of endogenous AngII may be beneficial for brain perfusion. In accordance, Tryambake et al. demonstrated that blood pressure lowering treatments increased CBF in older subjects with hypertension as well [377].

A beneficial effect of EM was detected in all AngII-infused mice regarding the structural connectivity. Here, FA in the hippocampus and optic tract was increased in EM-treated AngII-infused mice indicating an improved microstructural integrity, while after EM-treatment MD of the motor cortex of Sal-infused mice and of AngII-infused AD mice, and MD of the optic tract of AngII-infused WT mice was lowered. In accordance, Gons et al. showed that increased blood pressure is associated with a decrease in FA respectively an increase in MD [378]. De Leeuw et al. also demonstrated that long-standing hypertension increases the risk of developing white matter lesions [379]. Notably, a novel finding is the negative effect of EM on FC in all Sal-infused mice, but not in AngII-infused mice, see supplementary table 2 for a detailed description of the significant results. While all Sal-infused EM-treated mice showed a decreased FC compared with untreated mice, all AngII-infused EM-treated mice showed an increased FC compared with untreated mice. In addition, Füchtemeier et al. showed that Compound 21, an AngII receptor type 2 agonist, had opposing effects on spatial reference memory in hypoperfused and sham mice [380]. These effects need to be further investigated in future studies, but suggest that blockade of the angiotensin II receptors in absence of elevated AngII levels may have harmful effects on structural connectivity and cognition. Moreover, Henriksen et al. also showed in insulin resistant obese Zucker rats that irbesartan, another AngII receptor antagonist, improved insulin sensitivity and glucose tolerance in the skeletal muscle by increasing the protein expression of glucose transporter type 4, indicating the importance of measuring insulin and glucose blood levels [381] and the relationship with AngII in future studies. Furthermore, it will be interesting to investigate the possible role of
phosphorylated tau (pTau) in follow-up studies, because of the emerging role of pTau in pathology and behavior for both AD [382]. Glodzik et al. revealed that only in hypertensive subjects a longitudinal decrease in mean arterial pressure was related to memory decline and an increase in phosphorylated tau [383]. Another interesting aspect to investigate in future studies is how hypertension affects heart hypertrophy, and whether cognitive decline and decreased connectivity are indirect effects of heart diseases. In a systematic review Vogels et al. confirmed that heart failure is associated with a pattern of generalised cognitive impairment that includes primarily memory, attention, mental flexibility and global cognitive deficits [384].

In conclusion, we showed that chronic infusion of AngII increased SBP in both transgenic and WT mice, which could be reversed by EM-treatment. In addition, aging combined with AD pathology exacerbated the increase in SBP. Correspondingly cerebral perfusion was impaired in the AD mouse model. Furthermore, EM-treatment improved the white matter integrity combined with increased CBF and improved FC. AngII-induced hypertension impaired the functional connectivity in AD and WT mice comparably, indicating a negative role of hypertension on functional connectivity irrespective of AD pathology.

Our results suggest that elevated levels of AngII may indeed represent a mechanistic link between the risk factor hypertension and the clinical development of AD, however, AngII may not to be a causal factor for sporadic AD as it did not initiate amyloid pathology in this study. Rather, it may progress the clinical development of AD in people with amyloid accumulation through its negative effects on cerebral blood flow, functional connectivity and cognition. Recent human studies on sporadic AD indicate that amyloid accumulation increases with age to as much as 30-50% of the normal population over the age of 70, but can remain asymptomatic for decades [385-387]. We speculate that elevated levels of AngII (essential hypertension) may be a factor promoting the development of symptomatic disease. In addition, our study suggests that elevated AngII may also lead to abnormalities that may mimic AD (reduced functional connectivity leading to cognitive decline) which could imply that hypertension may lead to dementia that has similarity with AD and may be clinically diagnosed as such. In both scenarios however, counteracting the negative effects of elevated AngII will have a beneficial effect in reducing the prevalence of dementia. Gathering more insight in the actions of antihypertensive treatments on brain processes and the vascular system could support the development of effective tailor-made blood pressure-lowering treatments for AD patients.
Supplementary material

Supplementary material can be found here:
http://journals.sagepub.com/doi/full/10.1177/0271678X16667364#_i47
Improved spatial learning strategy and memory in aged Alzheimer AβPP$_{swe}$/PS1$_{dE9}$ mice on a multi-nutrient diet

Chapter 5 Improved spatial learning strategy and memory in aged Alzheimer AβPP<sub>swe</sub>/PS1<sub>dE9</sub> mice

Abstract

There is accumulating evidence showing that lifestyle factors like diet may influence the onset and progression of Alzheimer’s disease (AD). Our previous studies suggest that a multi-nutrient diet, Fortasyn, containing nutritional precursors and cofactors for membrane synthesis, viz. docosahexaenoic acid, eicosapentaenoic acid, uridine-mono-phosphate, choline, phospholipids, folic acid, vitamins B6, B12, C, E, and selenium, has an ameliorating effect on cognitive deficits in an AD mouse model. In the present study we analyzed learning strategies and memory of 11-month-old AβPP<sub>swe</sub>/PS1<sub>dE9</sub> (AβPP/PS1) mice in the Morris water maze (MWM) task performed after nine months of dietary intervention with a control diet or a Fortasyn diet to characterize diet-induced changes in cognitive performance. The Fortasyn diet had no significant effect on MWM task acquisition. To assess hippocampus-dependent learning, the strategies that the mice used to find the hidden platform in the MWM were analyzed using the swim path data. While on control diet during the fourth day of the MWM AβPP/PS1 mice used more often the non-spatial random search strategy, on Fortasyn diet the transgenic animals exhibited more chaining strategy than their wild-type littermates. During the probe trial, AβPP/PS1 mice displayed no clear preference for the target quadrant. Notably, in both transgenic and nontransgenic mice on Fortasyn diet the latency to reach the former platform position was decreased compared to the mice on control diet. In conclusion, this specific nutrient combination showed a tendency to improve searching behavior in AβPP/PS1 mice by increasing the use of a more efficient search strategy and improving their swim efficiency by decreasing the latency to reach the former platform position.
Introduction

The most common form of dementia is Alzheimer’s disease (AD). AD is characterized by brain atrophy and a gradual cognitive decline caused by neuronal death and loss of synapses in brain regions involved in learning and memory processes (e.g. temporal and frontal lobes) [182, 191]. During the course of AD, pathological hallmarks are the deposition of amyloid-β (Aβ) plaques into parenchymal and cerebrovascular tissue, the production of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein (NFT), and neuronal cell loss [388-390]. Already the early stages of AD are characterized by the accumulation of Aβ affecting specific brain regions like the forebrain and medial temporal lobe structures like hippocampus, amygdala and entorhinal cortex [180, 181, 391]. Almost all insoluble Aβ is accumulated within the neuritic plaques and cerebral vessel walls [182]. There are many theories proposing the cause of AD, and one of the most well-known ones is the amyloid cascade hypothesis. According to the amyloid cascade hypothesis, Aβ plaques are the leading cause of the neuronal cell death and the cognitive decline in AD [390, 392]. This hypothesis is under debate because Aβ plaques do not correlate well with the severity of the disease [194]. No correlation between neuronal, metabolic or synaptic loss with amount of amyloid plaques could be found [194, 393]. However, the amyloid theory still stands firm by recent findings suggesting that the soluble oligomers and not the Aβ plaques are the toxic forms of Aβ [394, 395]. Many large epidemiological studies have indicated that vascular disorders are risk factors for AD [396-398]. Furthermore, it has been shown that vascular risk factors can influence the development of AD pathology, and this led to the vascular hypothesis [194]. In elderly patients a decreased CBF was related to increased hippocampal and amygdalar atrophy [203, 399]. Therefore an impaired or reduced cerebral perfusion may play a role in the development of AD via decreased delivery of oxygen in energy-sensitive brain regions like the hippocampus, inducing neurodegeneration and subsequent cognitive decline [205]. Up to now, no effective treatment for AD is available. This calls for new approaches for treatment and prevention of AD. There is growing knowledge about lifestyle factors such as nutrition having an impact on the onset and development of AD in late life [400, 401]. Many studies have shown that Mediterranean diets may not only have a positive effect on vascular health, but also on cognitive symptoms in AD [402, 403]. Epidemiological studies indicate that intake of omega-3 fatty acids is correlated to a reduced risk of AD [404-406]. Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid of the omega-3 family (n3-PUFA) and an important component of neuronal phospholipid membranes via which membrane fluidity may be affected [407]. Animal studies show that the combined supplementation of dietary membrane precursors like DHA, EPA,
Chapter 5  Improved spatial learning strategy and memory in aged Alzheimer AβPPswe/PS1dE9 mice

UMP, and choline, stimulates the synthesis of neuronal and synaptic membranes through the production of phospholipids via the Kennedy cycle [408-410]. In addition, the dietary interventions were found to increase dendritic spine density, and to improve learning and memory [410-412]. The combined intake of nutritional precursors and cofactors in neuronal membrane synthesis known as Fortasyn Connect® was recently shown to improve memory performance in early AD [413, 414]. The findings are in line with observations that brains of AD patients show decreased concentrations of choline, phospholipids, and polyunsaturated fatty acids like DHA [415-417]. Notably, epidemiological studies have shown that the plasma levels of DHA, folic acid, vitamin B12, vitamin E, and vitamin C are also lower in AD patients compared to age-matched controls [418-420], suggesting that the dietary intake of these nutrients may be relatively insufficient. In behavioural neuroscience the Morris water maze (MWM) navigation task is used to study spatial memory and learning [421]. Cognition of mice can be studied with the common MWM test, but the qualitative aspects of learning cannot be assessed. Originally, Wolfer and Lipp have shown that different behavioral strategies are available in the context of spatial learning [184, 185, 187, 422]. So the analysis of the different behavioral search strategies to find the hidden platform in the Morris water enables the identification of the actual hippocampal search strategies.

In the current study, we investigated the effects of the specific nutrient combination Fortasyn, containing the dietary precursors and cofactors for membrane synthesis, viz. DHA, eicosapentaenoic acid (EPA), UMP, choline, phospholipids, folic acid, vitamins B6, B12, C, E, and selenium, on spatial learning and memory in 11-month-old male AβPPswe/PS1dE9 [AβPP/PS1] mice [423] and wild-type C57BL/6J littermates mice. To this end, we analyzed the different search strategies to find the hidden platform in the Morris water maze. A parameter-based algorithm was used to assess the qualitative aspects of learning [183-187]. Our study investigated whether Fortasyn may improve spatial learning of transgenic mice by facilitating the use of hippocampus-dependent search strategies.

Materials and Methods

Animals & diets
The AβPPswe/PS1dE9 founder mice were obtained from John Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. of Pathology) [423, 424] and a colony was first established at the University of Kuopio, Finland and thereafter a colony was bred at the Central Animal Facility at the Radboud University Nijmegen Medical Centre, The Netherlands. In short, mice were created by co-injection of chimeric mouse/human AβPPswe (mouse AβPP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial
AD pedigrees) and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transfected genes co-integrate and co-segregate as a single locus [423]. This line was originally maintained on a hybrid background by backcrossing to C3HeJ×C57BL/6J F1 mice (so-called pseudo F2 stage). For the present work, the breeder mice were backcrossed to C57BL/6J for 13 generations to obtain mice for the current study at the Central Animal Facility of the Radboud University Nijmegen Medical Centre, The Netherlands. Throughout the experiments, the animals were housed socially with a maximum of six animals per cage, 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 the Radboud University Nijmegen Medical Centre. For the present experiment, we used a total of 58 male mice, of which 36 wild-type littermates, and 22 AβPP/PS1 mice. From two months of age, all wild-type and transgenic littermates were separated into distinct groups and fed either a control diet, similar to normal rodent chow, or a specific Fortasyn diet containing precursors and cofactors in membrane synthesis, such as EPA, DHA, phospholipids, UMP, choline, vitamins B6, B9, B12, C and E, folic acid and selenium for 9 months (Table S1). Both diets were isoenergetic, based on AIN93M [425], pelleted and kept at -20°C until use.

Morris water maze
From 11 months of age, all mice were trained in the water maze task to assess spatial learning capabilities. Mice were tested in a circular pool (104 cm diameter) filled with water (21-22 °C; made opaque by the addition of milk powder). During the task mice had to learn to find a hidden platform in the North-East (NE) quadrant of the pool starting from four different positions defined by the four cardinal points (South, North, East and West). Four colored shapes on the room walls were presented as distant visual cues.

Task acquisition
Mice performed four acquisition trials (maximal swimming time 120 seconds, 30 seconds on the platform and an inter-trial interval of 60 minutes) per day for four days. After each trial mice were placed on a paper towel for additional drying and then put back into their home cages.

Probe trial
At the end of the fourth day, the platform was removed from the pool and the mice had to perform a single probe trial (swimming time 120 seconds; starting
point: South) to test the short-term visuospatial memory. Swim paths, total swim distance, swim velocity, relative time spent in each quadrant, and platform crossings were recorded and analyzed using EthoVision (EthoVision XT 7.0, Noldus Information Technology, Wageningen, The Netherlands).

**Data analysis of the MWM**

During task acquisition general spatial learning was assessed using classical water maze parameters like latency to reach the platform, swim path length and swimming velocity. The measured velocity in the acquisition was monitored by EthoVision (EthoVision XT 7.0, Noldus Information Technology, Wageningen, The Netherlands). This measured velocity represents the averaged velocity during swimming throughout a trial, and not the velocity averaged over the total length over the trial. Furthermore, relative time spent in each pool quadrant and platform area crossings were calculated. For heatmaps the pool was divided into 10x10 cm wide sectors allowing the presence probability of a mouse in each sector to be represented as heatmap-like occupancy plots [183].

**Classification and Qualitative Analysis of Search strategies**

Wolfer and Lipp have shown that there are different behavioral strategies in the context of spatial learning in the water maze [184, 185, 187, 422]. During subsequent trials mice proceed towards hippocampus-dependent and thus increasingly directed and precise search strategies (Figure 1A), indicating both effective selection of stable visual cues as well as organizing these cues into a coherent cognitive map. To differentiate between hippocampus-dependent and independent search strategies, swim paths were analyzed using a parameter-based classification of search strategies developed by Garthe *et al.* [183]. In summary, swim path data recorded with an Ethovision video tracking system (Ethovision XT 7.0, Noldus Information Technology, Wageningen, Netherlands) were used to record the time-tagged xy-coordinates of the animals. The coordinates were then analyzed using an algorithm implemented in Matlab. In summary, due to stress by a novel environment and situation, the mice first show a persistent swim along the wall of the pool including possible sporadic swims toward the center of the pool, called thigmotaxis (wall-hugging swim). The next strategy of the animals is the random search pattern. Here, the animals could swim in straight swims (zig-zag pattern) through the total pool, or in wide circular swims. During the next strategy the mice scan their environment for landmarks, known as the scanning strategy. The next search behavior is called chaining. Here, the mice use circular swimming (in anticlockwise or clockwise direction) at a fixed distance from the wall being equal to the distance between platform and wall of the pool. During chaining the mice use the visual cues or the height of the wall of the pool to determine the
distance of the platform from the wall. Chaining is considered as a non-spatial strategy, because here the mice do not really make use of information stored in their hippocampus. Chaining is a successor of the scanning technique and helps the mice to find the platform more effectively than using the non-spatially precise search strategies as random search and scanning. Thereafter, by making increasing use of the visual cues, the animals develop a precise and place-specific preference for the goal platform by using search strategies as directed search, focal search and direct swimming being more spatially precise search strategies. For a brief overview see table 1 and for a detailed description of search patterns recognized and the parameters used for classification, see Garthe et al. [183].

**Table 1 Short descriptions of the search strategies.**

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Short description</th>
</tr>
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<tbody>
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<td>Highly emotional response to stress; wall hugging</td>
</tr>
<tr>
<td>Random Search</td>
<td>Total pool surface is covered; zig-zag pattern or circular swims; no directional preference</td>
</tr>
<tr>
<td>Scanning</td>
<td>Scanning of environment for landmarks; no directional preference</td>
</tr>
<tr>
<td>Chaining</td>
<td>Successor of scanning; circular swimming at a fixed distance from the wall (same as between platform &amp; wall); preference for goal annulus (correct distance)</td>
</tr>
<tr>
<td>Directed Search</td>
<td>Use of distant visual landmarks; directional preference; hippocampus-dependent</td>
</tr>
<tr>
<td>Focal Search</td>
<td>Animals spend most of the time close to the platform; hippocampus-dependent</td>
</tr>
<tr>
<td>Direct Swimming</td>
<td>Direct navigation to the platform regardless of the starting position; hippocampus-dependent</td>
</tr>
</tbody>
</table>

**Statistics**

For the statistical analysis, SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) for Windows was used. Repeated-measures ANOVA for the total task acquisition phase using genotype and diet fixed factors was used to analyze swim path length, velocity and latency time to reach the hidden platform. Moreover, to evaluate different aspects of spatial learning we analyzed the swim patterns for each trial and also performance per day. Due to the high trial-to-trial variation we divided the task into mean performances per separate day and used the days as within-subject factor for repeated measures ANOVA. First an overall repeated-measures ANOVA was performed using genotype, diet and search strategy as between-subject factors and the days as within-subject factor to elucidate the effects of the between subject factors across the acquisition time. When this statistical test reveals an interaction between search strategies, genotypes and diets across days, subsequent splitting of the data was performed. Furthermore, the Morris water maze data were analysed for each separate day of the acquisition phase using univariate ANOVA to detect differences in genotype- and diet-interactions in the use of the different search strategies during each single day. Probe trial performance was assessed using univariate ANOVA with the genotype and diet as between-subject factors to evaluate the differences in the latencies to reach the platform, swim distance and velocity, as well as time spent in each pool quadrant.
All ANOVAs were performed with the Bonferroni correction. In all cases, when overall statistical analysis showed a significant difference, we used the post-hoc Tukey’s HSD test to analyze the separate groups. The statistical significance was set at $p \leq 0.05$. Furthermore, also an univariate ANOVA to show eventual genotype-diet interactions on the body weight was performed. To determine the relationship between genotypes, diets or body weight between the swimming distance during the total probe phase, a bivariate correlation with a Pearson correlation coefficient ($r$) was used.

**Results**

**Body weight**

Only genotype ($F(1,50)=22.3, p<0.000$) and not diet ($F(1,50)=0.4, p<0.528$) affected the overall body weight of the animals, but a genotype-diet interaction was measured ($F(1,50)=4.3, p<0.043$). The AβPP/PS1 mice on Fortasyn diet showed increased body weight compared to their wild-type littermates on Fortasyn (AβPP/PS1-Fortasyn: $41.4g \pm 4.1g$; WT-Fortasyn: $33.8g \pm 3.4g$) ($F(1,23)=24.6, p<0.001$), while in comparison with the transgenic animals on control diet the AβPP/PS1 mice on Fortasyn exhibited only a non-significant increase in body weight (AβPP/PS1-Fortasyn: $41.4g \pm 4.1g$; AβPP/PS1-control: $39.8g \pm 4.2g$) ($F(1,27)=3.5, p<0.074$). Furthermore, AβPP/PS1 mice on control diet also had a slightly higher body weight than the wild-type mice on control diet (AβPP/PS1-control: $39.8g \pm 4.2g$; WT-control: $36.9g \pm 4.2g$) ($F(1,27)=3.5, p<0.074$), although it did not reach statistical significance. The wild-type mice on Fortasyn showed a decreased body weight compared to the wild-type group on control diet (WT-Fortasyn: $33.8g \pm 3.4g$; WT-control: $36.9g \pm 4.2g$) ($F(1,31)=5.1, p<0.030$). A summary of the significant differences can be found in table 2.

Additionally, also bivariate relationships between weight, genotypes, diets and swimming distance during the total time of the probe phase were performed by using *Pearson correlation* coefficients. An overall relationship analysis showed that no statistical significant correlations existed between weight ($r=-0.122; p<0.393$), genotypes ($r=-0.117; p<0.410$) nor diets ($r=-0.241; p<0.085$) compared with swimming distance.

**Morris water maze – Qualitative aspects of spatial learning**

**Acquisition**

Spatial learning and memory was assessed with the MWM. AβPP/PS1 mice needed significantly more time to reach the platform compared to wildtype mice during all days as shown by overall ANOVA for repeated measures (WT: $38.0s \pm 1.8s$; AβPP/PS1: $...
47.7±2.2) (F(1,55)=11.8, p<0.001), although both genotypes successfully learned to navigate to the hidden platform during the MWM. During the acquisition phase AβPP/PS1 mice exhibited an increased swim distance and swim velocity to reach the target platform compared to their wild-type littermates (Distance: Figure 1A: F(1,55)=14.7, p<0.000; Velocity: Figure 1B: F(1,55)=10.4, p<0.002). No diet effects or genotype-diet interactions were observed (Figure 1A+B).

**Search strategy**

To evaluate different aspects of spatial learning we analyzed the swim patterns for each trial (Figure 2B+C), but also performed further analysis on each single day performance (Figure 2D+E). The parameter-based classification of search strategies revealed that both wildtype and transgenic mice, irrespective of the specific diet used, showed the full range of search strategies to find the hidden platform. Due to the high trial-to-trial variation we divided the task into average single day performances and used the days as within-subject factor for repeated measures ANOVA. Repeated-measures ANOVA applied to the acquisition phase
task revealed an interaction between days, different search strategies, genotypes and diets across time (Figure 2D+E: F(1,24)=2.0, p<0.003). This allowed us to perform single day analysis on each single search strategy performance by ANOVA with genotype and diet as between-subject factors. Therefore, we were allowed to split our dataset into genotypes and diets to analyze each corresponding between-subject factor. As it can be seen clearly in figure 2D+E each animal group exhibit substantial day-to-day variation.

1st day of MWM
Genotype-effects
During the first day of the MWM AβPP/PS1 mice on control diet used more random search than wild-type mice on control diet (Figure 2D: F(1,31)=9.7, p<0.004). In agreement, the use of the directed search strategy was decreased in transgenic mice on control diet compared to their wild-type littermates (Figure 2D: F(1,31)=4.5, p<0.043).

Diet-effects
The supplementation with the Fortasyn diet led to an increase of the use of random search in wild-type mice compared to the wild-type mice on control diet (Figure 2D+E: F(1,34)=8.4, p<0.006). At the same time, wild-type mice on Fortasyn exhibited a tendency to use less directed search than their control diet fed littermates (Figure 2D+E: F(1,34)=3.5, p<0.070).

2nd day of MWM
Genotype-effects
During the second day of the acquisition phase AβPP/PS1 mice on Fortasyn used more thigmotaxis than their wild-type littermates on the same diet (Figure 2E: F(1,23)=8.943, p<0.007). On Fortasyn AβPP/PS1 mice showed a tendency to use the directed search strategy less than their wild-type littermates (Figure 2E: F(1,23)=3.7, p<0.068).

Diet-effects
No diet-effects were found on this acquisition day.

3rd day of MWM
Genotype-effects
On the third day of the acquisition phase, AβPP/PS1 mice on control diet used more thigmotaxis than their nontransgenic littermates on the same diet (Figure 2D: F(1,31)=4.6, p<0.024). In addition, AβPP/PS1 on control diet mice showed a
decreased preference for directed search compared to their wild-type littermates on this diet \((Figure\ 2D:\ F(1,31)=12.7,\ p<0.001)\). AβPP/PS1 mice on Fortasyn demonstrated a tendency to use less directed search \((Figure\ 2E:\ F(1,23)=4.2,\ p<0.052)\), and they also displayed more thigmotaxis \((Figure\ 2E:\ F(1,23)=6.6,\ p<0.017)\) than their wild-type littermates fed with this diet.

**Diet-effects**
Throughout the third day, the transgenic animals on Fortasyn used tendentiously less scanning search strategy \((Figure\ 2D+E:\ F(1,20)=3.8,\ p<0.065)\) than their transgenic littermates on control diet.

**4th day of MWM**

**Genotype-effects**
During the fourth day AβPP/PS1 mice on control diet used more random search than wild-type animals \((Figure\ 2D:\ F(1,31)=8.4,\ p<0.007)\). Only on the fourth day the transgenic mice on control diet tended to use the chaining strategy more often than the wild-type mice on the same diet \((Figure\ 2D:\ F(1,31)=3.9,\ p<0.059)\). The directed search strategy was used significantly less by the transgenic mice on Fortasyn than by their wild-type littermates fed with the same diet \((Figure\ 2E:\ F(1,23)=15.9,\ p<0.001)\). Notably, the AβPP/PS1 mice on a Fortasyn diet performed the chaining strategy more often than their wild-type littermates on Fortasyn \((Figure\ 2E:\ F(1,23)=10.5,\ p<0.004)\).

**Diet-effects**
During the fourth day the AβPP/PS1 mice fed with Fortasyn used tendentiously more chaining than their transgenic littermates on control diet \((Figure\ 2E:\ F(1,20)=4.1,\ p<0.057)\). Moreover, during the last MWM day the AβPP/PS1-Fortasyn group showed less directed search than their transgenic littermates on control diet \((Figure\ 2E:\ F(1,20)=4.7,\ p<0.042)\).
Figure 2 Qualitative analysis of spatial learning in 11-month-old AβPP/PS1 (TG) and C57BL/6J wild-type mice on Fortasyn and Control diets. (A) Examples of search strategies detected with the classification algorithm used. (B) In the wild-type animals on Control diet a clear progression towards increasingly spatially precise strategies was measured from day 1 to day 4, while the transgenic mice on Control diet did not. (C) Both groups of mice (AβPP/PS1 and C57BL/6J wild-type) on Fortasyn showed a changed pattern of used search strategies compared to the corresponding group. (D+E) Also the averaged daily pattern of the relative time engaged in specific search strategies to solve the MWM task are shown to reduce the enormous trial to trial variation in the prevalence of the different behavioral strategies.

**Morris water maze – Reference memory in AβPP/PS1 mice**

**Probe**

The ability to remember the platform location in the pool area was measured as probe trial performance one hour after the last regular trial on the last day of the MWM experiment.

The overall univariate ANOVA revealed a significant genotype-diet interaction in swim distance during the first thirty seconds and also during the total probe phase (1st thirty seconds: F(3,55)=6.6, p<0.013; Total time: F(3,52)=4.4, p<0.040). During the total probe and in the first thirty seconds of the probe trial significant
genotype differences in mice on Fortasyn were found regarding the swim distance (Figure 3A+B: 1st thirty seconds: F(1,24)=9.9, p<0.004; Total time: F(1,22)=4.5, p<0.045). More specifically, AβPP/PS1 mice on the Fortasyn diet swam less than their wild-type littermates (Figure 3A+B). In addition, during the total probe trial and also in the first thirty seconds of the probe trial diet effects were found in transgenic animals (Figure 3A+B: 1st thirty seconds: F(1,21)=6.8, p<0.016; Total time: F(1,19)=7.0, p<0.016). In detail, Fortasyn fed AβPP/PS1 mice swam less than AβPP/PS1 mice on control diet (Figure 3A+B).

Figure 3 Probe phase of the MWM - decreased swim distance in AβPP/PS1 mice (TG) on Fortasyn. (A) Analyzing the first thirty seconds of the MWM probe data revealed that the AβPP/PS1-Fortasyn group exhibited a decreased swim distance compared to their wild-type littermates on the same diet but also compared to their transgenic littermates on control diet. (B) This effect was also detected during the total MWM probe.
In the probe trial AβPP/PS1 animals needed more time to reach the platform position for the first time than their wild-type littermates demonstrating an overall genotype effect (Figure 4A: F(1,47)=8.1, p<0.007). An overall diet effect was found in both groups of animals. In detail, latency to reach the platform for the first time was lower in all animals on a Fortasyn diet compared to control diet (Figure 4B: F(1,47)=5.2, p<0.027).

The statistical analysis for the first thirty seconds revealed overall genotype-effects for the time spent in the North-East (NE) target quadrant. In detail, transgenic animals spent less time in the NE target quadrant than their wild-type littermates (Figure 5D: F(1,54)=6.1, p<0.017).

In addition, during the total time the same genotype-effects as during the first thirty seconds of the probe were found. Wildtype-animals still showed a preference for the NE target quadrant compared to their transgenic littermates (Figure 5E: F(1,50)=19.5, p<0.000).
Figure 5 Probe phase of the MWM - Spatial memory measured in 11-month-old AβPP/PS1 (TG) and C57BL/6J wild-type mice, on control & Fortasyn diet. The occupancy plots, respectively heatmaps, reveal the presence probability of each experimental group during the first thirty seconds (A) and also the total time (B) of the MWM probe trial. Analyzing the first thirty seconds and also the total MWM probe trial revealed that the wild-type mice spent more time in the NE target quadrant than transgenic mice, indicating successful spatial learning of the position of the platform by the wild-type animals (D+E).

A summary of the significant differences is given in table 2.
Table 2 Summary of the significant differences. A=WT-control, B=WT-Fortasyn, C=AβPP/PS1-control & D=AβPP/PS1-Fortasyn

<table>
<thead>
<tr>
<th>Acquisition</th>
<th>Weight</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>4th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: WT-control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B: WT-Fortasyn</td>
<td>&lt;A</td>
<td>Random</td>
<td>&gt;A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C: AβPP/PS1-control</td>
<td>Random &gt;A</td>
<td>Directed</td>
<td>&gt;A</td>
<td>Random &gt;A</td>
<td></td>
</tr>
<tr>
<td>D: AβPP/PS1-Fortasyn</td>
<td>&gt;B</td>
<td>Thigmotaxis &gt;B</td>
<td>Thigmotaxis &gt;B</td>
<td>Directed</td>
<td>Chaining &gt;B</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Probe</th>
<th>Latency to reach platform (s)</th>
<th>Swim distance (1st 30 seconds)</th>
<th>Swim distance (Total time)</th>
<th>Target quadrant preference (1st 30 seconds)</th>
<th>Target quadrant preference (Total time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: WT-control</td>
<td>(A+B)+(C+D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B: WT-Fortasyn</td>
<td>(A+C)+(B+D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C: AβPP/PS1-control</td>
<td>(A+B)+(C+D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D: AβPP/PS1-Fortasyn</td>
<td>(A+C)+(B+D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Several studies revealed the use of diverse search strategies in rats and AD transgenic mice [185, 187, 422, 426-429]. However, until now no studies demonstrated dietary influences on the use of search strategies in wild-type and transgenic mice, making the present study unique in this field. In this study we hypothesized that the identification of the search strategies used in the MWM may help to study the effects of dietary intake on spatial memory and learning abilities in mice.

In the present study the influence of a specific nutrient combination registered as Fortasyn® Connect, and comprising DHA, EPA, phospholipids, choline, UMP, vitamin B12, B6, and folate, vitamins C and E and selenium, was studied on cognitive decline in the AβPP/PS1 mouse model. Our results suggest that Fortasyn induced beneficial effects on learning and memory in both AβPP/PS1 mice and wild-type animals. During the acquisition phase of the MWM, AβPP/PS1 mice on Fortasyn focused more on the chaining strategy than their transgenic littermates on control diet, this effect was even more visible during the last acquisition day. Furthermore, AβPP/PS1 mice and also their wild-type littermates on Fortasyn showed a decreased latency to swim to the former platform position during the probe trials compared to their control groups indicating an improved memory. Fortasyn stimulated the transgenic mice to use the chaining strategy thereby developing a highly efficient way to reach the platform that is not dependent on an intact hippocampus [430]. While chaining is usually considered as a coping...
strategy, it seems to help to compensate for the cognitive decline in AβPP/PS1 mice. Referring to the least-effort principle, for searching a hidden goal in a comparatively small pool chaining can become highly effective. Therefore, Fortasyn improved the search behavior of these AβPP/PS1 mice by facilitating the use of a chaining strategy. For the given experimental conditions this affects the overall efficiency of these animals to find the hidden goal. In accordance to our results, another study showed that the supplementation with long chain polyunsaturated fatty acids and extra additives enhanced the performance of the cerebral hypoperfused animals in MWM tests [431]. Notably, our results are also not at variance with other studies showing that fish oil containing diets improve spatial memory in AβPP/PS1 mice and ameliorate performance in hippocampus dependent spatial memory tests like the MWM [432-434].

During the probe trial of the Morris water maze in the present study, transgenic mice showed no clear preference for the target quadrant neither during the first thirty seconds nor the total time of the probe. While wild-type mice exhibited a clear goal preference during the first thirty seconds and also the total time of the probe. One possible explanation could be that the chaining search pattern during the fourth day of the MWM was the dominant search strategy of both transgenic groups. Moreover, the AβPP/PS1-Fortasyn group used this strategy even more often than their transgenic littermates on control diet. Again, chaining is considered as circular swimming at a fixed distance from the wall equally to the distance between platform and wall of the pool [422]. During the probe trial, the platform is removed from the pool and these aforementioned transgenic mice used the chaining strategy to find the platform resulting in serial visits and circular swimming. This searching behavior resulted in equal search times in all quadrants of the MWM pool during the probe trial in both transgenic groups and explains our findings that no clear preference for any quadrant could be found. Chaining is a normal part of the strategy pattern, which is increased in AD mice after the supplementation with Fortasyn resulting in zero quadrant preference, as observed in the probe trial, when the platform is removed.

The analysis of the probe phase of the MWM showed that all transgenic mice took more time to reach the platform position for the first time than their wild-type littermates indicating impaired memory. This supports the AD-like pathology of the AβPP/PS1 mice presenting cognitive decline and impaired memory [435]. Notably, all mice on Fortasyn diet showed a shorter latency to reach the former platform position in the probe trial than mice on control diet. In addition, during the probe phase AβPP/PS1 mice on Fortasyn swam also less compared to almost all other animal groups indicating an improvement of their swim efficiency. This specific nutrient combination was able to improve memory performance in transgenic and also nontransgenic mice on Fortasyn diet. From this latter
result we conclude that Fortasyn diet is beneficial irrespective of genotype due to an improvement in the latency time to reach the platform for the first time in both wild-type and transgenic mice. In a double-blind, placebo-Controlled human study using functional near IR spectroscopy (NIRS) to measure cerebral hemodynamics, the supplementation of a DHA-rich diet increased the cerebral blood flow in response to cognitive tasks [436], showing already that this results and ours are in line with each other. In AD, cerebral phospholipids [415, 416] and synaptic connections are reduced [437, 438] being thought to contribute also to the loss of cognitive functioning. In a randomized, double-blind human study a twelve weeks long supplementation with the medical food Souvenaid, containing Fortasyn® Connect, improved memory (delayed verbal recall) in mild AD patients [413]. Recently, Scheltens et al. demonstrated again that Souvenaid is well tolerated and improves memory performance in drug-naïve patients with mild AD [414]. Their EEG outcomes suggested also that Souvenaid had an effect on brain functional connectivity [414]. Our previous mouse studies have shown that feeding AβPP/PS1 mice specific nutrient-enriched diets can diminish the AD-like pathology [439, 440]. We now show that, in 15-month-old AD mice on the Fortasyn diet the amount of Aβ was decreased, the relative cerebral blood volume was slightly increased and the spatial memory of these mice was improved being again in accordance with our results. Other experiments have also shown that the membrane phospholipid synthesis is stimulated, the dendritic spine density is increased, and also learning and memory are improved, when the two synergistically acting components, DHA and UMP, are enriched in the diet [410-412].

In our present study we observed a decreased swim distance in the AβPP/PS1 mice on Fortasyn diet compared to their transgenic littermates on control diet and also to the wild-type mice on Fortasyn. Previous studies have shown that AβPP/PS1 mice were more active than wild-type mice in the open field test [434, 435]. In general hyperactivity seems to be a specific characteristic of many AβPP transgenic mice [441-443] and this effect may be related to elevated levels of anxiety in these animals [443, 444]. However, in the present study we did not find higher activity levels in the AβPP/PS1 mice, not even in the ones on control diet, rendering the possibility unlikely that this characteristic was of influence on the present observations. However, in the present study both AβPP/PS1 groups had higher body weights than the wild-type control groups, while the change in swimming distance was only observed in the AβPP/PS1 mice on Fortasyn diet. Despite their reduced swimming distance, the AβPP/PS1 mice on Fortasyn do show an improved task performance. It is possible that the adopted search strategy in these animals may have contributed to both observations.

In summary, our present results show that Fortasyn improved spatial learning and
memory in mice as evidenced by a reduced latency to find the hidden platform in the MWM. Importantly, AβPP/PS1 mice on Fortasyn diet showed a tendency to use more chaining than controls to master the task, albeit without relying on the hippocampus. Whether this adapted cognitive performance is due to a reduced AD pathology or to an improved ability to cope with such pathology needs further investigation.

**Supplementary material**

Supplementary material can be found here: http://dx.doi.org/10.3233/JAD-130179
A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice


¹ The authors contributed equally to the present work.
Abstract

APOE ε4 (apoE4) polymorphism is the main genetic determinant of sporadic Alzheimer’s disease (AD). A dietary approach (Fortasyn) including docosahexaenoic acid, eicosapentaenoic acid, uridine, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium has been proposed for dietary management of AD. We hypothesize that the diet could inhibit AD-like pathologies in apoE4 mice, specifically cerebrovascular and connectivity impairment. Moreover, we evaluated the diet effect on cerebral blood flow (CBF), functional connectivity (FC), gray/white matter integrity and post-synaptic density in aging apoE4 mice. At 10-12 months, apoE4 mice did not display prominent pathological differences compared to wild-type (WT) mice. However, 16-18 month-old apoE4 mice revealed reduced CBF and accelerated synaptic loss. The diet increased cortical CBF, amount of synapses, and improved white matter integrity and FC in both aging apoE4 and WT mice. We demonstrated that protective mechanisms on vascular and synapse health are enhanced by Fortasyn, independent from apoE genotype. We further showed the efficacy of a multi-modal translational approach, including advanced MR neuroimaging to study dietary intervention on brain structure and function in aging.
Introduction

Extensive research has been pursued in search for an effective therapy for Alzheimer’s disease (AD). However, no treatment is yet available nor seems near. Preventive approaches have therefore consistently emerged as key policy priorities in recently formulated dementia strategies. These approaches include modification of health-compromising behavior such as lifestyle and dietary intake that may lead to AD. For example, the Mediterranean diet (high consumption of fruit, vegetables and legumes, moderate consumption of fish, nuts and olive oil as the main source of fats), has been associated with a reduced risk of AD [445, 446] and with a lower mortality [447, 448]. The mechanisms via which diet influences the onset and progression of AD pathology are still under investigation. One possible mode of action is the beneficial effect of nutrients, such as omega-3 long-chain polyunsaturated fatty acids (n3-LCPUFAs) on the vascular system [448, 449].

Herewith n3-LCPUFAs will target the very early, asymptomatic phase of the disease, in which (cerebro)vascular impairment is the strongest contributor to the onset and progression of neurodegenerative traits of typical AD and dementia in general [450]. N3-LCPUFAs may diminish severity of vascular risk factors, like atherosclerosis [451], high blood pressure [452] and other cardiovascular diseases [451, 453-455], which are risk factors for AD as well. Other nutrients may instead directly protect synaptic integrity. For instance, the formation of phosphatidylcholine, the most common phosphatide in the brain and a major component of the synaptic membrane, is enhanced due to presence of its precursors in the diet [456, 457]. Several preclinical studies confirmed these findings, showing that animals supplemented with the combination of these membrane precursors showed increased levels of brain phospholipids, dendritic spines and neurite outgrowth, with beneficial effects on cognition [260, 458-461]. Based on these findings, a novel multi-nutrient supplementation diet called Fortasyn, comprising n3-LCPUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), besides other precursors and cofactors for membrane synthesis, such as uridine, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium, has been proposed for the dietary management of AD [462]. 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 nutrient combination [463-465]. Although it has been recognized that Fortasyn addresses specific nutritional needs in early AD and that it improves functional connectivity as assessed by EEG [466], other processes by which Fortasyn may influence the pathophysiology of AD need to be further elucidated, and more studies are required to confirm its efficacy.
Some studies suggested an interaction of the cholesterol transporter apolipoprotein (apoE) with lipid-based dietary intervention [467-469]. ApoE is a 34-kDa glycoprotein that exists in three isoforms: apoE-ε2, -ε3 and -ε4, which differ by one or two amino acid residues 112 and 158 [470]. This small difference strongly affects the conformation and the structure of apoE and influences its ability to bind lipids, receptors and amyloid-β (Aβ) [471]. ApoE polymorphic alleles have been identified as the main genetic determinants of AD risk; specifically, apoE-ε4 has been associated with increased toxicity, and loss of neuroprotective function in the pathogenesis of Alzheimer disease, dependent or independent from Aβ accumulation [53]. Importantly, some of these processes are directly or indirectly linked to an impaired vascular system [472-474]. The development of an appropriate animal model, targeting the murine APOE gene for replacement with the human APOE-ε4 (apoE-ε4/apoE4 mouse) [475], opened a window for new possibilities to characterize the apoE-ε4 phenotype and to study the effects of very early AD-like pathology development in relation with lipid-based treatment. ApoE4 carrier mice exhibit an altered lipid profile, with increased risk of atherosclerosis plaques formation [475]. Altered behavior and cognitive deficits have also been reported [476]. A previous study from our group showed that a long-term dietary intervention with the Fortasyn diet was able to reduce anxiety behavior in 10-month-old apoE4 mice [477]. Transgenic AD mice on the same diet showed restored cortical cerebral blood flow (CBF) and brain structural integrity compared to wild-type mice [461].

Following these promising findings, we hypothesize that a nutritional intervention with Fortasyn may be able to rescue or prevent the occurrence of early AD-like pathologies in apoE4 mice, such as cerebrovascular impairment and concomitant brain connectivity loss. To test this hypothesis, we evaluated the effect of the Fortasyn diet on cerebral and plasma levels of fatty acids and sterols, cerebral blood flow (CBF), gray and white matter integrity, functional connectivity (FC) and post-synaptic density during aging in 12- and 18-month-old apoE4 mice.

**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 medical center (Radboudumc). ApoE4 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 [478]. Resulting chimeras were backcrossed to C57BL/6J mice for 8 generations. The line was derived by embryo transfer and is maintained by inbreeding homozygous mice. For the present study,
male and female apoE4 breeder mice were used to generate homozygous apoE4 offspring (3rd generation). C57BL/6J wild-type mice (WT), obtained from our colony at the Radboudumc 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 Radboudumc, the Netherlands, approved all the protocols within this study (RU-DEC 2011-058).

**Diets and timeline of experimental design**

Starting at 2 months of age, mice were randomly divided in two groups; animals were fed either with control diet or Fortasyn diet that differed with respect to the presence of a specific combination of dietary precursors and cofactors, i.e. uridine, docosahexaenoic acid, eicosapentaenoic acid, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium (Table 1). Both diets were isocaloric and based on AIN-93M [479] with 5% fat. The control diet contained 1.9% soy oil, 0.9% coconut oil and 2.2% corn oil; the Fortasyn diet contained 0.1% coconut oil, 1.9% corn oil and 3.0% fish oil. The Fortasyn-based diet contains a specific multi-nutrient composition comprising nucleotides, omega-3 PUFAs, choline, B vitamins, phospholipids and antioxidants (Table 1). Diets were manufactured and pelleted by Ssniff (Soest, Germany) and stored at −20°C until use. The first group of mice underwent MR imaging at 11 (average age: 10.6 ± 0.1 months) to 12 months of age (average age: 12.3 ± 0.1 months) and was sacrificed immediately thereafter. The second group was scanned at 16 months of age (average age: 16.2 ± 0.1 months) and sacrificed at 18 months of age (average age: 17.9 ± 0.1 months). The time line of the experimental design is illustrated in figure 1. The sample size of minimal 6 mice (12-Month: WT-control n=9, WT-Fortasyn n=9, apoE4-control n=8, apoE4-Fortasyn n=10; 18-Month: WT-control n=10, WT-Fortasyn n=10, apoE4-control n=10, apoE4-Fortasyn n=6) per group was chosen based on formal calculation of power as described in the approved protocols (RU-DEC 2011-058).
Table 1. Compositions of the experimental diets used, based on AIN-93M [479] with minor revisions

<table>
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<tr>
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<tr>
<td>Vitamin mix (AIN-93-VX)</td>
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**Fats**

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<td>Coconut oil</td>
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<td>Fish oil</td>
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**Additions**

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<td>Folic acid (90 %)</td>
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<td>Cyanocobalamin (0.1 % in mannitol)</td>
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<td>Ascorbic acid (100 % pure)</td>
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Figure 1: Timeline of experimental design. Starting from 2 months of age, mice were randomly divided in two groups; animals were fed either with control diet or Fortasyn diet. The first group of mice underwent MR imaging (MRI) at 11 to 12 months of age and were sacrificed (†) immediately thereafter. The second group was sacrificed after MRI at 18 months of age.

MR imaging
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 Paravision 5.1 software between 8 am and 8 pm. 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, low-dose isoflurane was used (3.5% for induction and ~1.5% for maintenance), slightly adjusted throughout the experiment to maintain a fast and stable breathing frequency (>130 bpm). The mice were placed in a stereotactic device in order to immobilize the head. Body temperature was measured with a rectal thermometer and maintained at 37°C by a heated air flow device.

After standard adjustments and shimming, gradient echo (GE) T$_2^*$-weighted images covering the entire mouse brain were acquired for anatomical reference.

To study brain perfusion under resting conditions, we used a flow-sensitive alternating inversion recovery arterial spin labelling (FAIR ASL) technique [461, 480]. Briefly, 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. Relative cerebral blood flow (CBF) was measured in the cortex, in the hippocampus and in the thalamus based on [461].

Diffusion of water was imaged as described previously [228, 477]. In short, 22 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.

The diffusion tensor was estimated for every voxel using the PATCH algorithm [230]; mean water diffusivity (MD) and fractional anisotropy (FA) were derived from the tensor estimation following a protocol as described elsewhere [477]. MD and FA values were measured in several white matter (WM) and grey matter (GM) areas, which were manually selected based on an anatomical atlas [481].

The resting state fMRI (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). Mean and maximum displacement across the six degrees of freedom (along the x-, y-, and z-axes and on three rotation parameters pitch, roll, and yaw) were measured in each mouse. The mean SE-EPI images of each mouse were then used to generate a study-specific template through linear affine and nonlinear diffeomorphic transformation (ANTS. v1.9; http://picsl.upenn.edu/ANTS/). Visual inspection of the normalized dataset was performed to screen for possible normalization biases. On the template, 15 areas were selected in left and right hemisphere. The selected regions were based on previous work in functional connectivity in mice [231], and included: left and right dorsal hippocampus, left and right ventral hippocampus, left and right auditory cortex, left and right motor cortex, left and right somatosensory cortex, and left and right visual cortex. All cortical ROI were selected 1–2 voxels away from the edge of the cortex, to minimize the impact of susceptibility-weighted artifacts, which are more prominent in areas of different tissues interface (e.g., near the skull or near the ear canals). After motion regression, in-plane spatial smoothing (0.4 × 0.4 mm), linear detrending, and temporal high-pass filtering (cutoff at 0.01 Hz) were applied to compensate for small across-mouse misregistration and temporal low-frequency noise. FC group comparison between ROIs were calculated from the BOLD time series using total correlation and partial correlation analyses implemented in FSLNets (FSLNets v0.3; www.fmrib.ox.ac.uk/fsl). Pearson’s correlation values were Fisher transformed to Z-scores for group comparisons and statistical analysis.

**Brain tissue preparation**

Directly following the MR measurements at 12 and 18 months of age, anaesthetised mice were sacrificed by transcardial perfusion with 0.1M phosphate buffered saline (PBS). The perfused brains were cut sagittally and the right hemispheres were snap frozen in liquid nitrogen and stored at -80°C, before further biochemical processing. The left hemispheres were immersion fixated 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.

**Immunohistochemistry - PSD95**
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 Polyclonal rabbit anti-PSD95 antibody (1:2000; Abcam Cat# ab18258, RRID:AB_444362) using one complete series of brain sections. Immunohistochemistry was performed using standard free-floating labelling procedures, as described previously.

**Quantification**
The stained sections were analysed 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 analysed. 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, and Bethesda, Maryland, USA). The contrast was manually enhanced, following the same procedure for all digitized images, and the amount of tissue stained was measured with a threshold-based approach.

**Biochemical analyses**
Serum and brain sterol levels were measured by gas-chromatography-mass-spectrometry-selected-ion-monitoring (GC-MS-SIM) as described in detail previously [234]. The cerebellum of the right hemisphere was homogenized and sterols were extracted overnight by chloroform/methanol trimethylsilylation prior to GC-MS-SIM analysis [234]. Brain fatty acid analyses were performed with a part of the brain homogenate (olfactory bulb and part of frontal cortex), as described previously [477].

**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 effects of diet supply at two stages in which apoE4 mice may develop different neuropathological traits of AD, statistical analyses were performed separately for the two age-points.
Multivariate ANOVA (MANOVA) with Bonferroni corrections, using body weight as covariate when necessary, was conducted with between-group-factors genotype and diet to analyze possible differences in all the other parameters. If the Bonferroni post hoc test indicated a significant interaction between genotype and diet, the data were split for the concerning factor and thereafter analyzed again with the MANOVA. Statistical significance was set at $p \leq 0.05$. All data are expressed as mean ± SEM.

**Results**

Mortality rate in the apoE4 mice was normal until age 18 months (<8% total mortality rate) in both diet groups. None of the WT animals died during the experiment.

**Body weight**

**10-12 month-old mice**

Body weight was measured at 10 and 12 months of age (figure 2A). Statistical analysis revealed a significant genotype x diet interaction ($p=0.050$). ApoE4 and WT mice fed the Fortasyn diet were significantly heavier than animals on control diet (ApoE4: $F(1,18)=46.6$, $p<0.001$; WT: $F(1,17)=31.2$, $p<0.001$). ApoE4 mice on Fortasyn were significantly heavier compared to WT mice on Fortasyn ($F(1,17)=7.3$, $p=0.015$).

At the start of the MRI measurements a significant genotype x diet interaction on bodyweight was found ($p=0.018$). Again, all animals on Fortasyn diet showed an increased body weight compared to animals on control diet (ApoE4: $F(1,18)=50.1$, $p<0.001$; Wild-type: $F(1,17)=6.7$, $p=0.019$). ApoE4 mice on Fortasyn were heavier than wild-type mice on the same diet ($F(1,17)=7.6$, $p=0.013$).

**16-18 month old mice**

Body weight was measured at 16 and 18 months of age (figure 2B). In both measurements, ApoE4 mice weighed significantly less than WT mice (16 months: $F(1,33)=10.3$, $p=0.003$; 18 months: $F(1,33)=9.0$, $p=0.005$). Both at 16 and 18 months of age, all animals on Fortasyn were heavier than animals on control diet (16 months: $F(1,33)=5.6$, $p=0.024$; 18 months: $F(1,33)=4.5$, $p=0.042$).
Chapter 6

A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Figure 2: Body weight was measured in 10-12 month old (A) and 16-18 month old ApoE4 and wild-type mice (WT). (A) At 10 months of age, ApoE4 and also wild-type mice on Fortasyn were significantly heavier than on control diet (ApoE4: p<0.001; Wild-type: p<0.001). Only on Fortasyn diet a genotype effect was found showing a higher weight of ApoE4 mice compared with wild-type mice (p=0.015). Again at 12 months of age, all animals on Fortasyn diet showed an increased body weight compared to animals on control diet (ApoE4: p<0.001; Wild-type: p=0.019). ApoE4 mice on Fortasyn were heavier than wild-type mice on the same diet (p=0.013). (B) At 16 and also 18 months of age, ApoE4 mice were significantly lighter than wild-type mice (16-Month: p=0.003; 18-Month: p=0.005). Furthermore, all animals on Fortasyn were heavier than animals on control diet (16-Month: p=0.024; 18-Month: p=0.042).

Magnetic Resonance Imaging

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. In all measures, CBF was not significantly influenced by body weight.

In the 12-month-old mice, we detected a genotype×diet interaction in the thalamus (p=0.045). In detail, Fortasyn diet increased thalamic CBF (F(1,16)=5.0, p=0.040) more strongly in WT mice than in the apoE4 littermates (figure 3A). In the 18-month-old mice (figure 3B), CBF was decreased in the cortex (F(1,31)=4.4, p=0.044) and in the thalamus (F(1,31)=5.7, p=0.023) of apoE4 mice as compared to WT mice. Cortical CBF was increased by the Fortasyn diet in both WT and apoE4 mice (F(1,31)=4.7, p=0.038).
Chapter 6  A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Figure 3: Cerebral blood flow (CBF) as measured with a flow-sensitive MRI technique (FAIR ASL) at 12 months and 18 months of age in C57BL/6J wild-type control mice and ApoE4 transgenic mice on control diet or Fortasyn diet. Three regions of interest (ROI) on the left and right brain hemispheres were analysed: cortex, hippocampus and thalamus. (A) In the 12-month-old mice we detected a genotype×diet interaction in the thalamus ($p=0.045$). Fortasyn diet increased thalamic CBF ($p=0.040$) more strongly in wild-type mice than in the apoE4 littermates. (B) In the 18-month-old mice, CBF was decreased in the cortex ($p=0.044$) and in the thalamus ($p=0.023$) of apoE4 mice as compared to wild-type mice. Cortical CBF was increased by the Fortasyn diet in both wild-type and apoE4 mice ($p=0.038$).

**Diffusion Tensor Imaging**
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 apoE4 and non-transgenic WT mice (figure 4A).

**Fractional anisotropy**
In the 12-month-old mice, we did not detect any significant differences in white matter FA between the groups of mice (figure 4B).
At 18 months of age (figure 4C), Fortasyn fed mice showed a lower FA at -0.7mm in the corpus callosum compared to control fed mice ($F(1,31)=4.7$, $p=0.008$).

**Mean diffusivity**
In the 12-month-old mice, the MANOVA revealed a genotype×diet interaction for MD in the motor cortex ($p=0.024$, figure 4B).
In detail, WT mice on Fortasyn diet had higher MD in the motor cortex than wild-type mice on control diet ($F(1,14)=9.2$, $p=0.009$). Furthermore apoE4 mice on Fortasyn diet had a lower MD in the motor cortex than WT mice on Fortasyn ($F(1,17)=5.2$, $p=0.036$). Fortasyn diet decreased MD in the auditory cortex ($F(1,29)=4.9$, $p=0.034$), and in the somatosensory cortex ($F(1,29)=7.9$, $p=0.009$) compared to control diet, irrespective of genotype.
At 18 months of age (figure 4c), apoE4 mice displayed an increased MD in the auditory cortex (F(1,29)=5.6, p=0.025) compared to WT mice.

**Figure 4:** Quantitatively assessed diffusion tensor derived indices at 12 months and 18 months of age in C57BL/6J wild-type control mice and ApoE4 transgenic mice on control diet or Fortasyn diet. (A) Fractional anisotropy (FA) and mean diffusivity (MD) were measured for ROIs drawn in white and gray matter regions, respectively. (B) In 12-month-old mice, no differences in FA were observed. In the 12-month-old mice, the wild-type mice on Fortasyn diet had higher MD in the motor cortex than wild-type mice on control diet (p=0.009). Furthermore apoE4 mice on Fortasyn diet had a lower MD in the motor cortex than wild-type mice on Fortasyn (p=0.036). Fortasyn diet decreased MD in the auditory cortex (p=0.034), and in the somatosensory cortex (p=0.009) compared to control diet, irrespective of genotype. (C) At 18 months of age, Fortasyn fed mice had a lower FA at -0.7mm in the corpus callosum than Control fed mice (p=0.008). At 18 months of age, apoE4 mice displayed an increased MD in the auditory cortex (p=0.025).
A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Chapter 6

rsfMRI

Total correlation analyses
To compare the FC patterns between different genotypes and diets, rsfMRI data were statistically analyzed based on total correlation (figure 5) and partial correlation (figure 6).

At 12 months of age, multivariate ANOVA (MANOVA) revealed some significant genotype but no diet effects in apoE4 compared to WT mice. More specifically, apoE4 mice showed reduced FC between the right auditory cortex and the left dorsal hippocampus \(F(1,24)=5.1, \ p=0.033\), and also between the left visual cortex and the right auditory cortex \(F(1,24)=5.5, \ p=0.028\).

At 18 months of age, the MANOVA demonstrated several significant genotype and diet effects. In detail, apoE4 mice had significant lower FC between cortical and hippocampal regions, but also between cortical regions themselves. Notably, Fortasyn was able to increase the hippocampal FC, and also FC between the visual and retrosplenial cortex to the hippocampus.

The MANOVA revealed genotype×diet interactions in the left auditory cortex to retrosplenial cortex \(p=0.011\), in the right auditory cortex to right somatosensory cortex \(p=0.05\), and in the left somatosensory cortex to retrosplenial cortex \(p=0.025\). ApoE4 mice on control diet showed a reduced FC between left auditory cortex and retrosplenial cortex \(F(1,15)=17.9, \ p=0.001\), right auditory cortex and right somatosensory cortex \(F(1,15)=4.9, \ p=0.042\), and left somatosensory cortex to retrosplenial cortex \(F(1,15)=18.2, \ p=0.001\), which were not observed in ApoE4 mice on Fortasyn diet.

Moreover, compared to their transgenic littermates on control diet only apoE4 mice on Fortasyn displayed an increased FC between left auditory cortex to retrosplenial cortex \(F(1,11)=13.0, \ p=0.004\), right auditory cortex to right somatosensory cortex \(F(1,11)=9.9, \ p=0.009\), and left somatosensory cortex to retrosplenial cortex \(F(1,11)=10.2, \ p=0.009\).
A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Figure 5: Resting-state functional connectivity (FC) based on total correlation analyses of 15 regions of interest (ROI) in the mouse brain. Total correlation matrices of wild-type and apoE4 at 12 and 18 months of age, both on Fortasyn and control diets. At 12 months of age, multivariate ANOVA (MANOVA) revealed some significant genotype but no diet effects in apoE4 compared to wild-type mice, (figure 5B). More specifically, apoE4 mice showed reduced FC between the right auditory cortex and the left dorsal hippocampus (p=0.033), and also between the left visual cortex and the right auditory cortex (p=0.028). At 18 months of age, apoE4 mice had significant lower FC between cortical and hippocampal regions, but also between cortical regions themselves. Notably, Fortasyn was able to increase the hippocampal FC, and also FC between the visual and retrosplenial cortices to the hippocampus.
Partial correlation analyses
At 12 months of age, significant genotype and diet effects were shown using MANOVA (figure 6). In detail, apoE4 mice showed a reduced FC between left dorsal and ventral hippocampus (F(1,24)=4.7, \( p=0.040 \)). Additionally, animals on Fortasyn diet showed an increased partial correlation in the interhemispheric connection between left and right ventral hippocampus (F(1,24)=7.3, \( p=0.012 \)).

At 18 months of age, the MANOVA showed several significant genotype and diet effects (figure 6). ApoE4 mice had a reduced FC between the right auditory cortex and the right motor cortex (F(1,26)=16.4, \( p<0.001 \)). The Fortasyn diet increased FC between left and right motor cortices (F(1,26)=7.1, \( p=0.013 \)), but slightly reduced FC between left and right dorsal hippocampus (F(1,26)=4.4, \( p=0.045 \)). MANOVA also revealed a genotype×diet interaction between right auditory cortex to right somatosensory cortex, \( p=0.010 \). Fortasyn diet increased partial correlation in apoE4 mice compared to WT between right auditory cortex to right somatosensory cortex, (F(1,12)=8.1, \( p=0.015 \)).

**Postsynaptic density protein-95 (PSD-95)**
Density of postsynaptic densities were visualised and quantified immunohistochemically in various cortical and hippocampal areas with polyclonal rabbit anti-PSD-95. At 12 months, we did not observe significant differences between groups (figure 7). At 18 months, reduced PSD-95 levels were seen in the sensory cortex of apoE4 mice compared to WT mice (F(1,33)=5.8, \( p<0.021 \)). Fortasyn diet increased levels of PSD-95 in the sensory cortex (F(1,33)=6.7, \( p<0.014 \), CA3 area (F(1,31)=9.9, \( p<0.004 \)), and IML (F(1,31)=9.9, \( p<0.004 \)), irrespective of genotype. No genotype × diet interactions were observed.
A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Chapter 6

Figure 6: Resting-state functional connectivity (FC) based on partial correlation analyses of 15 regions of interest (ROI) in the mouse brain. (A) Total correlation matrices of wild-type and apoE4 at 12 and 18 months of age, both on Fortasyn and control diets. At 12 months of age, apoE4 mice showed a reduced FC pattern between left dorsal and ventral hippocampus (p=0.040). Additionally, animals on Fortasyn compared to control diet showed an increased partial correlation in the interhemespheric connection between left and right ventral hippocampus (p=0.012). (C) At 18 months of age, ApoE4 mice on both diets had a reduced FC between the right auditory cortex and the right motor cortex (p<0.001). The Fortasyn diet caused contrasting effects: a higher FC was found between left to right motor cortices (p=0.013), but a slightly FC reduction was revealed between left to right dorsal hippocampus (p=0.045). Fortasyn diet increased partial correlation in apoE4 mice compared to their wild-type littermates between left to right auditory cortex (p=0.015), and between right auditory cortex to right somatosensory cortex, (p=0.015). In wild-type mice FC between left somatosensory cortex to left auditory cortex (p=0.041) was higher in Fortasyn fed animals compared to control fed animals.
A dietary treatment improves cerebral blood flow and brain connectivity in aging apoE4 mice

Chapter 6

Figure 7: Levels of postsynaptic density-95 (PSD-95) in various brain areas in wild-type mice and apoE4 mice on either control diet or Fortasyn diet. (A) At 12 months, we did not find significant differences between groups. (B) At 18 months, reduced PSD-95 levels were seen in the sensory cortex of apoE4 mice compared to wild-type mice (p=0.021). Fortasyn diet increased levels of PSD-95 in the sensory cortex (p=0.014), CA3 area (p=0.004), and IML (p=0.004), irrespective of genotype. No genotype × diet interactions were observed.

Fatty acids
Fatty acid content was analysed in the brains of apoE4 and WT mice (supplementary material + supplementary table 1). At both 12 and 18 months of age, increased relative levels of omega-3 fatty acids (p=0.000), and an increased ratio of omega 3/6 were found in Fortasyn fed mice compared to their littermates on Control diet (p=0.000). At 18 months of age, ApoE4 mice displayed significantly increased relative arachidonic acid (p=0.022) and relative omega-6 content (p=0.038) compared to wild-type mice. For a detailed overview see the fatty acid section in the supplementary material and supplementary table 1.

Sterol levels
Sterol levels were determined in the blood plasma (serum) and in the cerebellum of the brain. The main findings are described below, for all detailed results see supplementary material and supplementary table 2 respectively 3.

Blood serum
At 12 months of age, apoE4 mice displayed increased level of the cholesterol precursor, dihydro-lanosterol, (p=0.000) compared to WT mice. At 12 months of age, apoE4 mice on control diet had higher levels of lathosterol (p=0.002) and lanosterol (p=0.003) compared to WT mice on control diet. At 18 months of age, apoE4 and WT mice on control diet displayed increased levels of campesterol (p=0.000), sitosterol (p=0.016), lanosterol (p=0.010), desmosterol (p=0.000), 24OH-cholesterol (p=0.002) and cholesterol (p=0.033) compared to apoE4 and WT mice on Fortasyn diet.
Cerebellum
At 12 months of age, apoE4 and WT mice on control diet demonstrated increased levels of lathosterol ($p=0.003$), campesterol ($p<0.000$), and lanosterol ($p=0.001$) in the cerebellum.

At 18 months of age, cerebellar cholesterol levels were unchanged in ApoE4 mice compared to WT mice ($p=0.614$). In control fed mice levels of campesterol and precursors of cholesterol, lathosterol and lanosterol, were higher than in Fortasyn fed mice (Campesterol, $p<0.000$; Lathosterol, $p<0.000$; Lanosterol, $p=0.011$).

Discussion

ApoE4 mice as model for the early asymptomatic phase in AD
In this study, we investigated the extent to which apoE4 mice display cerebrovascular flaws, synaptic loss and connectivity during aging. The ε4 allele of the apoE gene is strongly associated with sporadic AD [188]. Among several proposed mechanisms by which apoE4 promotes AD, there are indications that apoE4 is less effective in synaptic repair and remodelling processes compared to other isoforms [482, 483]. Moreover, apoE4 carriers are clearly more susceptible to vascular brain damage (eg. stroke, brain haemorrhage; [52-54] and they display aberrant functional connectivity [55]. Similarly, the apoE4 mouse line exhibits increased risk of developing vascular disorders and neuronal deficits due to altered cholesterol metabolism, especially when challenged with a high-fat diet [475]. Recently we also described spontaneous functional connectivity deficits in these mice, possibly associated with cerebral blood flow reduction [484]. Because these deficits seem to aggravate with aging we investigated this mouse line at two different ages.

At 10-12 months of age, apoE4 mice did not display many differences compared to WT animals. Despite some slight alterations in the sterol levels of 12-month-old apoE4 mice, all other measured parameters including cerebral blood flow and number of post-synapses were unaffected compared to WT animals. Previously, we have shown that at this age also cerebral blood volume, amount of pre-synaptic boutons and neurogenesis did not differ from WT mice [477]. The lack of cerebrovascular alterations like a reduced CBF at this age may explain the absence of pathologies.

However at 16-18 months of age, apoE4 mice revealed reduced CBF and accelerated neurodegeneration, which are typical features of prodromal AD [485-488]. Specifically, we detected reduced thalamic and cortical perfusion, reduced cortical post-synaptic density, increased cortical mean diffusivity (MD) and reduced fractional anisotropy (FA) in white matter tracts. Similar changes in brain diffusivity, as a biomarker for white and gray matter integrity, have been
reported in human -ɛ4 carriers [57], [56, 58, 59]. These structural modifications may be linked to the isoform-specific role of apoE in synaptic development, dendrite formation and axonal guidance, which in some extent may be impaired in apoE4 carriers [60]. Nevertheless, these results were not consistent across different ages, suggesting that changes in WM/GM microstructure properties may not directly reflect an associated AD-like pathology [489]. Moreover, at this age we measured a widespread reduction in functional connectivity at rest, which was previously reported [360].

Overall, in line with other studies in this mouse model [476], these findings suggest that the apoE4 mice spontaneously develop age- and apoE4-dependent accelerated brain pathology. This is in agreement with human studies on apoE4 carriers showing a faster decline of CBF during aging [490, 491], and reduced connectivity between cortical regions at old age [492-494]. However, the apoE4 mice exhibited relatively small genotype effects like CBF decline and reduced connectivity just at 18 months of age, and not at 12 months of age. Therefore, one could argue that an 18-month-old apoE4 mouse is at most similar to a 65-year-old still healthy, possibly only mildly impaired human apoE4 carrier, showing no signs of dementia yet and carrying no multiple risk factors and co morbidities. It has been found namely, that human apoE4 carriers often carry multiple risk factors (genetic modifiers, co-morbidities and lifestyle factors) that contribute significantly to synaptic integrity. The only risk factor included in our mice was age, combined with apoE4 genotype causing mild pathology.

A recent review on apoE-related biomarker profiles in the early phase of AD further elucidates this relatively novel concept of the –ɛ4 to be considered more as a vulnerability factor rather than a pathogenic factor [51]. Based on this idea, it is the interaction between these vulnerabilities and the age-related pathological events that may trigger synaptic loss, contributing to the development of AD. In our study, this aging-dependency of apoE4 seemed confirmed, as most of the biomarkers for brain deficits were identified only in the 18-month-old apoE4 group; representing an early stage of the disease, when complete early AD-like pathology is still not fully developed. Holding this hypothesis, the model becomes extremely attractive to study the effects of nutritional intervention as a preventive strategy against early AD-like pathology.

**Dietary intervention**

In the current experiment we fed the mice a specific multi-nutrient supplementation diet designed to ameliorate synapse loss and to reduce membrane-related pathology in AD by providing nutritional precursors and cofactors to support neuronal membrane formation and function [495]. This nutrient combination,
called Fortasyn, comprises uridine, docosahexaenoic acid, eicosapentaenoic acid, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium. Some of the components in these diets, such as omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs), have also been shown to improve vascular health [449, 496-499]. The results confirmed our initial hypothesis, that the Fortasyn diet has the potential to reduce the occurrence of vulnerabilities for AD by simultaneously improving cerebrovascular health and enhancing neuroprotective mechanisms. However it is also possible that the capacity of the diet to support membrane phospholipid synthesis could underlie both the synapse formation/functional connectivity and the effects on cerebrovascular functioning. Besides, the combination of phosphatide precursors like n3-LCPUFAs, uridine, and choline, has proven to synergistically increase the synthesis of synaptic proteins and phospholipids in the brain [458-460].

These findings confirm novel important mechanisms by which these diets may affect AD onset and development, similar to earlier findings in a transgenic AD mouse model [461]. Particularly, among the different parameters analyzed, the strongest and most consistent dietary effect in these studies involve the improvement of cerebrovascular health and functional connectivity. Several epidemiological studies and controlled trials showed a correlation between B-vitamins, n3-LCPUFAs and MUFA (like oleic acid from olive oil and nuts) and improvements in autonomic function, lowered blood pressure, reduced atherosclerosis, reduced total homocysteine and enhanced microvascular endothelium-dependent vasodilation processes [448, 500, 501].

All these factors may contribute to an improved functionality of the brain vasculature, with a beneficial effect particularly in apoE4 mice, in which these pathologies are aggravated. Interestingly, increased cortical CBF and levels of post-synaptic markers were found in both apoE4 and WT animals on Fortasyn diet; these findings suggest that the diet had a similar effect in both genotypes and its contribution is not determined by the apoE genotype. We have also shown that the Fortasyn diet affected brain fatty acid profiles in both genotypes (supplementary material, table 1), by decreasing the relative concentration of n6 fatty (notably arachidonic acid), which is significantly increased in the 18-month-old apoE4 mice compared to WT, and by increasing the relative concentration of n3 fatty acids (notably DHA) and monounsaturated fatty acids, especially oleic acid. These supplementary results indicate a replacement of n6 fatty acids from cell membranes in favour of n3-LCPUFAs, (reflected by the increased n3/n6 ratio) which has beneficial effects on membrane fluidity, and neuronal transmission and signalling [502-504]. Furthermore, we have shown that the Fortasyn diet similarly affected serum (supplementary material, table 2) and brain sterol level (supplementary material, table 3). In detail, in the plasma of aged animals on
Fortasyn we found decreased levels of cholesterol, but also of precursors like lanosterol and derivates of cholesterol like 24OH-cholesterol. Notably, in the cerebellum of aged animals on Fortasyn we detected a decreased concentration of the cholesterol-precursor lanosterol and increased level of a derivate of cholesterol, 24S-hydroxycholesterol. In the brain, the enzyme, cholesterol 24S-hydroxylase, converts cholesterol to 24S-hydroxycholesterol. This mechanism is the most important pathway for the elimination of brain cholesterol and the maintenance of brain cholesterol homeostasis [505-508]. These results are also in line with our previous study using another AD mouse model overexpressing Aβ. Here, another cholesterol precursor, lathosterol, was decreased in the brain of Fortasyn fed AD and WT mice, while again an increased cerebral level of the derivate of cholesterol, 24S-hydroxycholesterol, was measured in Fortasyn fed AD and WT mice [215]. Our data indicate a higher elimination rate of brain cholesterol in Fortasyn fed mice. This may also explain the improved white matter integrity and preserved functional connectivity in both WT and AD mice fed with Fortasyn. In support of this hypothesis, it has been demonstrated that animals fed with a diet containing uridine monophosphate (UMP), n3-LCP UFAs and choline, showed increased levels of brain phospholipids, dendritic spines and neurite outgrowth [458-460].

Conclusions

Overall, the study presented here further proved that two simultaneous protective mechanisms on vascular and synapse health are both enhanced by the specific Fortasyn diet and may strengthen each other synergistically, independent from the apoE genotype. The beneficial effect of these diets is suggested to be caused by increased production of phospholipids to sustain synaptic genesis and repair processes [458, 509]. In this and other recent experiments, we showed that a strong effect of these diets also involves the amelioration of cerebrovascular health. Although decreased cerebral perfusion has been recognized as an early and important contributor to AD pathology and cognitive decline [510], we believe that this aspect is not sufficiently considered in human nutritional intervention studies. In our mice, we have detected most of the pathological effects of apoE4 just at 18 months of age, and therefore, most of the beneficial effects of the diet were just present at 18 months of age. It is important to stress that our apoE4 mouse model only represents susceptibility to cognitive impairment and just like in human apoE4 carriers multiple risk factors are required in combination with apoE4 to precipitate disease pathology. For future research it would therefore be interesting to study the effect of the Fortasyn diet in older (24 months of age) apoE4 mice, in apoE4 mice with induced apoE4 co-morbidities like hypertension,
stroke, or in apoE4 mice on high fat diet or in female apoE4 mice, resembling more closely the human susceptibility to AD. For example, in clinical studies on brain atrophy and clinical decline among cognitively normal older individuals and individuals with mild cognitive impairment and Alzheimer disease it has been shown that the presence of apoE4 significantly accelerated rates of decline, and women in all cohorts had higher rates of decline than men [511]. Additionally, a preclinical study revealed that expression of human apoE4 renders aged mice fed with a western-type diet more susceptible to sensorimotor deficits upon stroke indicating an altered functional outcome following stroke in apoE4 carriers [512]. Furthermore, this study demonstrated the value of a multi-modal approach, including advanced MR neuroimaging tools, for detecting changes in brain structure and function with respect to dietary intervention.

**Supplementary material**

Supplementary material can be found here: https://www.hindawi.com/journals/np/2016/6846721/sup
A specific dietary intervention to restore brain structure and function after ischemic stroke


\(^1\) The authors contributed equally to the present work.
Abstract

Occlusion of the middle cerebral artery (MCAo) is among the most common causes of ischemic stroke in humans. Cerebral ischemia leads to brain lesions existing of an irreversibly injured core and an ischemic boundary zone, the penumbra, containing damaged but potentially salvageable tissue. Using a transient occlusion (30 min) of the middle cerebral artery (tMCAo) mouse model in this cross-institutional study we investigated the neurorestorative efficacy of a dietary approach (Fortasyn) comprising docosahexaenoic acid, eicosapentaenoic acid, uridine, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium as therapeutic approach to counteract neuroinflammation and impairments of cerebral (structural+functional) connectivity, cerebral blood flow (CBF), and motor function. Male adult C57BL/6j mice were subjected to right tMCAo using the intraluminal filament model. Following tMCAo, animals were either maintained on Control diet or switched to the multicomponent Fortasyn diet. At several time points after tMCAo, behavioral tests, and MRI and PET scanning were conducted to identify the impact of the multicomponent diet on the elicited neuroinflammatory response, loss of cerebral connectivity, and the resulting impairment of motor function after experimental stroke. Mice on the multicomponent diet showed decreased neuroinflammation, improved functional and structural connectivity, beneficial effect on CBF, and also improved motor function after tMCAo. Our present data show that this specific dietary intervention may have beneficial effects on structural and functional recovery and therefore therapeutic potential after ischemic stroke.
Introduction

Stroke is the second leading cause of morbidity and mortality [513], and the most common cause of serious adult long-term disability in the United States [514, 515]. Ischemic strokes have been the target of many failed drug trials [516] and only few treatment options are available, but many patients still show significant poststroke disabilities [517-519]. Until now, thrombolysis and thrombectomy are the common treatments for ischemic stroke, but not many patients benefit from these therapies due to the narrow therapeutic window and complexity of administration [515, 520].

There is therefore an urge for restorative interventions supporting better recovery and improving quality of life. Damaged brain networks have been shown to be responsible for absence of recovery or even for progressive disease, leading to increased mortality [18]. Furthermore, 87% of all strokes are ischemic [521], indicating that specific treatments should aim to improve impairments in cerebral blood flow (CBF) [516]. Therefore, stroke studies are needed investigating the effects of stroke and its interaction with a novel treatment on parameters being alternated after a stroke CBF, structural and functional connectivity, neuroinflammation, neuro- and synaptogenesis, vascular density, cognition, and behavioral parameters (motor coordination, activity etc.). Novel approaches targeting CBF, neuroinflammation, and brain networks may provide new avenues for stroke treatment [522]. In particular, dietary approaches could facilitate recovery after stroke [523, 524] because it has been shown that increased adherence to a Mediterranean-style diet is associated with a lowered risk of ischemic stroke and myocardial infarction [163]. Following MCA occlusion, mice fed a rapeseed oil-enriched diet revealed a decreased mortality rate, lowered levels of lipid peroxidation, and a reduced infarct size [166]. Also low fish consumption may already decrease the incidence of ischemic stroke [157]. In women, intake of omega-3 long-chain polyunsaturated fatty acids (n3-LCPUFA) is associated with a lowered risk of total stroke, while dietary cholesterol has been found to be positively associated with risk of total stroke and cerebral infarction [162].

In several other studies n3-LCPUFA have shown to diminish severity of vascular risk factors, like atherosclerosis [451], high blood pressure [525], and other cardiovascular diseases [451, 526-528]. Ozen et al. demonstrated a protective effect of n3-LCPUFA against cerebral ischemia in rats, showing a reduced number of apoptotic neurons in the prefrontal cortex when fed a standard diet combining eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [160]. Moreover, combined administration of DHA and UMP improved learning abilities in rats [529] and combined administration of DHA and EPA improves (cerebral) vascular health in human [530, 531]. Supplementation of DHA and choline to normal adult
gerbils also enhanced performance on several maze tests, being even further enhanced by coadministering UMP [532]. Additionally, in several studies a specific combination of nutrients called Fortasyn, comprising precursors and cofactors for membranes (DHA, EPA, uridine monophosphate (UMP), choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium), has been shown to support synergistically neuronal membrane synthesis [533, 534], increasing levels of brain phospholipids, dendritic spines and neurite outgrowth, cerebral blood flow, and to beneficially affect cognition [260, 294, 459, 535, 536]. Recently, we showed that this latter Fortasyn diet increased cortical CBF and synaptic density, and improved white matter integrity and functional connectivity (FC) in aging apoE4 mouse. Furthermore, we demonstrated that protective mechanisms on vascular and synapse health are enhanced by Fortasyn, independent of apoE genotype [346]. In another AD murine model, we showed that this multi-nutrient supplementation diet improved cerebrovascular health and protected against neuronal degeneration [224]. This neurorestorative potential of Fortasyn has recently been obtained in a rodent model of spinal cord injury as well [537]. Thus, in present experiment we wanted to investigate the possible efficacy of this multicomponent diet as therapeutic intervention after transient ischemic stroke on both structural and functional parameters. Using state-of-the-art imaging techniques, we measured CBF, and the neuroinflammatory response after ischemia. Notably, upon activation during the neuroninflammatory response, microglial cells express the translocator protein (TSPO) [538, 539]. In vivo positron emission tomography (PET) for imaging of microglial activation has focused on a variety of radiolabeled compounds binding to TSPO. In particular, the second generation TSPO tracer $N,N$-diethyl-2-(2-(4-([18F]fluoroethoxy)phenyl)-5,7-dimethylpyrazolo[1,5-$a$]pyrimidin-3-yl)acetamide ([18F]DPA-714) has been assessed for temporal and spatial evaluation of inflammatory alterations in models of cerebral ischemia, experimental autoimmune encephalomyelitis and cancer [540].
Material and methods

Animals and diets (Nijmegen, The Netherlands; Münster, Germany)
In total 48 3–4 months old C57BL/6J mice (Nijmegen, 24 mice: Harlan Laboratories Inc., Horst, the Netherlands; Münster, 24 mice 3–4 months old in-house bred C57BL/6J mice) were used for this cross-institutional, randomized, and double-blind controlled study (blinded for investigators and outcomes assessor) conducted at the preclinical imaging center (PRIME) of the Radboudumc (Nijmegen, the Netherlands) and the European Institute for Molecular Imaging (EIMI, Münster, Germany). Before tMCAo, the animals were housed socially with a maximum of six animals per cage, with room temperature at 21°C, and artificial 12:12h light: dark cycle (lights on at 7 a.m.). After stroke induction, the mice were housed separately to control the food intake of the experimental diets (see below). Food and water were available ad libitum. Samples sizes were determined by power analysis during the animal ethics dossier application. The experiments (Nijmegen) were performed according to Dutch federal regulations for animal protection and were approved by the Veterinary Authority of the Radboud university medical center, Nijmegen, The Netherlands. Experiments (Germany) were in accordance with the German Law on the Care and Use of Laboratory Animals and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz of North Rhine-Westphalia. All experiments (Nijmegen & Münster) of this international multicenter preclinical study were exerted in accordance to the ARRIVE [214] and the (updated) STAIR guidelines [541-543]. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Before tMCAo all mice were fed a standard Control diet (Ssniff rm/h V1534-000, Bio Services, Uden, The Netherlands). Starting right after tMCAo, mice were randomly divided in two groups; animals were fed either Control or Fortasyn diet that differed with respect to their fatty acid composition and some additional nutrients. All diets were isocaloric and based on AIN-93M [425] with 5% fat. The Control diet contained 1.9% soy oil, 0.9% coconut oil and 2.2% corn oil. The Fortasyn diet contained 0.1% coconut oil, 1.9% corn oil and 3.0% fish oil. The Fortasyn diet contains a specific multi-nutrient composition comprising uridine, omega-3 PUFAs, choline, B vitamins, phospholipids and antioxidants as specified in Table 1. To assure intercenter-comparability and accordance to the ARRIVE and (updated) STAIR guidelines in this study, all experiments were performed in line with the following predefined experimental rules. In this experiment we used a study design with pre-set endpoints for all participating centers (Münster & Nijmegen). Both diets were manufactured and pelleted by Ssniff (Soest, Germany) and stored at −20°C until use. All mice underwent behavioral tests before and after tMCAo. 35 days poststroke, MRI measurements were performed.
and all animals were euthanized after completion of the study. Post mortem immunohistochemical procedures were performed on all brains. The time line of the experimental design is illustrated in figure 1. No potential disparities in verum/control group allocation are given. The random allocation of each mouse into its test group was performed by a blinded researcher. To insure intercenter-comparability, all surgeries were performed by the same experienced researcher (D. R.) in Münster, Germany, and in Nijmegen, the Netherlands. For all surgeries, the same type of filaments (70SPRePK5, Doccol Corp., Sharon, MA, USA) for the occlusion of the MCA were used in Münster, Germany, and in Nijmegen, the Netherlands and the same batch of diets were tested in both centers. Furthermore, in Nijmegen, the Netherlands, all imaging and behavioral procedures were performed by the same researchers. In accordance, in Münster, Germany, all imaging procedures were performed by the same investigator. Moreover, all analyses of behavioral, imaging, and immunohistochemical data were performed by blinded researchers.

**Figure 1. Study design.** Behavioral and MRI studies were performed in Nijmegen, the Netherlands. After a transient occlusion of the middle cerebral artery (tMCAo) for 30 min, mice were divided into two dietary groups (Control or Fortasyn). At 7 and 14 days post tMCAo all mice underwent MRI. In between, all mice were tested on motor and cognitive impairments via several behavioral tests, like the Open field, Rotarod, Pole test, Prepulse inhibition (Ppi), grip strength test, and novel object recognition test (ORT). After MRI, all brains were processed for immunohistochemical stainings. PET imaging studies were conducted in parallel in Münster, Germany. $[^{18}F]$DPA-714 PET was conducted 7, 14 and 35 d after tMCAo in two dietary groups (Control or Fortasyn). T2w MRI for anatomical localization of stroke was conducted 14 d post tMCAo. The group sizes at the start and at the end of the experiments performed in Nijmegen, the Netherlands, and in Münster, Germany, are given. The group sizes shown in brackets indicates the number of mice used for the behavioral or imaging procedures and quantification. The dashed line indicates the period of time of exclusion of mice due to i.e. surgical problems, human endpoint.
Transient Middle Cerebral Artery Occlusion Surgery (Nijmegen, The Netherlands; Münster, Germany)

3 months old, male C57BL/6J mice underwent right tMCAo (30 minutes), using an intraluminal occlusion model as described elsewhere with minor modifications [544, 545]. This stroke model of transient occlusion of the MCA mimics one of the most common types of ischemic stroke in patients [544, 546]. The MCA was transiently occluded for 30 min. This ischemia time leads to moderate pathological changes within the infarct core, perilesional and remote regions [547-549]. In accordance with human stroke this reperfusion model involves a substantial degree of reperfusion via collateralization through the circle of Willis and leptomeningeal collaterals, and via early clot lysis [550]. In summary, mice were anesthetized with 1.5% isoflurane (Abbott Animal Health, Abbott Park, IL, USA) in a 2:1 air and oxygen mixture. After preparing the right common carotid artery, a 7-0 monofilament (tip diameter 190 to 200 μm, coating length 2 to 3 mm, 70SPRePK5, Doccol Corp., Sharon, MA, USA), was inserted in the common carotid artery and positioned at the point where the MCA branches out. Successful occlusion was maintained for 30 minutes before retracting the filament allowing reperfusion. Reduction in blood flow was monitored intraoperatively via a Laser Doppler probe (Nijmegen, moorVMS-LDF2, Moor Instruments, UK; Münster, Periflux 5000, Perimed Instruments, Järfälla, Sweden) attached to the skull of the mouse. Using the Laser Doppler Flow measurement, we considered an induction of ischemia as valid, when during the occlusion a decrease in regional CBF of more than 80% of baseline values at filament insertion is reached (inclusion criterion).

Mice were kept under 1.5% isoflurane anesthesia in a 2:1 air and oxygen mixture throughout the procedure. Body temperature was maintained at physiological levels with a custom built heating pad. Exclusion criteria were: a decreased motor activity (<50% of the baseline measurements combined from the baseline values of each behavioral test) or extreme weight loss (>20% within three consecutive days). Using a T2-weighted RARE sequence to measure lesion size, all animals showed a comparable lesion size and no dietary effect on lesion size (data not shown). In all animals N=3 Control animals and n=4 animal on Fortasyn diet died during first week poststroke in Nijmegen, the Netherlands. N=2 Control animals and n=1 animal on Fortasyn diet died during the experiments in Münster, Germany.

Behavioral and cognitive tests (Nijmegen, The Netherlands)

Open field
Locomotion and explorative behavior were evaluated for 10 minutes in the open field prior to the tMCAo, and also 3 and 23 days poststroke as previously described [215, 224, 345, 551]. Using EthoVision XT10.1 (Noldus, Wageningen,
The Netherlands), locomotion was automatically recorded. The floor of the arena was divided into center, periphery, and corners. The frequency of entering these zones was measured automatically. In addition, exploration was manually scored (walking, sitting, wall leaning, jumping, rearing, grooming) and analyzed as described previously [305, 552].

**Grip strength test**

Grip strength test was performed at 14 days poststroke using a grip strength meter (Grip-Strength Meter, 47200, Ugo Basile, Italy) to determine forelimb and also total limb (fore- and hindlimbs combined) muscle strength. Each mouse was trained prior to tMCAo to perform this test. The grip strength meter was arranged horizontally on the table. While holding the tails, the mice were lowered towards the grip strength meter to enable to grasp the grip trapeze with the forepaws or the grid with both their fore- and hindpaws. After grasping the trapeze or the grid, the mice were pulled backwards till the grasp was released. The test was repeated five consecutive times first with the trapeze and one hour later with the grasping grid. For each session the average value of the peak force (in gf) was calculated from the last three trials [553] and the first two trials were used as acclimatization. Additionally, trials were only analyzed, in which the mouse grasped with either two forepaws (trapeze) or all four paws (grid).

**Pole test**

The pole test was performed at day 15 poststroke, as previously described [554]. The mice were placed head upward just below the top of a vertical rough-surfaced pole (diameter 2.5 cm; height 60 cm) and then allowed to descend. This procedure was repeated five times. Each mouse was trained prior to tMCAo to perform this behavioral test. The time taken to turn completely downward and the rotation direction were measured manually, while the time to reach the floor was determined in EthoVision XT10.1 (Noldus, Wageningen, The Netherlands) to measure downward velocity (cm/s). Laterality index is determined by the averaged turn direction of each mouse (0= left/ contralateral turn, 1= right/ ipsilateral turn). A laterality index of 0.5 indicates that no lateralization is present, while a laterality index of 0.0 respectively 1.0 suggests a laterality of more contralateral respectively ipsilateral turns. Results for downward velocity and rotation time are displayed as the mean of minimal three successful trials. The first acquisition trial was excluded from the statistical analysis and used as acclimatization trial.
MRI protocol (Nijmegen, The Netherlands)

MRI measurements were performed at 7 and 35 days poststroke 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 operating on Paravision 5.1 software platform (Bruker, Karlsruhe, Germany). 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 receiving (Bruker BioSpin). For the imaging procedure, the animals were anesthetized with isoflurane (3.5% for induction and 1.8% 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 monitored with a rectal probe and maintained at 37°C with heated airflow. Respiration of the animal was monitored using a pneumatic cushion respiratory monitoring system (Small Animal Instruments Inc, NY, USA). First gradient echo images were acquired using previously described image parameters [224].

Cerebral blood flow
MR perfusion data were acquired under resting conditions using established methods with a flow-sensitive alternating inversion recovery (FAIR) technique [226, 295]. Regional perfusion was evaluated in the cerebral cortex (all cortical regions above corpus callosum), hippocampus, thalamus [224] accordingly to the atlas of Franklin and Paxinos [227]. To calculate regional CBF we used the same protocol as previously described [224].

Diffusion tensor imaging
Diffusion of water was imaged as described previously [347, 555]. In short, 22 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/mm² (b0 images; 5x) and 1000 s/mm² were used and diffusion-sensitizing gradients were applied along 30 non-collinear directions in three-dimensional space. The diffusion tensor was estimated for every voxel using the PATCH algorithm [230]; mean water diffusivity (MD) and fractional anisotropy (FA) were derived from the tensor estimation following a protocol as described elsewhere [555]. MD and FA values were measured in several white matter (WM) and grey matter (GM) areas, manually selected based on an anatomical atlas [227], as described previously in [346].
**Resting state fMRI**

The resting state fMRI (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/; [556]). Mean and maximum displacement across the six degrees of freedom (along the x-, y-, and z-axes and on three rotation parameters pitch, roll, and yaw) were measured in each mouse. The mean SE-EPI images of each mouse were then used to generate a study-specific template through linear affine and nonlinear diffeomorphic transformation (ANTS. v1.9; http://picsl.upenn.edu/ANTS/). Visual inspection of the normalized dataset was performed to screen for possible normalization biases. On the template, 14 areas were selected in left and right hemisphere. The selected regions were based on previous work in functional connectivity in mice [231], and includes: left and right dorsal hippocampus, left and right ventral hippocampus, left and right auditory cortex, left and right motor cortex, left and right somatosensory cortex, and left and right visual cortex. All cortical ROI were selected 1–2 voxels away from the edge of the cortex, to minimize the impact of susceptibility-weighted artefacts, which are more prominent in areas of different tissues interface (e.g., near the skull or near the ear canals). After motion regression, in-plane spatial smoothing (0.4 × 0.4 mm), linear detrending, and temporal high-pass filtering (cut-off at 0.01 Hz) were applied to compensate for small across-mouse misregistration and temporal low-frequency noise. FC group comparisons between ROI were calculated from the BOLD time series using total correlation and partial correlation analyses implemented in FSLNets (FSLNets v0.3; www.fmrib.ox.ac.uk/fsl). Pearson’s correlation values were Fisher transformed to Z-scores for group comparisons and statistical analysis.

**[^18F]DPA-714 Positron Emission Tomography (PET, Münster)**

During image acquisition, mice were anesthetized with 1.5% isoflurane (Abbott Animal Health) in pure oxygen. For tracer application, the lateral tail vein was cannulated using a 26 Ga catheter (Vasculon Plus, BD, Heidelberg, Germany) connected to a 15-cm polyethylene tubing (27 Ga, Smith Medical, Kent, UK).[^18F]DPA-714 was prepared following the procedure described previously with a radioactive purity of 99% and a decay corrected yield (rcy) of 25.1 ± 4.9% [557]. Mice were injected with 15.7 ± 1.5 MBq of[^18F]DPA-714 and imaged consecutively 7 (n=5/group), 14 (n=8/group), and 35 (n=8/group) days poststroke. Only animals with complete imaging set (n=5 group) could be taken into consideration for repeated measurement ANOVA analysis. N=2 animals per group without measureable infarction and DPA-714 PET changes were excluded from the analysis. PET data were acquired 45-55 min p.i. on a high-resolution small animal PET scanner (32 module quadHIDAC, Oxford Positron Systems Ltd., Oxford, UK)
with uniform spatial resolution (1 mm FWHM (full-width at halfmaximum)) over a large cylindrical field-of-view (165 mm diameter, 280 mm axial length). Images were reconstructed with a one-pass list mode expectation maximization algorithm with resolution recovery. CT images were acquired on a small animal CT scanner (Inveon, Siemens Medical Solutions, Knoxville, TN, USA) with a spatial resolution of 80 μm. For data analysis, CT images were coregistered to PET, followed by co-registration to MRI data using a landmark-based approach as described previously [545]. Magnetic Resonance Imaging studies were performed with a 9.4-T small animal MR scanner with 20 cm bore size (Bios-Spec 94/20; BrukerBioSpin MRI GmbH, Ettlingen, Germany). The system was operated using the software ParaVision 5.1. (BrukerBioSpin MRI GmbH). Anatomic T2w RARE brain images were obtained in three imaging planes (28 planes, slice thickness 0.5 mm, in-plane resolution 78 μm (repetition time 3,000 to 5,500 ms, echo time 50 ms, rare factor 16, 6 averages, 14 to 28 contiguous slices, slice thickness 0.5 mm, field of view 20 mm2, 256 matrix, in-plane resolution 78 μm², scan time 5 to 9 minutes, respectively). Image analysis was conducted with the VINCI software (Version: 4.19.0; http://www.nf.mpg.de/vinci3/) [558] by drawing a volume of interest (VOI) over the infarcted area depicted by T2w MRI. A duplicate VOI was mirrored to the contralateral hemisphere and served as control. Mean and maximum lesion-to-background (Lmean/Bmean and Lmax/Bmean) ratios were calculated.

**Immunohistochemical procedures (Nijmegen, The Netherlands; Münster, Germany)**

After the last scanning session, the mice were sacrificed by transcardial perfusion using 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. The brains were harvested and stored separately. The brains were postfixed overnight in 4% paraformaldehyde at 4°C and transferred to 0.1M PBS containing 0.01% sodium azide the next day. One part of the brain (Bregma: -0.7 to -4.36) was cut in 30 μm frontal sections using a sliding microtome (Microm HC 440, Walldorf, Germany) equipped with an object table for freeze-sectioning at -60°C. 24 Hours before cutting, the brains were transferred in 30% sucrose in 0.1M phosphate buffer. 8 Series were cut and stored in 0.1M PBS with 0.01% sodium azide so multiple immunohistochemical stainings could be performed. All sections were stained in one session to minimize differences in staining intensity.

In total three stainings were performed for vascular integrity measured via glucose transporter-1 (GLUT-1), for activated microglia via ionized calcium-binding adapter molecule 1 (IBA-1) as indicator for neuroinflammation, and for immature
neurons (measure for neurogenesis) with antibodies against doublecortin (DCX) on free-floating brain sections on shaker tables at room temperature. Immunohistochemistry was performed using standard free-floating labelling procedures, using previously described protocols [559]. The GLUT-1 amount was visualized using polyclonal rabbit anti-GLUT-1 antibody (1:40,000, Chemicon AB 1340, Chemicon International, Inc., Temecula, CA, USA) and as secondary antibody donkey anti-rabbit biotin (1:1500 Jackson ImmunoResearch, West Grove, PA, USA).

For IBA-1, as primary antibody against IBA-1 polyclonal goat anti-IBA-1 (1:3000; Abcam) and for DCX, polyclonal goat anti-DCX (1:8000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a primary antibody to assess neurogenesis. For both as secondary antibody donkey anti-goat biotin (1:1500; Jackson ImmunoResearch, West Grove, PA, USA) was used.

From a more frontal part of the brain tissue (Bregma: -0.10 to 0.98) was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in paraffin according to a standard protocol. Synaptophysin (SYN; measure for synaptic plasticity) immuno reactive presynaptic boutons with antibodies against synaptophysin and postsynaptic density (measure for synaptic plasticity) with antibodies against postsynaptic density protein 95 (PSD-95) were demonstrated immunohistochemically on 5 μm thin paraffin sections.

We used monoclonal rabbit anti-synaptophysin clone EP1098Y (1:500; Abcam Inc., Cambridge, UK) as a primary antibody to visualize synaptophysin in presynaptic boutons. For PSD-95, polyclonal rabbit anti-PSD95 (1:800; Abcam Inc.) was used as a primary antibody to reveal the postsynaptic density. After washing 3 times with 0.1M PBS, for both the synaptophysin and PSD-95 staining, the secondary antibody used was donkey anti-rabbit biotin (1:200; Jackson ImmunoResearch), followed again by 3 washes, Vector ABC-Elite (1:50) and the above mentioned DAB-Ni incubation.

Immunohistochemistry for TSPO (1:250, NBP1-95674, Novus Biologicals, Cambridge, UK) and GFAP (1:1000; Abcam Inc., Cambridge, UK) was performed on paraffin embedded slices. The slides were boiled in citrate buffer for antigen retrieval (pH 6; 30 minutes), before being treated with blocking solution for 30 minutes (1%BSA and 0.5% Triton-X in PBS), Primary antibodies were subsequently incubated with the primary antibodies (4°C, overnight). TSPO immunoreactivity was visualized through a multi-step process, including incubation with a biotinylated goat anti-rabbit (1:800, 45 minutes, B21078, Life Technologies, Darmstadt, Germany), followed by HRP-Streptavidin incubation (20 minutes, K1016, DAKO, Hamburg, Germany) and 3,3-Diaminobenzidine (D-5637, 5 min, Sigma, Hamburg, Germany). Sections were counterstained with hematoxylin, dehydrated, cleared
with xylol and mounted using Entellan (Merck, Darmstadt, Germany). For double immunohistochemistry slides were incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:800, A-21206, Life Technologies, Carlsbad, CA, USA), or Alexa Fluor 555 conjugated anti-goat (1:800, A-21432, Life Technologies, Carlsbad, CA, USA). Slides were mounted in DAPI containing mounting medium (Vectashield, H-1500, Vector Laboratories, Burlingame, CA, USA). Images were acquired with a combined fluorescence-light microscope (Nikon Eclipse NI-E, Nikon, Tokyo, Japan).

**Quantification (SYN and PSD-95)**
The stained sections (Bregma: 0.02 to 0.62) were analyzed using a Zeiss Axioskop microscope equipped with hardware and software of Microbrightfield. Brain regions were based on the mouse brain atlas of Paxinos and Franklin [227] and quantified in four regions of the hippocampus: left cortex, right cortex, left caudate putamen, and right caudate putamen. The relevant regions were digitized with a 40x objective and using Stereo Investigator software for finding the right location for the image. The quantification of the images was performed using ImageJ (National Institute of Health, Bethesda, MD, USA).

**Quantification (GLUT-1, IBA-1 and DCX)**
Brain sections (Bregma: -1.46 to -2.30) were preselected for quantification accordingly to the atlas of Franklin and Paxinos [227]. Quantification was done on images at a 5x objective using an Axio Imager A2 (Zeiss Germany). ImageJ (National Institute of Health, Bethesda, MD, USA) was used to analyze the regions of interest (GLUT-1+IBA-1: Cortex, hippocampus and thalamus; DCX: Hippocampus, and the subventricular zone, called SVZ).

**Statistics**
For the statistical analysis, IBM SPSS 22 software (IBM Corporation, New York, NY, USA) was used. Multivariate ANOVA or univariate ANOVA both with Bonferroni corrections (using Repeated measures ANOVA, when necessary, for example open field and PET data) was conducted with between-group-factor diet and if necessary also time to analyze possible differences in all the other parameters. Statistical significance was set at $p \leq 0.05$: #, $0.05 < p < 0.08$ (tendency); *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Degrees of freedoms, f-values and p-values are given for each statistical analysis. All data are expressed as mean ± SD.
Results

A summary of all experimental results is given in table 1.

**Table 1. Summary of all significant results.** Used abbreviations: CBF (cerebral blood flow), DTI (diffusion tensor imaging), FA (fractional anisotropy), MD (mean diffusivity), rsfMRI (resting state functional MRI), Total (total correlations), Partial (partial correlations), IHC (Immunohistochemistry), GLUT-1 (glucose transporter-1), IBA-1 (ionized calcium-binding adapter molecule 1), DCX (doublecortin), SYN (synaptophysin), PSD-95 (postsynaptic density protein 95).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
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<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td>↓ in both groups at 70; ↑ in both groups at 350</td>
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<tr>
<td><strong>Food intake</strong></td>
<td>↑ in both groups at 350; (↑) at 70→350 in Fortasyn mice</td>
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<tr>
<td><strong>Distance</strong></td>
<td>↓ in both groups at 3D</td>
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<td><strong>Velocity</strong></td>
<td>↓ in both groups at 3D</td>
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<td><strong>Frequency of entering:</strong></td>
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<td><strong>Corners</strong></td>
<td>↓ in both groups at 3D</td>
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<tr>
<td><strong>Periphery</strong></td>
<td>↓ in both groups at 3D; ↑ in both groups at 230</td>
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<tr>
<td><strong>Center</strong></td>
<td>↓ in both groups at 3D; (↑) in both groups at 230</td>
</tr>
<tr>
<td><strong>Moving</strong></td>
<td>↓ in both groups at 3D; ↑ at 230 in Fortasyn mice</td>
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<tr>
<td><strong>Manual scoring (frequency):</strong></td>
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<tr>
<td><strong>Leaning</strong></td>
<td>↓ in both groups at 3D</td>
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<td><strong>Rearing</strong></td>
<td>↓ in both groups at 3D</td>
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<td><strong>Grooming</strong></td>
<td>(↑) in both groups at 3D</td>
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<tr>
<td><strong>Sitting</strong></td>
<td>↑ in both groups at 230</td>
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<td><strong>Grip strength test</strong></td>
<td>Grid (All limbs) (↑) in Fortasyn mice</td>
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<td><strong>Pole test</strong></td>
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Food intake and body weight (Nijmegen, The Netherlands)

Body weight of both dietary groups decreased poststroke, while it increased significantly over time, comparing the first with the second to fifth week poststroke in all mice, for further details see supplemental material and supplemental figure 1A.

The daily food intake increased over time on both diets in all mice. Fortasyn diet tended to increase food intake compared to Control diet, for further details see supplemental material and supplemental figure 1B.

Behavioral and cognitive tests (Nijmegen, The Netherlands)

Open field

Open field was performed for 10 minutes to measure locomotion (total walking distance, velocity), frequency of zone enters (corners, center, periphery), exploration frequency (walking, wall leaning, rearing, sitting) and grooming, prior to tMCAo (baseline), 3 and 23 days after tMCAo.

Both diet groups exhibited a decreased total distance walked (Figure 2A; F(1,12)=87.8, p<.001) and velocity (Figure 2B; F(1,12)=84.2, p<.001) three days poststroke compared to baseline.

The frequency of entering the three zones of the open field (Figure 2C), corners (F(1,12)=61.5, p<.001), periphery (F(1,12)=55.9, p<.001), and center (F(1,12)=35.3, p<.001) were also decreased in both diet groups comparing before and three days poststroke. From three to 23 days poststroke, both diet groups increased their frequency of entering the center (F(1,13)=81.9, p<.001) and periphery (F(1,13)=4.1, p<.065) again.

Measured via manual scoring (Figure 2D), frequencies of walking (F(1,12)=39.4, p<.001), wall leaning (F(1,12)=28.2, p<.001), and rearing (F(1,12)=17.4, p<.002) were decreased, and frequency of grooming (F(1,12)=3.7, p<.079) was increased in both diet groups comparing before with three days after tMCAo. In contrast, from 3 to 23 days poststroke, mice showed an increase in frequency of sitting (F(1,13)=5.1, p<.042), and decrease in frequency of grooming (F(1,13)=11.1, p<.006).

Notably, a diet effect was shown over time on frequency of walking (F(1,13)=8.1, p<.014). Only mice on Fortasyn diet walked more often overtime (F(1,13)=10.2, p<.019). 23 days poststroke, Fortasyn fed mice walked more frequently than Control fed mice (F(1,13)=4.6, p<.052, trend).

Grip strength test

The grip strength test was performed to quantify muscle strength (of forepaws respectively of fore- hindlimbs combined) at 14 days poststroke.
No diet effects in muscle strength of the forepaws was found (Figure 2E). The grasping grid revealed that mice on Fortasyn diet tended to have higher muscle strength than mice on Control diet ($F(1,13)=4.5, p<.057$).

**Figure 2** Effects of Fortasyn on: locomotion (A+B), time spent in different zones (C), and exploration in open field measured prior to tMCAo (PO), and 3 and 23 days poststroke (E) grip strength test 14 days poststroke g-f gram-force (F-H) motor coordination determined via pole test 15 days poststroke. Values represent mean±SD. (A+B) Both diets exhibited decreased total distance walked ($p<.001$) and velocity ($p<.001$) comparing before, and three days poststroke. (C) Frequency of entering corners ($p<.001$), periphery ($p<.001$), and the center ($p<.001$) were decreased in both diet groups comparing before, with three days poststroke. From three to 23 days after tMCAo, both diet groups increased frequency of entering center ($p<.001$) and periphery ($p<.065$). (D) Frequencies of walking ($p<.001$), wall leaning ($p<.001$), and rearing ($p<.002$) were decreased. All mice showed increased frequency of sitting ($p<.042$), and decreased frequency of grooming ($p<.006$) poststroke. Mice on Fortasyn walked more at 23 days compared to 3 days poststroke ($p<.019$). At 23 days poststroke, Fortasyn fed mice tended to walk more frequently than Control fed mice ($p<.052$). (E) No diet effect was found in muscle strength of the forepaws (trapeze). Measuring total limb strength (grasping grid), revealed higher muscle strength on Fortasyn tended ($p<.057$). (F) No diet effects were observed concerning velocity to descend along the pole. (G) Control fed mice needed a longer turning time than Fortasyn fed mice when placed head-upwards on top of the pole ($p<.010$). (H) Mice fed Control diet displayed an ipsilateral bias to fully turn head-down ($p<.058$, trend).
Pole test
Motor coordination was measured with the pole test: turning time, rotation direction, and the downwards walking velocity. No differences were detected between the dietary groups concerning velocity to descend along the pole (Figure 2F, F(1,14)=0.2, p<.909). Mice on Control diet needed more time to turn than mice on Fortasyn diet when being placed head-upwards on top of the pole (Figure 2G, F(1,14)=9.0, p<.010). The turning phase requires fine-tuned and coordinated movements demanding on the potential motor coordination [554]. Notably, mice fed Control diet tended to turn more frequently to the right to fully turn head-down than mice fed Fortasyn diet indicating reduced laterality in the Fortasyn fed mice (Figure 2H, F(1,14)=4.3, p<.058).

Cerebral blood flow (Nijmegen, The Netherlands)
CBF was measured with flow-sensitive alternating inversion recovery (FAIR) MRI; from a series of echo planar imaging (EPI)-images in three different regions of interest (ROI): Cortex, hippocampus and thalamus. Each ROI was drawn double blind. One representative high-resolution voxel-wise analyzed CBF image for each dietary group at 7 and 35 after tMCAo is shown (Figure 3B). CBF was analyzed for each ROI in the unaffected (contralateral/ left) and affected (ipsilateral/ right) hemisphere separately for each dietary group at 7 and 35 poststroke (Figure 3A). Furthermore, loss of CBF in the affected ROI was calculated as the difference in CBF between right and left hemispheric ROI relative to left hemispheric ROI (Figure 3C).

7 days poststroke CBF in the right cortex (Control, F(1,12)=46.9, p<.001; Fortasyn, F(1,12)=38.8, p<.001), right hippocampus (Control, F(1,12)=148.2, p<.001; Fortasyn, F(1,12)=34.6, p<.001), and right thalamus (Control, F(1,12)=12.3, p<.005; Fortasyn, F(1,12)=60.7, p<.001) were lower than corresponding left ROI in mice for both diets. At this time point, mice on Fortasyn demonstrated higher CBF in the right affected cortex than mice on Control diet (Fortasyn, F(1,12)=7.7, p<.017). 35 days poststroke only mice on Control diet demonstrated lower CBF in right cortex (F(1,12)=5.2, p<.043), right hippocampus (F(1,12)=9.4, p<.010), and in right thalamus (F(1,12)=8.1, p<.015) compared to corresponding left ROI. Contrarily, mice on Fortasyn showed only a decreased CBF in right thalamus compared to left thalamus (F(1,10)=17.3, p<.002). Moreover, Fortasyn mice had a higher CBF than Control mice in left cortex (F(1,11)=5.4, p<.040), right hippocampus (F(1,11)=5.2, p<.045), and also the left thalamus (F(1,11)=7.4, p<.020) over time. We also investigated CBF over time from 7 days to 35 days poststroke. While animals on Control diet showed only a decreased CBF in left cortex (F(1,12)=17.7, p<.002) and in left hippocampus (F(1,12)=3.7, p<.079) over time, animals on Fortasyn diet did not show these decreases (left cortex, F(1,11)=1.4, p<.256; left hippocampus, F(1,11)=.0, p<.886), and had an increased CBF in the right
A specific dietary intervention to restore brain structure and function

Chapter 7

hippocampus (F(1,11)=7.6, p<.019) and right thalamus (F(1,11)=6.1, p<.032) over time. At 7 days poststroke, Fortasyn mice exhibited a lower decrease in cortical CBF than Control mice (F(1,12)=7.7, p<.017). This beneficial effect was also seen at 35 days poststroke in the hippocampus of Fortasyn fed mice compared with Control diet fed mice (F(1,11)=5.5, p<.040). From 7 to 35 days poststroke both dietary groups showed a less impaired CBF in cortex (Control, F(1,12)=10.9, p<.007; Fortasyn, F(1,11)=5.5, p<.040) and hippocampus (Control, F(1,12)=6.7, p<.024; Fortasyn, F(1,11)=10.1, p<.009), while this improvement was also revealed in the thalamus of Fortasyn mice only (Control, F(1,12)=2.4, p<.147; Fortasyn, F(1,11)=7.9, p<.018).

Figure 3. Cerebral blood flow (CBF) determined with flow-sensitive alternating inversion recovery (FAIR) MRI 7 and 35 days after tMCAo in cortex, hippocampus and thalamus of mice on Fortasyn or Control diet. Values represent mean±SD. (B) Representative high-resolution voxel-wise analyzed CBF image at 7 and 35 poststroke. (A) 7 days poststroke CBF in right cortex (Control, p<.001; Fortasyn, p<.001), right hippocampus (Control, p<.001; Fortasyn, p<.001), and in right thalamus (Control, p<.005; Fortasyn, p<.001) were lower than corresponding left ROI for both diets. Mice on Fortasyn showed increased CBF in right affected cortex (p<.017). 35 days poststroke, Control mice demonstrated lower CBF in right cortex (p<.043), right hippocampus (p<.010), and right thalamus (p<.015) compared to left ROI, while Fortasyn mice demonstrated decreased right thalamic CBF (p<.002) and a higher CBF than control mice in left cortex (p<.040), right hippocampus (p<.045), and also left thalamus (p<.020) at day 35. Control mice showed a decreased left cortical (p<.002) and left hippocampal (p<.079) CBF over time, Fortasyn fed animals showed increased CBF.
in right hippocampus ($p<.019$) and right thalamus ($p<.032$) over time. (C) Fortasyn mice exhibited a less diminished cortical CBF at 7 days poststroke ($p<.017$) and in hippocampal CBF at 35 days poststroke ($p<.040$). CBF recovery was shown in cortex (Control, $p<.007$; Fortasyn, $p<.040$) and hippocampus (Control, $p<.024$; Fortasyn, $p<.009$), and thalamus (Fortasyn, $p<.018$).

**Diffusion tensor imaging (Nijmegen, The Netherlands)**

Quantitative assessment of diffusion tensor derived indices (fractional anisotropy, FA, *Figure 4B*; and mean diffusivity, MD, *Figure 4C*) was performed for ROI drawn in white and gray matter regions to assess diet and time effects 7 and 35 days poststroke.

**Fractional anisotropy**

Fortasyn mice showed a trend of an increased FA in the right Motor Cortex (MC) compared to Control ($F(1,22)=4.2, p<.053$) at 7 and 35 days poststroke. Notably, a diet effect was found over time in the ROI containing the right Caudate, Putamen and Globus Pallidus (Cpu+GP, $F(1,22)=3.8, p<.065$). More specifically, only mice on Fortasyn showed an increase in FA in the right Cpu+GP over time ($F(1,11)=1.2, p<.044$), while mice on Control diet did not show this increase.

All mice showed increased FA over time poststroke in the left Corpus Callosum (CC, $F(1,22)=3.8, p<.065$), left Hippocampus (HC, $F(1,22)=5.6, p<.028$), right HC ($F(1,22)=9.0, p<.007$), and left optic tract ($F(1,22)=4.1, p<.055$).

7 days poststroke in both dietary groups a lower FA was measured in the right caudate Cpu+GP compared with Cpu+GP in the left hemisphere (Control, $F(1,12)=6.4, p<.027$; Fortasyn, $F(1,12)=9.1, p<.011$).

While Fortasyn mice showed no significant decrease of FA in the right ROI compared with the corresponding left ROI at 35 days poststroke, Control mice showed a lower FA in the right visual cortex (VC, $F(1,10)=6.5, p<.030$) compared to left VC.

**Mean diffusivity**

Again, at 7 and 35 days poststroke Fortasyn mice had a lower MD in the right MC than Control mice ($F(1,22)=4.0, p<.059$, trend). Notably, a diet effect was found over time in the left Cpu+GP ($F(1,22)=3.4, p<.078$): only mice on Control diet showed a non-significant decreased MD in the left Cpu+GP over time ($F(1,11)=4.2, p<.066$). In all mice, MD tended to decrease over time in the Fornix ($F(1,22)=3.8, p<.065$).

While Fortasyn mice showed no MD increase in the right hemisphere ROI compared with the corresponding left hemisphere ROI at 35 days poststroke, Control mice showed a higher MD in the right Cpu+GP ($F(1,10)=9.9, p<.030$) compared to left Cpu+GP.
Figure 4. Quantitatively assessed diffusion tensor derived indices at 7 + 35 days poststroke in mice fed Fortasyn or Control diet. Fractional anisotropy (FA) and mean diffusivity (MD) were measured for ROI drawn in white and gray matter regions (AUC=auditory cortex, CC=corpus callosum, F=fornix, HC=hippocampus, MC=motor cortex, OT=optic tract, SSC=somatosensory cortex, VC=visual cortex, Cpu+GP=caudate putamen+globus pallidus). Values represent mean±SD. (A) Representative high-resolution set of three (Bregma: 0.38, -1.34, -2.92) FA images is shown for each dietary group 7 and 35 days poststroke. (B) Fortasyn mice showed a higher FA in the right MC than Control mice (p<.053) 7 and 35 days poststroke. Only mice on Fortasyn showed an increased FA in right Cpu+GP over time (p<.044). In all mice, FA increased from day 7 to day 35 poststroke in left CC (p<.065), left HC (p<.028), right HC (p<.007), and left OT (p<.055). Both dietary groups demonstrated a lower FA in right Cpu+GP compared to left Cpu+GP (Control, p<.027; Fortasyn, p<.011) 7 days poststroke. Control mice showed a lower FA in right VC (p<.030) compared to left VC. (C) Fortasyn mice showed a lower MD in the right MC than Control mice overtime (p<.059, trend). Only Control fed mice showed a non-significant decrease in MD in left Cpu+GP over time (p<.066). In all mice, MD seemed to decrease again from day 7 to day 35 poststroke in the F (p<.065). Only Control mice showed a higher MD in right Cpu+GP (p<.030) than in left Cpu+GP.
Resting state fMRI (Nijmegen, The Netherlands)
To compare functional connectivity (FC) patterns at 7 and 35 days poststroke and on different diets, rsfMRI data were statistically analyzed based on total (Supplemental figure 2A-C) and partial correlation (Figure 5A-C) in twelve ROI including: left and right dorsal hippocampus (DH), left and right ventral hippocampus (VH), left and right auditory cortex (AU), left and right motor cortex (M1), left and right somatosensory cortex (S1), and left and right visual cortex (V1).

Total correlation analyses
With total correlations no significant diet effects nor time effects (Supplemental figure 2B,C) were measured.

Partial correlation analyses
Partial correlation analysis accentuates the direct connectivity between two ROI, while regressing the temporal BOLD signal from all other ROI. For the partial correlations we found significant diet effects at 7 and 35 days poststroke (Figure 5B).
At 7 days after tMCAo Fortasyn mice showed a decreased FC between left and right DH (F(1,8)=11.2, \( p < .011 \)), and between right DH and left AU (F(1,8)=5.4, \( p < .050 \)). In contrast, already at 7 days poststroke Fortasyn exhibited an increased FC between right AU and right V1 (F(1,8)=6.2, \( p < .038 \)).
At 35 days poststroke Fortasyn mice showed only a decreased FC between left DH and left V1 (F(1,8)=5.7, \( p < .044 \)), while FC between right VH and left AU (F(1,8)=5.5, \( p < .048 \)), and between right AU and left M1 (F(1,8)=6.8, \( p < .032 \)) was increased in mice fed the Fortasyn diet.
For the partial correlations we found significant time effects in mice on both diets (Figure 5C). Main findings were less negative effects over time in animals fed Fortasyn than animals fed the Control diet. Furthermore, a beneficial effect on FC between left and right M1 was revealed only in Fortasyn mice.
On Control diet, FC was lowered between left DH and right S1 (F(1,8)=11.6, \( p < .010 \)), between left VH and right AU (F(1,8)=6.2, \( p < .038 \)), between right VH and left AU (F(1,8)=22.9, \( p < .002 \)), and between right AU and left M1 (F(1,8)=6.5, \( p < .035 \)), while FC was heightened between right DH and left VH (F(1,8)=5.9, \( p < .041 \)), between right AU and left DH (F(1,8)=13.9, \( p < .006 \)), and between left V1 and left M1 (F(1,8)=5.9, \( p < .041 \)).
On Fortasyn diet, FC was decreased only between left DH to left VH (F(1,8)=5.3, \( p < .050 \)) and right AU to left V1 (F(1,8)=9.1, \( p < .017 \)). Notably, mice on Fortasyn had a higher FC between left and right M1 over time (F(1,8)=6.8, \( p < .032 \)).
Figure 5. Resting-state functional connectivity (FC) based on partial correlation analyses (A) in the brains of mice fed Fortasyn or Control diet 7 and 35 days poststroke. FC was measured between 12 ROI: dorsal hippocampus (DH), ventral hippocampus (VH), auditory cortex (AU), motor cortex (M1), somatosensory cortex (S1), and visual cortex (V1). (B) 7 days poststroke Fortasyn mice showed decreased FC between DH and right DH (p<.011), and between right DH and left AU (p<.050). Fortasyn mice exhibited an increased FC between right AU and right V1 (p<.038) 7 days poststroke. At 35 days after tMCAo Fortasyn mice showed decreased FC between left DH and left V1 (p<.044), while FC between right VH and left AU (p<.048), and between right AU and left M1 (p<.032) was increased in Fortasyn mice. (C) On Control diet, FC was lowered over time between several ROI, e.g. left DH and right S1 (p<.010), while FC was increased between left V1 and left M1 (p<.041). On Fortasyn diet, FC was decreased between left DH to left VH (p<.050) and right AU to left V1 (p<.017). Fortasyn fed mice had a higher FC between left and right M1 over time (p<.032).

PET results (Münster, Germany)

N=24 mice underwent tMCAo surgery and were intra-individually followed 7d, 14 d, and 35 d poststroke by PET using the TSPO ligand [\(^{18}\)F]DPA-714. [\(^{18}\)F]DPA-714 signal appeared within and around the infarct as determined by T2w-MRI (Figure 6A). In accordance, immunohistochemical data showed good spatial agreement as well as reduced TSPO levels in the Fortasyn group (Figure 6B). Double staining immunohistochemistry for TSPO and GFAP indicates astrocytes as source of TSPO at late time points after stroke (Figure 6C).

Investigation of diet effects over time revealed that [\(^{18}\)F]DPA-714 mean uptake ratios (Figure 6D) were significantly reduced from 7 to 35 d poststroke in the Fortasyn group (Lmean/Cmean: 1.43+/-.016; n=5) when compared to Control animals.
(Lmean/Cmean: 1.58+/-0.16; n=5; F(1,8)=7.1, p<.029). Similarly, comparison of the maximum $^{18}$F]DPA-714 radio uptake ratios (Figure 6E) highlighted reduced $^{18}$F]DPA-714 uptake in the Fortasyn diet group from 7 to 35 d poststroke (Lmax/Cmean: 1.57+/-0.23; n=5) as compared to the Control diet group (Lmax/Cmean: 2.21+/-0.20; n=5; F(1,8)=9.4, p<.016).

**Immunohistochemical procedures**

In the supplemental material (supplemental figures 3-7) for each immunohistochemical staining a set of photos is given showing photos of each animal group in each ROI.

**GLUT-1 staining (Nijmegen, The Netherlands)**

All brains were processed for immunohistochemical staining with GLUT-1 antibody (glucose transporter-1). In order to reveal the changes in total GLUT-1 amount as well as in capillary density, we measured relative GLUT-1+ area and counted manually the amount of GLUT-1+ blood vessels in the cortex, hippocampus, and thalamus (Figure 7A-C). In the supplemental material no diet effects were found for total GLUT-1 amount in the cortex, hippocampus, and thalamus (Figure 7B). Notably, Fortasyn mice had more GLUT-1 positive blood vessels in the cortex than Control mice (Figure 7C, F(1,13)=5.8, p<.024), especially in the right cortex (F(1,13)=5.6, p<.034) indicating a higher vascular density.

**IBA-1 staining (Nijmegen, The Netherlands)**

The brains of all mice were immunohistochemically stained with an antibody against ionized calcium-binding adapter molecule 1 (IBA-1, Figure 7D). IBA-1 is expressed by microglia, but also by phagocytes in general (monocytes and macrophages). We measured relative IBA-1+ area and the amount of IBA-1+ cells in the cortex, hippocampus, and thalamus (Figure 7D-F).

Compared to the left thalamus, the right thalamus revealed an increased relative IBA-1+ area (Figure 7E, F(1,26)=12.2, p<.002) and also an increased amount of IBA-1+ cells (Figure 7F, F(1,26)=21.8, p<.001) in mice on both diets. While mice on Fortasyn seemed to have a decreased relative IBA-1+ area in the cortex (F(1,28)=4.0, p<.057) and the hippocampus (F(1,24)=4.1, p<.054) compared with mice on Control diet, a non-significant increased relative IBA-1+ area was seen in the thalamus of Fortasyn mice (F(1,26)=3.5, p<.074). In addition, mice on Control diet had more IBA-1+ cells in their right hippocampus than mice on Fortasyn (Figure 7F, F(1,12)=5.5, p<.037), and also all mice on Fortasyn showed less IBA1+ cells in the total hippocampus than mice on Control diet. (Figure 7F, F(1,24)=4.8, p<.039).
Chapter 7  A specific dietary intervention to restore brain structure and function

A

T2w MRI

14 days post tMCAo

35 days post tMCAo

F[18]FDG-PET - T2w MRI fusion

%ID/cc

2.75

0

Control diet

Fortasyn diet

B

35 days post tMCAo

Control diet

Fortasyn diet

TSPO

C

TSPO

GFAP

TSPO GFAP DAPI

D

E

Days after tMCAo

Log$_{10}$ B$_{max}$

Days after IMCAo

Log$_{10}$ B$_{max}$

Control  Fortasyn
Figure 6. Positron Emission Tomography (PET) of microglial activation. (A) Representative intra-individual MR images and fusion images over time with PET of mice fed with the Control diet (left) versus mice fed with the Fortasyn diet (right). Fortasyn fed mice showed a reduced uptake of $[^{18}F]$DPA-714 in the infarction after 35 days. (B) Comparison of $[^{18}F]$ DPA-714 with TSPO immunohistochemistry reveals reduced TSPO staining in Fortasyn animals (Scale bar: 1000µm and 50µm). (C) Irrespective of the dietary intervention, TSPO (red) is also expressed by astrocytes (green) 35d after tMCAo (Scale bar: 100µm. (D) Values represent mean+SD. $[^{18}F]$DPA-714 mean uptake ratios were significantly reduced from 7 to 35 d after tMCAo in the Fortasyn group when compared to Control animals (p<.029). (E) Comparison of the max $[^{18}F]$DPA-714 radio uptake ratios highlighted reduced $[^{18}F]$DPA-714 uptake in the Fortasyn diet group from 7 to 35 d (p<.016).

DCX staining (Nijmegen, The Netherlands)
Immature neurons were visualized in all mice with a polyclonal antibody against doublecortin (DCX). As a measure for neurogenesis, DCX+ cells were counted in the subgranular zone of the hippocampus (Figure 7G+H) and DCX+ area was measured also in the subventricular zone (SVZ, Figure 7I+J).
No diet effects were found in the hippocampus. In the right SVZ Fortasyn fed mice had more DCX+ area than in their left SVZ (Figure 7J, F(1,12)=4.9, p<.047), and than Control fed mice in the same ROI (Figure 7J, F(1,12)=5.4, p<.039).

SYN and PSD-95 staining (Nijmegen, The Netherlands)
The amount of presynaptic boutons was visualized with a monoclonal antibody against synaptophysin (SYN). Whereas, postsynaptic density was stained with a polyclonal antibody against postsynaptic density protein 95 (PSD-95) reflecting synaptic function. Both pre- (Figure 7K-M) and postsynaptic (Figure 7N-P) density were measured in the cortex and caudate putamen (Cpu).
No diet effects were found in the cortex and Cpu for both the relative SYN+ and PSD-95+ area. All mice showed a decreased relative SYN+ area in the right Cpu (Figure 7M, F(1,25)=9.2, p<.006) compared to corresponding left ROI.
All mice had a lowered PSD-95+ area in the right cortex (Figure 7O, F(1,26)=11.3, p<.003) and also in the right Cpu (Figure 7P, F(1,24)=35.5, p<.001) compared to corresponding left ROI.

TSPO immunohistochemistry (Münster, Germany)
Spatial comparison of $[^{18}F]$DPA-714 with immunohistochemistry for TSPO at 35 days after stroke revealed good spatial agreement. However, reduced TSPO immunohistochemistry was observed in Fortasyn treated animals (Figure 6B). Interestingly, 35 d after stroke astrocytes (GFAP) also expressed TSPO. (Figure 6C).
Figure 7. Immunohistochemical stainings for glucose transporter-1 (GLUT-1, A-C), ionized calcium-binding adapter molecule 1 (IBA-1, D-F), doublecortin (DCX, G-J), synaptophysin (SYN, K-L) and Postsynaptic Density-95 Protein (PSD-95, N-P) on brains of Fortasyn and Control fed mice 35 days after tMCAo. Values represent mean±SD. (C) Fortasyn mice had more GLUT-1+ blood vessels in the cortex than Control fed mice (p<.024), especially in the right cortex (p<.034) indicating a higher vascular density. (E) Mice on Fortasyn seemed to have a decreased relative IBA-1+ in cortex (p<.057) and hippocampus (p<.054) compared to Control fed mice. (E+F) The right thalamus showed a larger IBA-1+ area (p<.002) and more IBA-1+ cells (p<.001) than the left thalamus in mice on both diets. (F) Control fed mice had more IBA-1+ cells in the right hippocampus than Fortasyn fed mice (p<.037), whereas Fortasyn mice showed less IBA1+ cells in total hippocampus compared to Control (p<.039). (G-J) DCX+ cells were counted in the subgranular zone of the hippocampus (G+H) and in the subventricular zone (SVZ) (I+J). (J) Fortasyn fed mice had more DCX+ area in the right SVZ than in left SVZ (p<.047), and in the same ROI of Control fed mice (p<.039). (M) All mice showed a decreased relative SYN+ area in the right caudate putamen (Cpu, p<.006) compared to the left Cpu. (O+P) All mice had a lowered PSD-95+ area in the right cortex (p<.003) and in the right Cpu (p<.001) compared to the corresponding left ROI.
Discussion

The present data show that the specific nutrient combination Fortasyn may enhance recovery from ischemic stroke in mice, as evidenced by improved cerebral blood flow, reduced loss of functional connectivity, and improved motor performance. Relevant processes for recovery after stroke, such as neurogenesis, angiogenesis, and neuroinflammation, were positively affected by the Fortasyn diet.

Cerebral hemodynamics

CBF was increased already at seven and also at 35 days poststroke in mice on Fortasyn diet. Notably, CBF dropped over time in several brain regions in Control mice from 7 to 35 days poststroke, while this decrease was not seen in Fortasyn mice. These results are in accordance with our previous research showing that this nutrient combination is able to improve cerebral perfusion in mice models of (vascular risk factors for) Alzheimer’s disease (AD) [224, 346]. In addition, improved vascular density due to an increased angiogenesis was found in the cortex of Fortasyn fed mice. This improved vascular health may explain the beneficial effects of Fortasyn on cerebral hemodynamics after the ischemic event.

DTI and rsfMRI

In the present study we investigated brain diffusivity with DTI as an imaging biomarker for white and gray matter integrity. FA is a marker of the degree of myelination and fiber density of white matter (WM), while MD characterizes an inverse measure of the membrane density and is sensitive to cellularity, edema, and necrosis in grey matter (GM) [348-350]. Here, we revealed an impaired WM microstructure at seven days poststroke in regions containing the Caudate Putamen and Globus Pallidus of both dietary groups. Notably, microstructural integrity of WM was restored in Fortasyn mice over time in affected regions containing Caudate Putamen and Globus Pallidus of the affected hemisphere. Moreover, at 7 and 35 days poststroke a slight beneficial effect on WM microstructure of the right Motor cortex was also seen only in Fortasyn mice. Also an improved GM microstructure in the right Motor cortex of Fortasyn animals at seven and 35 days, and an impaired GM integrity (an increase in MD) in regions containing the right Caudate Putamen and Globus Pallidus of only mice on Control diet was measured. Thus, Fortasyn may limit the damage to existing structures and may also support the regeneration of lost connectivity. Comparable results were found in other studies showing that intracerebral injection of EPA is able to stimulate the expression of myelin proteins in rat pup brain [560]. Partial correlations of functional connectivity data revealed several beneficial diet effects.
already at 7 and also at 35 days poststroke. Fortasyn diet increased FC between several brain regions, and over time Fortasyn limited loss of FC compared to Control diet. Notably, Fortasyn mice showed an improved interhemispheric FC between the motor cortices supporting the therapeutic value of Fortasyn already after 35 days. The importance of dietary composition for developing and maintaining distributed brain systems was shown in another preclinical rsfMRI study showing that n3-LCPUFA are crucial for functional connectivity and large-scale system organization in rhesus macaque brains [561]. Additionally, in a 24-week randomized, controlled, double-blind, parallel-group, multi-country study de Waal et al. explored the effect of a nutritional intervention with Fortasyn on brain activity-based networks. They showed that the nutritional intervention preserved the organization of brain networks in patients with mild AD within 24 weeks potentially via counteracting the progressive network disruption over time in AD [562]. In addition, in a recent study Fortasyn ameliorated deficits in FC in a mouse model for asymptomatic AD [346]. Notably, in a longitudinal observational clinical study acquiring rsfMRI data 4 times over a period of 6 months Park et al. demonstrated that FC of the ipsilesional M1 with the contralesional thalamus, supplementary motor area, and middle frontal gyrus at onset was positively correlated with motor recovery at 6 months after stroke [563].

**Molecular alterations**

The combination of phosphatide precursors like n3-LCPUFA, uridine, and choline, has proven to synergistically increase the synthesis of synaptic proteins and phospholipids in the brain [459, 535, 536]. In our study, a decreased amount of pre- and postsynaptic markers in the ipsilateral cortex and caudate putamen was observed indicating a significant negative effect of experimental stroke on synthesis and maintenance of synaptic proteins. Kawakita et al. demonstrated that DHA effectively promotes neurogenesis both in vitro and in vivo [564]. Notably, even in an experimental murine stroke model our specific nutrient combination was able to increase neurogenesis. Here, we measured via DCX a significant enhanced neurogenesis in the right (affected) border zones of the lateral ventricle in Fortasyn fed animals indicating accelerated (neuronal) membrane synthesis.

**Neuroinflammation**

$[^{18}\text{F}]$DPA-714 is a ligand for the 18 kDa translocatorprotein (TSPO), which is highly expressed by microglial cells upon neuroinflammatory stimuli. Previous studies have shown that the majority of PET signal is due to increased expression of TSPO by microglia [545, 565]. Therefore, $[^{18}\text{F}]$DPA-714 has now already been used in a variety of clinical studies in neurological diseases [566]. However, due to the lack of specific markers and antibodies reliably distinguishing microglia and
macrophages it cannot be excluded that macrophages contribute to the signal. We and others have shown that under rather chronic conditions astrocytes faintly express TSPO (see Figure 7C and [567]), whereas at early-intermediate time points (24h-14 days) after stroke astrocytes seem not play an important role [545]. A decreased neuroinflammation/ microglial activation was observed with Fortasyn for up to 35 days post stroke by PET imaging. The beneficial function of Fortasyn was likely mediated by a reduction of IBA-1 positive cells. IBA-1, which is expressed in activated microglia and macrophages, was reduced at 35 days post stroke in the cortex and the hippocampus of Fortasyn mice. Notably, Harvey et al. could not show an effect of single administration of DHA on IBA-1 at three, 7, and 21 days after TBI on neuroinflammation in post-traumatic brain injury (TBI) in Sprague Dawley rats, indicating that a combination of factors may enhance diet function. However, they also showed that DHA may be able to facilitate the polarization of microglia or macrophages into an anti-inflammatory and reparative microglia phenotype [568]. The reduced neuroinflammatory response might also explain the beneficial effects on functional and structural connectivity. Several recent studies indicated that microglia are crucially involved in processes of synaptic remodeling and plasticity in the healthy brain [569-571]. In future preclinical stroke research the impact of Fortasyn on glial cells needs to be studied, since DHA as single component by itself is already able to modulate glial cell activity by regulating gene expression and release of inflammatory mediators, as well as serving as substrate for specialized pro-resolving mediators [572-574].

**Motor function**

Fortasyn mice tended to have more grip strength with all four limbs compared to control mice. This effect could partly be due to the beneficial effects of the Fortasyn diet on cerebral regions involved in motor functions. During the turning phase of the pole test set of fine-tuned and coordinated movements are required [554]. In the current study mice on Control diet needed more time to turn on the pole than mice on Fortasyn diet. In addition, Control mice showed a lateral bias at 15 days poststroke which was not present in Fortasyn mice. These latter results are in accordance to the recent study of Bourourou et al. Here, the dietary supplementation with the omega-3 precursor, alpha-linolenic acid, was more effective in improving motor coordination in a mouse model of ischemic stroke than intravenous treatment [575]. Notably, at 23 days poststroke all Fortasyn mice walked more often than the Control mice and also more often than at three days poststroke indicating an improved locomotion. These latter results are in line with other studies demonstrating that DHA treatment has beneficial effects on locomotor activity [18, 346]. While Cao et al. focused only on the preventive effect of DHA treatment and Hong et al. on the acute phase after stroke, in our study we
now finally get one step further towards effective acute treatment for recovery after stroke which is still lacking for many stroke patients. The pole and grip test were not performed at multiple time points after tMCAo like the Rotarod or open field. Future studies should repeat these motor tests at multiple time points to obtain additional indications of the long-term effects of Fortasyn on motor performance at later time points poststroke. Particularly, rodents are well known to be able to compensate functional differences rather than presenting true recovery after an experimental stroke [576]. Notably, an example of functional compensation in our study is the improved functional connectivity over time in our control mice. Here, control mice also showed an improved functional connectivity between several brain regions, being not present in animals on Fortasyn diet. Here, we were able to demonstrate beneficial dietary effects already at 7 and also at 35 days post experimental stroke. Future studies should also focus on more severe stroke models to study the beneficial effects of multi-nutrient interventions. We showed improvement of long-term outcome using a specific combination of nutrients. Notably, in the current experiments no effect on body weight was detected, but all Fortasyn mice seemed to have a higher food intake during all 35 days after an experimental stroke.

We did not find diet effects on cognition in the novel object recognition test (ORT) measured from 22 to 24 days poststroke. This absent effect is in line with previous research of Wang et al. who demonstrated a significantly reduced preference at 7 and 14 days following stroke, but normal values between 21 and 28 days post-stroke, within the ORT [577]. Therefore in future research, the ORT should be performed earlier after the induction of the experimental stroke to reveal subtle dietary effects.

Summary and conclusions

This international, multicenter preclinical study was exerted according to ARRIVE and STAIR guidelines [214, 541-543] revealing that multimodal neuroimaging combined with behavioral analysis is an excellent approach to assess brain and motor function recovery after stroke. In addition, this experimental setup shows the beneficial effects of an academic-industry collaboration and an industry-relevant translational research covering many aspects and complexities of a multicenter multimodal paradigm [578, 579]. In conclusion, our data indicate that a post-stroke intervention with the specific nutrient combination of Fortasyn is able to support recovery after ischemic stroke, by improving cerebral circulation due to an improved vascularization and increased angiogenesis, protecting white and gray matter integrity, restoring functional connectivity, increasing
neurogenesis, decreasing the neuroinflammatory response, and improving motor skills and muscle strength. No therapeutic intervention is available for stroke yet, but the broad range of structural and functional therapeutic benefits observed in the present study indicates clear therapeutic potential of the Fortasyn nutrient combination to support functional recovery after ischemic stroke. This recent study investigating a specific dietary approach which has been tested in several mouse models of (risk factors) AD, is the first step in the development of tailor-based dietary treatments against stroke. Increased adherence to a Mediterranean-style diet lowers the risk of ischemic stroke [163], and future studies should therefore start to focus on optimization of multicomponent combinations targeting dietary components of the Mediterranean diet to improve clinical outcome after a stroke.

**Supplementary material**

Supplementary material can be found here:
http://www.thno.org/v07/p0493/thnov07p0493s1.pdf
Summarizing discussion and concluding remarks
Summarizing discussion

Due to the increasing life expectancy, dementia is developing into one of the major public health problems in the aging world population [189, 190]. Alzheimer’s disease (AD) and vascular dementia (VaD) are responsible for most cases of dementia [2, 3]. The production of Aβ peptides – deposited in plaques - is increased in familial forms of AD and is thought to be the primary driving force in non-familial (sporadic) AD pathogenesis [21]. Although this amyloid cascade hypothesis is still the dominant theory for the pathogenesis of AD, it is under debate for years whether the Aβ plaques and the NFTs are the main cause of neurodegeneration in AD [22]. To underline this, studies revealed that the density of senile Aβ plaques in brains of non-affected patients may resemble the large amount of Aβ found in brains of AD patients [23, 24]. Epidemiological and clinical studies demonstrated that AD and VaD share common vascular related risk factors such as hypertension, diabetes, hyperlipidemia, cerebrovascular disease, and arrhythmia [91-96, 192, 193]. Furthermore, it has been shown that vascular risk factors can influence the development of AD pathology (the vascular hypothesis) [194]. AD, VaD, and stroke share hypertension as a common risk factor, while stroke in itself is also a risk factor for the development of AD and VaD. The risk of incident dementia is elevated in patients with ischemic stroke [580], but even mild dementia and cognitive impairment are related to an increased incidence of stroke among subjects age 75 years old and over [581]. The apolipoprotein E ε4 allele (apoE4) represents another strong genetic (vascular) risk factor for AD as it is associated with increased cardiovascular risk factors [12, 50]. Recently, research is focusing on the very early, asymptomatic phase of the disease, in which (cerebro)vascular impairment may be the strongest contributor to the onset and progression of neurodegenerative traits of typical AD and dementia in general [450]. Preventive approaches have therefore consistently emerged as key policy priorities in recently formulated dementia strategies. These approaches include modification of health-compromising behavior such as lifestyle and dietary intake that may lead to AD. Contrarily, the Mediterranean diet (MD; high consumption of fruit, vegetables and legumes, moderate consumption of fish, nuts and olive oil as the main source of fats), has been associated with a reduced risk of AD [445, 446] and with a lower mortality [447, 448]. Notably, in the Doetinchem Cohort Study high intake of some subgroups of fruits and vegetables (i.e. nuts, cabbage and root vegetables) was associated with better cognitive function at baseline and/or smaller decline in in middle-aged individuals [582]. Moreover, a meta-analysis showed that interventions aiming at adopting a MD dietary pattern for at least 1 year reduced both the systolic blood pressure (BP) and diastolic BP levels in individuals with normal BP or mild hypertension [583]. Among persons
at high cardiovascular risk, a MD supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events [584]. Moreover, this latter MD supplemented with nuts was able to exert a beneficial effect on the risk of depression in patients with type 2 diabetes [585]. In accordance with the beneficial effect on hypertension, another meta-analysis revealed that even only a very moderate fish consumption protects against the incidence of ischemic stroke [157]. Docosahexaenoic acid (DHA), one of the main components in fish oil besides eicosaepentanoic acid (EPA), both being omega-3 long-chain polyunsaturated fatty acids (n3-LCPUFAs), has proven its benefits as therapeutic approach for focal ischemic stroke [517, 586]. Other dietary nutrients may instead directly protect synaptic integrity. For instance, the formation of phosphatidylcholine, the most common phosphatide in the brain and a major component of the neuronal membrane, is promoted due to presence of its precursors in the diet [456, 457]. Several preclinical studies confirmed these findings, showing that animals supplemented with the combination of these membrane precursors showed heightened levels of brain phospholipids, dendritic spines and neurite outgrowth, with beneficial effects on cognition [260, 458-461]. Therefore, a novel multi-nutrient supplementation diet called Fortasyn (Fortasyn®Connect, medical name Souvenaid), containing DHA and EPA, besides other precursors and cofactors for membrane synthesis, such as uridine, choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium, has been proposed for the dietary management of AD [462]. Although it has been demonstrated that this nutritional supplement addresses specific nutritional deficiencies in early AD and that it may improve functional connectivity as assessed by EEG [466] and MRI [461], other processes by which it may have an impact on the pathophysiology of AD need to be further investigated, and more studies are required to confirm its efficacy. This thesis aimed to elucidate the underlying pathological processes of major vascular risk factors hypertension, apoE4 and stroke during very early development of neurodegenerative processes in AD using several mice models. Another aim of this thesis was to investigate whether vascular risk factors have the potential to accelerate the course of neurodegenerative processes in AD. In addition, this thesis also intended to investigate the possible capacity of antihypertensives and specific multi-nutrient diets to serve as preventive or treatment against AD-like symptoms and vascular risk factors in the very early stage of AD.
Familiar Alzheimer’s Disease/ Genetic Risk Factors

Aβ

As mentioned above, the excessive production of Aβ proteins and the consecutive formation of Aβ plaques is a pathological hallmark of the familial forms of AD. During the course of AD, pathological hallmarks are not only the deposition of Aβ plaques into parenchymal and cerebrovascular walls, but also the production of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein (NFT), and neuronal cell loss [388-390]. Already during the early stages of AD, the accumulation of Aβ affects specific brain regions like the forebrain and medial temporal lobe structures like hippocampus, amygdala and entorhinal cortex [180, 181, 391]. This increased Aβ production might also lead to cerebral amyloid angiopathy (CAA) which exacerbates cerebrovascular degeneration [587-589].

Studies on MCI and AD patients [246] revealed an impaired cerebral blood flow (CBF) in the early phase of AD, which may possibly be caused by an accumulation of Aβ in the vessel walls or in close vicinity of the blood vessels [204, 247, 248]. The pathophysiological process of AD can be divided into several stages, including the very early phase showing no pathology being followed by the preclinical phase wherein the Aβ accumulation, the synaptic dysfunction, and tau-mediated neuronal injury is promoted [590]. The next pathological stage of AD is the MCI stage involving impairment of brain structures accompanied by cognitive decline [590]. These stages indicate that increased vascular and parenchymal Aβ deposition is accompanied by a diminished CBF leading to impaired cognition. Furthermore, the impaired vasoreactivity observed in our transgenic mice is also a clinical hallmark of AD [249]. Aβ is able to directly enhance the vasoconstriction of the cerebral arteries and to stimulate selected constrictor responses, resulting in a reduced CBF [247]. In addition, deposition of Aβ in the cerebral microvessels promotes vascular pathology and dysfunction [250-252]. In this thesis we also used an Aβ-overexpressing AD-like mouse model, the double transgenic AβPP/PS1 mouse [213, 291]. This AD-like mouse model tested in studies described in chapter 2 to chapter 5 of this thesis resembles a mouse model for cerebral amyloidosis and familial early-onset AD [212, 213]. In chapter 2 we aimed to elucidate the underlying vascular origin of the very early phase of neurodegenerative processes in AD, therefore we investigated the relation between vascular parameters (systolic blood pressure (SBP), cerebrovascular density, cerebral perfusion and vasoreactivity), functional and structural connectivity, and postmortem markers for neuroinflammation, neurogenesis, postsynaptic density, and levels of fatty acids and sterols, and their relationship with cognition in the AβPP/PS1 mouse model. This study aimed to reveal how vascular parameters like SBP are involved in the AD pathology of aging AβPP/PS1 mice. These aging 16 to
18-month-old AβPP/PS1 mice showed an increased SBP linked to a declined regional CBF. Furthermore, using advanced MRI techniques, decline of functional and structural connectivity was revealed in the AD-like mice in relation to impaired cognition, increased locomotor activity and anxiety-related behavior. In accordance, also in chapter 3, we investigated cerebral hemodynamics in younger 12-month old, normotensive and hypertensive AβPP/PS1 mice, and showed that the hypertensive AD-model mice had an impaired CBF in the hippocampus (and a slightly reduced CBF in the thalamus). Hypertension as a major vascular risk factor is able to induce AD-like symptoms like an impaired CBF. Aging and AD genotype lead to more AD-like symptoms due to declining CBF and possibly related energy metabolism of the aging brain. In aging rats, structural abnormalities of cerebral microvessels [591-593] like microvascular fibrosis, and basement membrane thickening were observed [592, 594]. Notably, these age-related, degenerative capillary changes can be accelerated by chronic hypertension [595]. These pathological microvascular alterations combined with a lowered cerebral perfusion can block nutrient transport to and consequent metabolic activity of the brain [596] contributing to impaired cognition [597], as seen in our aged AD mice. In accordance, this AD-like mouse model suffers from early-impaired CBF, but also shows abnormalities in the cortical microvasculature [242-245], additionally presenting cognitive decline and impaired memory [435]. All untreated (normotensive and hypertensive) 12-month old AβPP/PS1 mice demonstrated impaired cerebrovascular circulation (chapter 4). Notably, in chapter 4 we investigated whether AngII-induced hypertension causes neurovascular dysfunction leading to cognitive decline independent of AD pathology. Moreover, we also tested if AngII-induced hypertension adds to the cognitive effects of AD or even is able to mimic AD in absence of underlying AD pathology in 12-month old normotensive and hypertensive AβPP/PS1 mice compared to their age-matched WT littermates. Here, we found again that transgenic AD-model mice had an impaired cerebral blood flow. Paris et al. showed that soluble Aβ peptides can enhance vasoconstriction induced by endothelin-1 (ET-1), which is an endogenous vasoconstrictor [299]. Moreover, in WT and AD model mice cerebral autoregulation remained intact resembling the human AD situation [598]. The severity of disturbances in cerebral hemodynamics is significantly lower in AD compared to VaD [599]. Notably, cerebral vasomotor reactivity to hypercapnia is reduced in AD [600]. In chapter 4 we now further investigated the effect of AngII-induced hypertension in our 12-month-old AD-like model mice using advanced MRI techniques, the Morris water maze (MWM), and immunohistochemical stainings. In particular, at 12 months of age, SBP of AD mice was increased compared to WT littermates. In addition, these hypertensive 12-month-old AD mice presented an impaired functional connectivity (FC)
comparable to the FC at 18 months of age indicating the impact of hypertension on FC in AD-model mice. With resting-state fMRI (RsfMRI) spontaneous neural activity through the blood-oxygen-level-dependent (BOLD) signal change [601] representing FC can be analyzed. Impaired FC is a clinical symptom found in AD patients as well [206-208] and additionally, a link between the incidence of white matter lesions and the severity of the underlying AD pathology like cognitive impairment has also been reported [209-211]. In accordance, a lowered connectivity between cortical regions was detected in older hypertensive AD patients relative to older non-hypertensive AD patients [602]. Furthermore, the aforementioned hyperactivity present in our aged 18-month-old AβPP/PS1 mice seems to be a specific characteristic of many AβPP transgenic mice [441-443] including the AβPP/PS1 mouse, being potentially related to elevated levels of anxiety in these animals [443, 444]. Anxiety is also a clinical symptom of dementia [603]. Post mortem analyses demonstrated also an enhanced neuroinflammation, and both lowered synaptogenesis and neurogenesis in these aged 18-month-old AβPP/PS1 mice (chapter 2). Aβ-plaques in AD are engulfed by activated microglia [271]. In accordance to the study of Minogue et al., an age-associated dysregulation of microglial activation is coupled to an enhanced blood-brain barrier permeability in this mouse model after 14 months of age [268]. In the early pre-plaque stages of transgenic AD mice and, to a much lesser extent, in old WT mice highly activated microglia have been found [267-271]. As mentioned above, all these pathological changes in the AβPP/PS1 mouse model lead to an impaired cognition as found in 18-month-old transgenic mice (chapter 2). In accordance, induced hypertension in 12-month-old AβPP/PS1 mice, was followed by a decreased spatial learning capacity during the MWM indicating a diminished cognitive function like in aged transgenic AD model mice. Additionally, we analyzed the different search strategies to find the hidden platform in the MWM using a parameter-based algorithm to assess the qualitative aspects of learning (chapter 5) [183-187]. In behavioural neuroscience the MWM navigation task is used to examine spatial memory and learning [421]. Cognition of mice can be studied with the common MWM test, but the qualitative aspects of learning cannot be assessed. In our study described in chapter 5 we investigated the use of spatial learning of transgenic mice by analyzing the hippocampus-dependent search strategies. Here, we demonstrated that AβPP/PS1 mice used the “chaining” search pattern during the fourth day of the MWM as their dominant search strategy. During the “chaining” strategy, the mice use circular swimming at a fixed distance from the wall being equal to the distance between platform and wall of the pool. Therefore, they utilize the visual cues or the height of the wall of the pool to determine the distance of the platform from the wall. Several studies revealed the use of diverse search strategies in rats and AD transgenic mice [185, 187, 422, 426-429]. In
accordance to our results, impairment in spatial working memory was demonstrated in a six-arm radial water maze in another transgenic mouse model for AD however showing a retained plasticity to choose alternative search strategies like the chaining search strategy [604]. In addition, our transgenic AD-like mice did not show clear preference for the target quadrant, indicating an impaired spatial memory. This supports the AD-like pathology of the AβPP/PS1 mice presenting cognitive decline and impaired memory [435]. The present AβPP/PS1 mouse model used in the studies described in chapter 2 to chapter 5 showed that an increased SBP is present already in younger, 10- to 12-month-old, transgenic mice, but that induced hypertension promotes a cerebrovascular dysfunction accompanied by an impaired FC and cognition. In contrast, in aged animals increased SBP, impaired cognition, increased locomotor activity, and an anxiety-related behavior was concomitant with impairments in cerebral hemodynamics, and loss of structural and functional connectivity. Therefore, this AD murine model could serve as translational tool for the development of treatments to inhibit neurodegenerative diseases like AD already in the prodromal phase of the disease, also because of its vulnerability to a vascular risk factor like hypertension already at younger age.

ApoE4
As mentioned above, familial early-onset AD (< 1% of all AD cases [388, 605]) is primarily caused by the overproduction of Aβ due to mutations in either the Aβ precursor protein (AβPP) and/ or in genes encoding presenilin 1 or presenilin 2 being crucial components of the γ-secretase complexes responsible for cleavage and release of Aβ [606]. The apolipoprotein E ε4 allele (apoE4) represents another strong genetic (vascular) risk factor for sporadic AD [188]. ApoE is a 34-kDa glycoprotein existing in three isoforms: apoE -ε2, -ε3 and -ε4; differing by one or two amino acid residues 112 and 158 [470]. In detail, apoE, produced mainly by astrocytes, plays a crucial role in cholesterol transport and clearance of Aβ [606]. This small difference of the three isoforms affects the conformation and structure of apoE which changes its potential to bind lipids, receptors and Aβ [471]. Specifically, apoE4 is related with increased neurotoxicity accompanied by a loss of neuroprotective function in the pathogenesis of Alzheimer disease, dependent or independent from Aβ accumulation [606]. Replacement of the murine APOE gene with the human APOE-ε4 (apoE-ε4/apoE4 mouse) [475], creates a novel appropriate animal model to examine the apoE4 phenotype and to measure the effects of very early AD-like pathology development. ApoE4 carrier mice have an altered lipid profile, with increased risk of atherosclerosis plaques formation [475], accompanied by an altered behavior and an impaired cognition [476]. Especially when challenged with a high-fat diet, these transgenic apoE4
mice demonstrate an altered cholesterol metabolism combined with heightened risk of developing vascular disorders and neuronal deficits [475]. The translational character of the apoE4 mouse makes it an extremely attractive model to study the effects of apoE4 on CBF, synaptogenesis and connectivity during aging (chapter 6). No genotype effects on cerebral blood volume, amount of presynaptic boutons, nor neurogenesis were observed in younger 12-month-old apoE4 mice compared to their age-matched WT littermates, while these transgenic mice exhibited an impaired spatial memory at that age [235]. These latter results are in line with the results described in chapter 6 using age-matched apoE4 and WT mice. Here, no effects of apoE4 were detected on all measured parameters including cerebral blood flow and number of post-synapses, despite some slight alterations in the sterol levels and an impaired FC. However, in aged 16- to 18-month-old apoE4 mice an impaired cerebrovascular circulation, a reduced cortical post-synaptic density, and a disturbed white and gray matter integrity in white matter tracts was found. Additionally, these apoE4 mice had a significant lower FC between cortical and hippocampal regions, but also between cortical regions themselves. Recently, we also showed spontaneous functional connectivity impairments in these mice, possibly associated with cerebral blood flow reduction [484]. In a recent study of Luo et al., a decreased interhemispheric FC in apoE4 versus apoE3 carriers was observed [607]. In accordance, in young, middle-aged, and elderly human apoE4 carriers an impaired CBF and cerebral glucose metabolism has been found in cerebral regions being affected during the pathological changes in AD [490]. Furthermore, during aging, apoE4 carriers show a faster decline in CBF than apoE3 carriers, which demonstrates that APOE genotype differentially impacts cerebrovascular function across the lifespan and that it may modify the relationship between CBF and cognition indicating a potential mechanism in the development of AD [608]. In 9 months old apoE4 mice we could not detect impact of genotype on spatial learning and memory [235], but as mentioned above these apoE4 mice exhibited an impaired cerebral hemodynamics at 18 months of age [484]. These latter results elucidate that the apoE4 mice is mirroring the asymptomatic/preclinical phase of AD [590]. These human and our mouse data show the relationship between the apoE4-genotype and aging accelerating the course of neurodegenerative processes in AD like impaired structural and functional connectivity, CBF, and cognition. Therefore, this transgenic apoE4 mouse model demonstrates also several clinical symptoms of AD during aging, making it a suitable animal model to study neurodegenerative diseases already in the prodromal phase of the disease. With our study we could show that apoE4 is not only a strong genetic risk factor for AD. Notably, apoE4 represents also an association with increased cardiovascular risk factors [12, 50] like the impaired CBF in our aged apoE4 model mice indicating that apoE4 is also a major vascular
risk factor for AD. Contributing to an increased risk of AD, the apoE4 genotype is synergistically linked to atherosclerosis, peripheral vascular disease, or type 2 diabetes [606, 609, 610], being by itself already vascular risk factors for AD.

Vascular Risk Factors

Hypertension

Hypertension is considered to be one of the leading causes of morbidity and mortality worldwide [611, 612]. Among all vascular related risk factors, hypertension is the most common cardiovascular risk factor [49, 108-115], being associated with all (other) key markers of AD such as of Aβ deposition, neurofibrillary tangles, and brain atrophy [114]. In several studies [95, 297] an association between midlife elevated BP compared to late-life elevated BP is found with cognitive impairment and AD [298]. Further evidence supporting this link between hypertension and AD comes from preclinical and clinical studies in which it is argued that antihypertensives may reduce the development of AD [196, 197]. A recent study showed that hypertension negatively affects the expression of tau in an animal for tauopathies as well as that of Aβ in an AD mouse model [195]. Other studies demonstrated that hypertension also triggers neuroinflammation prior to Aβ deposition and that chronic hypertension could lead to an impaired blood-brain barrier permeability with deposition of Aβ in brain tissue [315, 316]. Hypertension via promoting the development of atherosclerotic plaques, is able to alter the cerebral blood vessel structure and function [613]. Being a component of the renin-angiotensin system (RAS), Angiotensin II (AngII) is a candidate for the hypothesized mechanistic link between hypertension and AD [285]. AngII plays a crucial role in BP regulation [286], causing vasoconstriction and elevated BP by binding to the AngII-receptor type1 (AGTR1) and AngII-receptor type2 (AGTR2) [286, 287]. It is thought to be a key mediator of the clinical syndrome of essential hypertension [319], through chronically induced vasoconstriction, increased aldosterone secretion, increased sympathetic tone, and cardiac and vascular hypertrophy [320, 614]. To examine the effect of hypertension on WT and AD mice, we used the so called slow pressor model of hypertension because of its similarities to some forms of AngII– dependent hypertension in humans (chapters 3 and 4) [615-617]. In both studies, AD and their WT littermates received systemically a chronic infusion of either AngII (hypertension) or saline (normotension) delivered by subcutaneously implanted micro-osmotic pumps [217] starting from 10 months of age till 12 months of age. In chapter 3 chronic AngII-infusion elevated systolic BP (SBP) in both AβPP/PS1 and WT mice. Notably, in both hypertensive and normotensive conditions AβPP/PS1 mice had a higher SBP than their WT littermates. Another
aim of this study was to determine the relation between AngII-induced hypertension and cerebral circulation. Hypertensive AD mice exhibited an impaired CBF in the hippocampus and thalamus. Using the same hypertension and AD model, chapter 4 focuses more on the pathological changes during AD accompanied with hypertension and vice versa. We chronically infused WT mice with AngII to investigate if AngII can initiate AD pathology, or whether AngII promotes AD-like changes in absence of AD pathology. Therefore, we investigated post-mortem brain pathology and metabolic changes, and in vivo cerebrovascular, FC, and cognitive changes in WT mice. We detected that AngII-induced hypertension did not induce all aspects of AD pathology in WT mice. However, AngII-induced hypertension caused an impaired FC in these WT mice. In accordance, hypertensive patients show deficits in the white matter and functional connectivity in frontal and parietal lobes, associated with cognitive decline [618]. Moreover, in clinical studies a decreased FC is detected in AD patients [207, 208, 619]. In healthy young adults a family history of hypertension was related to subtle changes in visuospatial attention combined with a lowered task-related activation in several brain regions during this visuospatial working memory task without the accumulation of Aβ [620]. The second aim of the study was to determine whether AngII-induced hypertension can exacerbate AD pathology, and to study possible negative interactions between AngII and AD pathology (chapter 4). Notably, confirming the findings of chapter 3, AD mice showed a higher SBP at 12 months of age. At 12 months of age, only hypertensive AβPP/PS1 mice exhibited an impaired FC accompanied by lowered spatial learning capacity. Our results demonstrate impaired FC in AngII-infused mice. In agreement with a recent study, a reduced connectivity between cortical regions was found in older hypertensive AD patients relative to older non-hypertensive AD patients [602]. Furthermore, this diminished cognition present in our hypertensive AD model mice is in line with another study in a rat model of hypertension revealing an impaired memory and learning [368]. No effects of the AngII-induced hypertension was found on structural connectivity using DTI and its related parameters, fractional anisotropy (FA) and mean diffusivity (MD). In contrast, increased blood pressure has been associated with a decrease in FA respectively an increase in MD [378] indicating impaired white and grey matter integrity. Moreover, de Leeuw et al. also demonstrated that long-standing hypertension increases the risk of developing white matter lesions [379]. Nevertheless, in our study, AngII was infused only for two months starting from 10 months of age. This short duration of AngII-infusion can be a reason for the unaffected structural connectivity measured via DTI, but many changes in functional parameters like FC and cognition. Another explanation could be the increased amount of GLUT-1 measured in the thalamus of AngII-infused mice as a compensatory mechanism
leading to this unaffected structural connectivity. GLUT-1 is a marker for vascular endothelial cells and cerebral metabolism [324]. In several animal studies, myocardial ischemia increased the synthesis of GLUT-1 mRNA and protein levels in both ischemic and also non-ischemic cardiac regions indicating also a potential rescue mechanism for myocardial ischemia [370, 371]. Notably, McCall et al. revealed an overexpression of GLUT-1 in microvessels after the onset of global cerebral ischemia in the rat brain [372]. Moreover, Gerhart et al. and Urabe et al. also showed also an overexpression of GLUT-1 in the cerebral cortex in two ischemia and reperfusion animal models [373, 374]. Notably, without inducing experimental hypertension, AβPP/PS1 mice exhibited an increased SBP accompanied by a decreased cortical and thalamic CBF, an impaired hippocampal vasoreactivity, and a diminished cognition as described in chapter 3. Long-standing hypertension stimulates atherosclerosis and vascular remodeling leading to increases in wall thickness. Arterial stiffness and severe atherosclerosis can lead to an increase in pulse pressure [117]. An increased pulse pressure is correlated with a higher risk of AD in older adults [117]. In the Rotterdam study the presence of atherosclerotic plaques or wall thickening has been associated with AD and vascular dementia [109]. In line with our research, Toth et al. demonstrated that hypertension could induce an impaired cerebrovascular autoregulation [254], cerebromicrovascular injury and neuroinflammation in aging mice. Hypertension itself worsens vascular dysfunctions and aggravates pathological processes, like endothelial dysfunction, vascular remodeling, inflammation, and arterial stiffness. All these pathological processes are crucial players in the development of hypertension itself [621]. Moreover, Cifuentes et al. revealed that hypertension advances the development of AD-like structural and functional alterations, partially through cerebral vascular impairment and reduced nitric oxide production [375]. With this thesis we wanted to unravel the impact of hypertension as a major vascular risk factor of AD on the development of neurodegenerative processes in AD. The second aim was, whether hypertension is able to accelerate the course of neurodegenerative processes in AD. In chapter 4 we showed for the first time that a reduction in FC could be introduced in mice via AngII-induced hypertension, also even in the absence of a classical AD pathology (amyloidosis). Using an experimental hypertension mouse model measuring the SBP (chapters 3 and 4), helped us to demonstrate that hypertension promotes and exacerbates pathological processes like cerebrovascular impairments, a diminished FC, and an impaired cognition, without affecting and stimulating Aβ deposition already in younger animals. Our results (chapters 3 and 4) indicate that heightened levels of AngII may indeed represent a mechanistic link between the risk factor hypertension and the clinical development of AD. However, AngII may not be a causal factor for sporadic AD as it did not initiate amyloid pathology in this study. Rather, it may is
involved in the clinical development of AD in people with amyloid accumulation through its negative effects on CBF, FC and cognition. Notably, also in aged AD model mice (chapter 2) we detected an elevated SBP, deteriorations in cerebral hemodynamics resulting in an impaired cognition and structural and functional connectivity, increased locomotor activity, and anxiety-related behaviour being all symptoms of clinical AD. These latter results elucidate that hypertension and vascular impairment play important roles in the very early stage of AD but whether these are causative factors or just a contributors aggravating the disease progress, remains to be investigated.

Stroke
Another vascular risk factor for dementia is stroke. Global stroke incidence was calculated at 16 million cases in 2005, and is projected at 23 million by 2030 [622]. Stroke is the second leading cause of morbidity and mortality [513]. Many stroke patients show a gradual but continuous deterioration following a single stroke lesion [16] being characterized clinically by cognitive and behavioral dysfunction. 87% of all strokes are ischemic [521], indicating that specific treatments should target CBF impairments [516]. Stroke is a major vascular risk factor for dementia [623]. The accumulation of AβPP and Aβ 1-42 demonstrated in patients with multi-infarct dementia emphasizes the overlap and relationship between AD and VaD [124-126]. In animal models of cerebral ischemia a relationship between the AβPP and cerebral ischemia was demonstrated [124, 125, 127]. Notably, in a combined AD and cerebral ischemia rat model the presence of Aβ increased the infarct size, neuroinflammation and also cognitive deficits [133]. A highly sensitive fluorescent RT-PCR assay revealed a significant increase in the peripheral blood expression of AβPP mRNA levels among patients who suffered from recent stroke [128]. A correlation between the density of cortical microinfarcts (CMIs) and the degree of cerebral amyloid angiopathy (CAA) was found in a postmortem analysis of human brains [129]. After subjecting AβPP/PS1 to microstrokes, Garcia-Alloza et al. showed that also vice versa stroke is able to accelerate Aβ deposition via interference with amyloid clearance pathways revealing a fast increase in Aβ plaque burden and CAA in the region surrounding the infarction [130]. As mentioned above, the risk of incident dementia is high among patients with ischemic stroke [580], but also already mild dementia and cognitive impairment are associated with an increased incidence of stroke among subjects age 75 years old and over [581]. Ischemic strokes have been the target of many failed drug trials [516] and only few treatment options are available, and therefore the a large amount of patients suffers from significant poststroke disabilities [517-519]. There is therefore an urge for restorative interventions supporting better recovery and improving quality of life. In chapter 7 we used a transient middle cerebral artery
occlusion model as mouse model for stroke. Here, 3 months old, male C57BL/6J mice underwent right tMCAo (30 minutes), using an intraluminal occlusion model [544, 545]. This stroke model of transient occlusion of the MCA mimics one of the most common types of ischemic stroke in patients [544, 546], as mentioned above accounting for 87% of all stroke cases [521]. The MCA was transiently occluded for 30 min. This ischemia time leads to moderate pathological changes within the infarct core, perilesional and remote regions [547-549]. In accordance with human stroke, this reperfusion model reveals a substantial degree of reperfusion via collateralization through the Willis’ circle and leptomeningeal collaterals, and via early clot lysis [550]. Here (chapter 7), after an induced experimental stroke, mice demonstrated a decrease in body weight. Moreover, after the experimental stroke these mice walked less and slower, leaned and reared less, and showed a decreased explorative respectively an increased anxious phenotype in the open field. Using advanced imaging techniques, these stroke mice exhibited 7 days after stroke increased neuroinflammation, impaired cerebrovascular function accompanied by a diminished structural and functional connectivity in the tMCAo affected cerebral hemisphere, being all clinical hallmarks and risk factors for dementia [624]. Notably, in the stroke control mice CBF dropped over time in several brain regions in 7 to 35 days poststroke indicating the impact of aging on clinical outcome after stroke. In detail, we revealed an impaired WM microstructure at seven days poststroke in regions containing the Caudate Putamen and Globus Pallidus. In accordance to our results, Firbank et al. found in human studies, that the poststroke dementia group has more vascular pathology indicated by white matter hyperintensities (WMH) volumes and lower cortical perfusion than the poststroke no dementia group [625]. In contrast, in the AD group a reduced cerebral perfusion specifically in the parietal and prefrontal area, consistent with previous studies [626], but no increase in WMH volumes was found [625]. Revealing enhanced neuroinflammatory response in the stroke affected hemisphere, our results are in line with current research showing that inflammation is a crucial key player in the pathogenesis of ischemic stroke and other forms of ischemic brain injury [625]. In our research the tMCAo mouse model (chapter 7) has shown several AD-like symptoms making it a highly translational model to study the interaction of stroke and AD. In our thesis we aimed to reveal the underlying vascular origin of stroke during very early development of neurodegenerative processes in AD using several mice models. Here (chapter 7), we showed that in our tMCAo mouse model an impaired cerebral blood flow is accompanied by a worsened structural and functional connectivity, and an enhanced neuroinflammmatory response indicating the influence of stroke on the development of AD-like symptoms.
Treatments

**Antihypertensives**

With this thesis we aimed to increase the knowledge of antihypertensive actions on the cerebrovascular system in AD and on AD-like symptoms, herewith possibly supporting the development of effective, tailor-made blood pressure lowering treatments for AD patients. Preclinical and clinical studies reveal that antihypertensives may reduce the development of AD [196, 197]. Specifically, AngII receptor blockers (ARBs) have been associated with a lowered risk of AD [278, 279]. In the last decade many studies with contradictory results have been performed. To illustrate this point: no significant effects of active treatment on the incidence of dementia [281, 282] were found compared to placebo as shown in two randomized, placebo-controlled studies, the ‘Study on Cognition and Prognosis in the Elderly’ (SCOPE; candesartan/ hydrochlorothiazide vs placebo) and the ‘Systolic Hypertension in the Elderly Program’ (SHEP; chlorthalidone/ atenolol/ reserpine vs placebo). Contrarily to the examples above, two other randomized, placebo-controlled studies named ‘Systolic Hypertension in Europe’ (Syst-Eur) and ‘Perindopril Protection Against Recurrent Stroke Study’ (PROGRESS), showed beneficial effects on the incidence of dementia and cognitive decline [280, 283, 284]. The Syst-Eur study revealed that active treatment with nitrendipine, enalapril, and/or hydrochlorothiazide lowered the incidence of dementia [280, 283]. Results from the ‘Observational Study on Cognitive function And SBP Reduction’ (OSCAR) revealed that Eprosartan (ARB) is associated with preservation or improvement of cognitive performance [627]. A case control analysis within the UK showed that patients on ARBs and angiotensin converting enzyme inhibitors (ACE-Is) had a respectively 53% and 24% reduced risk of AD, compared to patients on other anti-hypertensive medications [278]. To analyze the impact of antihypertensives on hypertension and to differentiate between the effect of AngII and elevated SBP per se (chapter 3), we also investigated the intervention by using two different antihypertensives: 1) the diuretic hydrochlorothiazide (HCT) and 2) the ARB eprosartan mesylate (EM). Both EM and HCT were able to lower the SBP in saline-infused and AngII-infused mice. Notably, AngII-induced hypertension was reduced by EM and HCT to near-normal values only in WT mice, and not in AβPP/PS1 mice. EM and HCT were not effective in lowering SBP and were not able to restore the decrease in hippocampal and thalamic CBF in our (hypertensive) AD-model mice, possibly by the vasotoxic effects of the present Aβ. EM was even less effective in reducing SBP than HCT. In contrast, Kume et al. revealed that Telmisartan, another ARB like EM, was able to elevate regional CBF in hypertensive patients, as opposed to those treated
with amlodipine [314]. In our study, after one month of untreated hypertension a clinical dosage of EM was supplemented to the mice only for one month indicating that the duration of treatment was probably too short. This is similar to results of a clinical trial, in which normotensive mean values were reached in 75.5% hypertensive stroke patients just after three months of treatment with EM, [628]. Another possible explanation for the decreased impact of EM on SBP and CBF could be that EM directly targets Aβ by inhibiting its accumulation. It has been shown that another ARB, Valsartan, was able to attenuate oligomerization of Aβ peptides into high-molecular-weight oligomeric peptides [307]. Due to increased Aβ production in AβPP/PS1 mice, EM molecules may be bound to Aβ peptides. Therefore, less EM molecules will be free and able to inhibit AngII activity mediated by its receptor, and, therefore, less EM is able to bind to the AngII receptor blocking its antihypertensive effect. This mechanism may explain the genotype effect of EM in our study in which EM decreased SBP in AβPP/PS1 mice in a less effective manner than in their WT littermates. As described in the previous chapter, SBP was also lowered in animals treated with EM. Treatment of hypertension with EM enhanced hippocampal CBF. This beneficial effect was shown in all mice, and was not restricted to transgenic animals only, indicating that either blood pressure lowering or blocking effects of endogenous AngII may be beneficial for brain perfusion. In accordance, blood pressure lowering treatments heightened CBF in older subjects with hypertension as well [377]. Furthermore, after EM-treatment we also detected an improved microstructural integrity measured via DTI in sal-infused mice and AngII-infused AD mice. Notably, a novel finding is the negative effect of EM on FC in all Sal-infused mice, but the beneficial effect in AngII-infused mice. While all saline-infused EM-treated mice had an impaired FC compared with untreated mice, all AngII-infused EM-treated mice showed an improved FC compared with untreated mice. This reveals the positive effect of EM as antihypertensive treatment on CBF, microstructural integrity of white and grey matter, and concomitant FC. In accordance to our results, the secondary longitudinal data analysis of the Ginkgo Evaluation of Memory Study demonstrated that diuretics, ARBs, and ACE-Is in addition to and/or independently of mean SBP, were associated with a lowered risk of AD dementia in participants with normal cognition, while only diuretics were associated with reduced risk in participants with MCI [629]. Nevertheless, not all AD-like pathological changes like an impaired cognition or CBF could be ameliorated by EM. These results are in line with the SCOPE and SHEP studies showing also no effect of antihypertensives on the incidence of dementia [281, 282]. Our results indicate the necessity to gather more insight in the actions of antihypertensive treatments on brain processes and the vascular system being involved in pathological processes of AD leading
to a better development of more effective tailor-made blood pressure-lowering treatments for early stage AD patients. In addition, our data also elucidate that the usage of only antihypertensives is not leading to a reduction of all AD-like symptoms.

Diet (preventive/therapeutic approach)

Extensive research has been pursued in search for effective therapies for AD. Till now, no effective treatment is available nor seems near. Preventive approaches have therefore consistently emerged as key policy priorities in recently formulated dementia strategies including modification of health-compromising behavior such as sedentary lifestyle and high fat, high caloric dietary intake leading potentially to AD. Lately the Mediterranean diet has been associated with a reduced risk of AD [445, 446] and with a lower mortality [447, 448]. The mechanisms via which diet influences the onset and progression of AD pathology are still under investigation. One possible mode of action is the beneficial effect of nutrients, such as omega-3 long-chain polyunsaturated fatty acids (n3-LCPUFAs) on the vascular system [448, 449]. Several epidemiological studies and controlled trials showed a correlation between B-vitamins, n3-LCPUFAs and monounsaturated fatty acids and improvements in autonomic function, reduced blood pressure, decreased atherosclerosis, lowered total homocysteine and enhanced microvascular endothelium-dependent vasodilation processes [448, 500, 501]. Atherosclerosis [451], high blood pressure [452] and other cardiovascular diseases are risk factors for cardiovascular disease and AD and are diminished by n3-LCPUFAs [451, 453-455]. Based on these findings, a novel multi-nutrient supplementation diet called Fortasyn, comprising n3-LCPUFAs, DHA and eicosapentaenoic acid (EPA), besides other precursors and cofactors for membrane synthesis, such as uridine monophosphate (UMP), choline, phospholipids, folic acid, vitamins B12, B6, C, and E, and selenium, has been proposed for the dietary management of very early stages of MCI and AD [462, 562]. To investigate the possible capacity of such a specific multi-nutrient diet to serve as preventive or treatment against AD-like symptoms and vascular risk factors for AD, we studied the effect of this specific multi-nutrient supplementation diet in chapter 6. This diet is designed to ameliorate synaptic loss and to reduce membrane-related pathology in AD by providing nutritional precursors and cofactors to support neuronal membrane formation and function [495] in younger and aged apoE4 model mice. These mice resemble patients in the prodromal phase of AD. Here, we hypothesized that this nutritional intervention with Fortasyn may be able to inhibit or prevent the occurrence of early AD-like pathologies such as cerebrovascular impairment and concomitant brain connectivity loss. In apoE4 mice, particularly, the strongest and most consistent dietary effect in these studies
involve the improvement of cerebrovascular health and FC indicated by an increased cortical CBF and levels of post-synaptic markers in both apoE4 and WT animals on Fortasyn diet. Concomitant with the preserved FC in both WT and AD mice fed with Fortasyn, we also observed an improved white matter integrity. In support with our findings, it has been demonstrated that animals fed with a diet containing a part of the diet composition, UMP, n3-LCPUFAs and choline, showed increased levels of brain phospholipids, dendritic spines and neurite outgrowth [458-460]. Our results show that this Fortasyn diet is a preventive multi-targeting approach against impairments in the cerebral vasculature, connectivity and underlying pathological mechanisms like neurogenesis and neuroinflammation.

In addition, in the study described in chapter 5, we investigated the impact of Fortasyn on spatial learning and memory in 11-month-old male AβPP/PS1 mice and WT littermates. To this end, we analyzed the different search strategies to find the hidden platform in the Morris water maze to assess the qualitative aspects of learning using a parameter-based algorithm [183-187]. We investigated whether Fortasyn may improve spatial learning of transgenic mice by facilitating the use of hippocampus-dependent search strategies. Here, Fortasyn induced beneficial effects on learning and memory in both AβPP/PS1 mice and wild-type animals. During the MWM acquisition, Fortasyn promoted the AD model mice to use the chaining strategy thereby developing a highly efficient way to find the platform being independent of an intact hippocampus [430]. While chaining is considered to be a coping strategy, it seems to help to compensate for the cognitive decline in these AβPP/PS1 mice. In accordance, our results are concordant with other studies showing that fish oil containing diets improve spatial memory in AβPP/PS1 mice and ameliorate performance in hippocampus dependent spatial memory tests like the MWM [432-434]. In these AβPP/PS1 mice we also measured the effect of Fortasyn on behavior, neurogenesis [630], cerebrovascular health, and other neuroprotective mechanisms [461]. Fortasyn had an anxiolytic effect, restored neurogenesis to normal WT values, enhanced cerebral perfusion, and protected against neurodegenerative processes in both grey and white matter improving also spatial learning capacity (chapter 5) in these AD mice. Till now, only two randomized controlled clinical trials have revealed improvements in the delayed verbal recall task and better cognitive performance in mild AD patients supplemented with this nutrient combination [463-465]. In contrast, a double-blind randomized controlled trial in patients with mild-to-moderate AD demonstrated that 24-week use of Souvenaid (Fortasyn®Connect) did not slow cognitive decline in these patients [631]. In addition, in a recent exploratory 24-week, double-blind, randomized, controlled electro- and magnetoencephalography study also no interventional effects of Souvenaid on brain activity could be detected in mild AD patients [632]. This
Fortasyn diet contains besides DHA and EPA, other precursors and cofactors for membrane synthesis [462]. DHA, alone or combined with EPA, contribute to improved memory function in older adults with mild memory complaints [633]. Combinations of EPA and DHA (EPA ≥ 60%) were effective against primary depression [634]. Notably, supplementation with EPA and/or DHA improved verbal fluency and attention in patients who had only very mild dementia or AD or presented apoE4 negative genotype [635]. But in AD patients, EPA and/or DHA supplementations did not reduce cognitive decline rates [635]. Some observational studies demonstrated that higher dietary intake or heightened serum levels of folate and fish/fatty acids accompanied with low serum levels of homocysteine were associated with a reduced risk of incident AD and dementia, while other studies showed no association [500]. Besides, the combination of phosphatide precursors like n3-LCPUFAs, uridine, and choline, has proven to synergistically increase the synthesis of synaptic proteins and phospholipids in the brain [458-460]. In addition, a meta-analysis of randomized controlled trials has proven, that the addition of EPA+DHA reduces SBP [636]. All the results of single component diets are inconsistent. Although it has been recognized that Fortasyn addresses specific nutritional needs in early AD and that it improves functional connectivity as assessed by EEG [466], other processes by which Fortasyn may influence the pathophysiology of AD need to be further elucidated, and more studies are required to confirm its efficacy. Therefore, the combination of our results from a multi-nutrient dietary approach and epidemiological data of dietary patterns on risk reduction of AD [637] indicates a greater opportunity of intervention in the very early phase of AD. This has been proven in our two mouse models of the early AD phase showing several beneficial effects. The data presented in this thesis regarding the impact of such a multicomponent diet on AD is encouraging for planning and exerting future clinical studies. Future studies developing multicomponent diets against AD should focus on the cerebral vascular impairment in the latent phase of AD, thereby protecting functional and structural connectivity, combined with improved cognition to develop tailor-based and personalized preventive treatments against the progression of AD, already during the very early phase of this disease. Therefore, these studies should further elucidate the impact and efficacy of Fortasyn and Mediterranean type dietary compositions on the pathophysiology of AD.

In both studies described in chapters 5 and 6 we investigated the effect of such a specific nutrient combination diet as a preventive approach on pathological processes in AβPP/PS1 and apoE4 mouse models representing both mouse models of (vascular) risk factors for AD. For each study, male transgenic mice and their WT littermates were assigned to the different diet groups from 2 months of age, until the end of the experiment. Nevertheless, ischemic strokes have been the target
of many failed drug trials [516] and only few treatment options are available, but many patients still show significant poststroke disabilities [517-519]. Until now, thrombolysis and thrombectomy are the common treatments for ischemic stroke, but not many patients benefit from these therapies due to the narrow therapeutic window and complexity of administration [515, 520]. There is therefore an urge for restorative therapeutic interventions supporting better recovery and improving quality of life. Novel approaches targeting several early underlying processes like CBF, neuroinflammation, and brain networks may provide new avenues for stroke treatment [522]. In particular, dietary approaches could facilitate recovery after stroke [523, 524] because it has been shown that increased adherence to a Mediterranean-style diet is associated with a lowered risk of ischemic stroke and myocardial infarction [163]. Among persons at high cardiovascular risk, a MD supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events [584]. Moreover, this latter MD supplemented with nuts had a beneficial effect on the risk of depression in patients with type 2 diabetes [585]. Therefore, in chapter 7 we elucidated the possible efficacy of this multicomponent diet as therapeutic intervention after transient ischemic stroke, another major vascular risk factor of AD on both structural and functional parameters. Using state-of-the-art imaging techniques, we measured CBF, and the neuroinflammatory response after ischemia. Our data described in chapter 7 indicate that a post-stroke intervention with the specific nutrient combination of Fortasyn is able to support recovery after ischemic stroke, by improving cerebral circulation due to an improved vascularization and increased angiogenesis, protecting white and gray matter integrity, restoring functional connectivity, increasing neurogenesis, decreasing the neuroinflammatory response, and improving motor skills and muscle strength. The present data show that the specific nutrient combination Fortasyn may enhance recovery from ischemic stroke in mice, as evidenced by improved cerebral blood flow, reduced loss of functional connectivity, and improved motor performance. Relevant processes for recovery after stroke, such as neurogenesis, angiogenesis, and neuroinflammation, were positively affected by the Fortasyn diet. In accordance, this neurorestorative potential of Fortasyn has recently been obtained in a rodent model of spinal cord injury as well [537]. Comparable results regarding structural and functional connectivity were found in other studies showing that intracerebral injection of EPA is able to stimulate the expression of myelin proteins in rat pup brain [560] and that n3-LCPUFA are crucial for functional connectivity and large-scale system organization in rhesus macaque brains [561]. Additionally, Fortasyn preserved the organization of brain networks in patients with mild AD within 24 weeks potentially via counteracting the progressive network disruption over time in AD [562]. This specific multi-nutrient approach has proven to counteract several
pathological alterations in several mouse models of (vascular) risk factors of AD as either a preventive or a therapeutic dietary approach (Chapters 5 to 7). Nevertheless, even low fish consumption may already decrease the incidence of ischemic stroke [157]. In women, intake of n3-LCPUFAs is related to a lowered risk of total stroke, while dietary cholesterol has been found to be positively associated with risk of total stroke and cerebral infarction [162]. As mentioned above, n3-LCPUFAs have shown to diminish severity of vascular risk factors, like atherosclerosis [451], high blood pressure [525], and other cardiovascular diseases [451, 526-528]. N3-LCPUFAs have a protective effect against cerebral ischemia in rats, presented by a reduced number of apoptotic neurons in the prefrontal cortex when fed a standard diet combining EPA and DHA [160]. Moreover, combined administration of DHA and UMP improved learning abilities in rats [529] and combined administration of DHA and EPA improves (cerebral) vascular health in human [530, 531]. These studies described in chapters 5 to 7 investigating a specific dietary approach which has been tested in several mouse models of (risk factors) AD, is the first step in the development of tailor-based dietary treatments against dementia. Future studies should therefore start to focus on optimization of multicomponent combinations targeting simultaneously several underlying processes of the early pathological processes in dementia and its (vascular) risk factors to develop effective preventive or therapeutic approaches. These future studies should evolve personalized approaches adapted to the individual pathological problems like an increased neuroinflammatory response etc..

Concluding remarks

The elucidation of the underlying source of major vascular risk factors like hypertension, apoE4 and stroke during very early development of neurodegenerative processes in AD using several mice models was the first aim of this thesis. Hypertension has proven to be associated with cerebrovascular impairment already at young age in AD model mice leading to reductions in structural and functional connectivity accompanied by an impaired cognition. Notably, aged AD mice developed an increased SBP concomitant with several AD-like pathological changes, like impairment in cerebral hemodynamics resulting in an impaired cognition, structural and functional connectivity, increased locomotor activity, and anxiety-related behavior without ANGII-induced hypertension. Notably, also in aged transgenic apoE4 mice an impaired cerebrovascular circulation, reduced cortical post-synaptic density, disturbed white and gray matter integrity in white matter tracts, and a lowered FC were detected. In conclusion, the (vascular) risk factors being present and/or induced in our mouse models have proven to be involved in the very early
development of neurodegenerative processes in AD. Therefore, all mouse models used for this thesis are animal models for the asymptomatic phase of AD, but possessing the potential to be challenged by (vascular) risk factors to develop and to accelerate the course of neurodegenerative processes in AD. Another aim of this thesis was to reveal the possible capacity of antihypertensives and specific multi-nutrient diets to serve as preventive or treatment against AD-like symptoms and vascular risk factors for AD. Despite their blood-lowering actions, we showed that antihypertensives like EM have beneficial effects on pathological processes of AD like counteracting the impaired CBF, and the reduced structural and functional connectivity. Therefore, it is important to improve development of more effective tailor-made blood pressure-lowering treatments for AD patients and to increase awareness for hypertension as a risk factor for AD. Furthermore, we demonstrated the value of a multi-modal approach, including advanced MR neuroimaging tools, for detecting changes in brain structure and function with respect to dietary intervention. The investigated multicomponent diet showed several beneficial effects as a preventive but also as a therapeutic approach on pathological alterations in mouse models for (vascular) risk factors for AD. Future studies should therefore focus on optimization of multicomponent combinations comprising dietary components of the Mediterranean diet to improve clinical outcome after a stroke to lower the incidence of stroke accompanied by a reduction of the incidence of dementia. Moreover, all tests were performed in relative young animals. Therefore, future studies should repeat all studies in older mice. In accordance, Toth et al. demonstrated that hypertension in aging mice could induce an impaired cerebrovascular autoregulation, cerebromicrovascular injury and neuroinflammation [254]. Also in our stroke study we used relatively young mice to evaluate our specific dietary approach against pathological changes of the experimental ischemic stroke. It has been confirmed that aged animals react differently to an induction of an experimental stroke [638]. Furthermore, for apoE4 [511, 639], hypertension [640], and also for stroke [641] sex differences have been revealed. Therefore, it is of utmost importance to study gender differences regarding to all of these risk factors of AD by testing also female mice during lifespan. All in all, vascular risk factors for AD have several negative effects on the very early development of neurodegenerative processes involved in AD, but these risk factors can be challenged and blocked by an effective tailor-based preventive or therapeutic approach lowering the incidence of AD. As our experimental results and epidemiological data have shown that treatment with only antihypertensives or single-component diets is not sufficient to fully counteract underlying processes of the early pathological processes in dementia. Therefore, future research should focus on preventive/therapeutic approaches using personalized multinutrient dietary approaches targeting pathological
processes like neuroinflammation, impaired CBF, cognitive impairments, and other clinical symptoms of dementia. Future studies should evaluate a mixed preventive approach containing cognitive and physical exercise [642, 643], in combination with a personalized multicomponent diet during the very early phases of AD. Moreover, these mixed preventive approaches need to be personalized for each individual dementia patient to develop an optimal prevention and to cover each individual genetic make-up.
Nederlandse samenvatting
Nederlandse samenvatting

Wereldwijd vormt dementie een groot gezondheidsprobleem mede door de hogere levensverwachting van mensen [189, 190]. De ziekte van Alzheimer (AD) en vasculaire dementie (VaD) zijn de twee meest voorkomende vormen van dementie bij ouderen [2, 3]. In de familiale variant van AD is de productie van het amyloid-β (Aβ) eiwit verhoogd door een genetische afwijking, dit resulteert in de karakteristieke Aβ eiwit ophopingen in het brein. Ook in de niet-familiaire (sporadische) variant van AD worden de Aβ overproductie en ophopingen daarvan als drijvende kracht gezien [21]. De Aβ cascade hypothese is nog steeds de meest dominante theorie voor de pathogenese van AD, echter staat ter discussie of de Aβ ophopingen en de klussen van draadvormige eiwitten (neurofibrillaire tangles) in de hersencellen de hoofdoorzaak van de neurodegeneratie in AD zijn [22]. Zo hebben bijvoorbeeld verschillende studies laten zien dat de hoeveelheid Aβ ophopingen in de hersenen van niet-Alzheimer patiënten net zo hoog kan zijn als in de hersenen van AD patiënten [23, 24]. Verder hebben epidemiologische en klinische studies aangetoond dat AD en VaD gemeenschappelijke vasculaire risico factoren hebben, zoals een hoge bloeddruk (hypertensie), diabetes, hyperlipidemie zoals verhoogd cholesterol waarden in je bloed, cerebrovasculaire aandoeningen, en hartritmestoornissen [91-96, 192, 193]. Dus vasculaire risicofactoren kunnen invloed hebben op het ontwikkelen van de AD pathologie [194]. Verder hebben AD, VaD en beroerte, hypertensie als een gemeenschappelijke risicofactor, en is het krijgen van een beroerte ook een risicofactor voor het ontwikkelen van AD en VaD [582].

Het apolipoproteine E4 gen (apoE4) levert een grote genetische (mede via het vasculair systeem) bijdrage aan de ontwikkeling van AD. Dit gen is betrokken bij het cholesterol metabolisme [12, 50]. Het AD onderzoek richt zich steeds meer op de vroege, asymptomatische fase van de ziekte, waarin (cerebro)vasculaire aandoeningen zich manifesteren [449]. Vanuit de wetenschappelijke gemeenschap is er met toenemende mate belangstelling voor het ontwikkelen van preventieve benaderingen om AD te bestrijden. Deze strategieën hebben als doel om het aantal AD incidenten te verlagen door onder andere aanpassingen op het gebied van voeding en levensstijl. Een Mediterraan dieet (MD), wat een hoge consumptie van fruit, groenten en peulvruchten, matige consumptie van vis, noten en olijfolie in houdt, wordt in verband gebracht met een verlaagd risico op AD [444, 445] en met een lagere mortaliteit [446, 447]. In de Doetinchem Cohort Studie werd bijvoorbeeld aangetoond dat een verhoogde inname van groenten en fruit (o.a. noten, koolsoorten en knolgewassen) geassocieerd met een betere cognitieve functie [584]. Een meta-analyse toonde aan dat interventies met een MD voor minstens 1 jaar zowel de systolische als de diastolische bloeddruk kunnen
Nederlandse samenvatting

Chapter 9

verlagen bij mensen met normale bloeddruk of milde hypertensie [585]. Ook vermindert een MD het ontstaan van ernstige cardiovasculaire gebeurtenissen bij mensen met een hoog cardiovascular risico [586]. Naast het gunstig effect op de bloeddruk, werd in een andere studie gevonden dat een matige visconsumptie beschermt tegen het ontwikkelen van herseninfarcten [157].

Doxosahexaeenzuur (DHA) en eicosapentaæenzuur (EPA) behoren tot de omega-3 meervoudig onverzadigde vetzuren (n3-LCPUFAs) en zijn hoofdcomponenten van visolie. Verschillende studies hebben aangetoond dat DHA een positief effect kan hebben bij het herstel van herseninfarcten [516, 588]. Andere voedingsstof fers zoals bijvoorbeeld fosfatiden kunnen direct de synaptische integriteit beschermen. De aanwezigheid van fosfatiden kan de vorming van fosfatidycholine stimuleren, wat de meest voorkomende fosfatide in de hersenen is en een belangrijke component vormt voor de neuronale membranen [455, 456]. Deze bevindingen werden door verschillende preklinische studies bevestigd. Als reactie op deze bevindingen werd er een specifiek dieet ontwikkeld speciaal voor AD patiënten. Dit dieet is verrijkt met DHA en EPA, naast andere voorlopers en cofactoren betrokken bij de membraansynthese zoals uridine, choline, fosfolipiden, foliumzuur, vitamines B12, B6, C en E en selenium [461]. Verschillende studies hebben aangetoond dat Fortasyn de verlaagde concentraties van specifieke voedingscomponenten kan aanvullen in beginnende AD en ook dat het de functionele connectiviteit kan worden verbeterd zoals gemeten met EEG [465] en MRI [460].

Dit proefschrift heeft als focus om met behulp van verschillende muismodellen de onderliggende pathologische processen van vasculaire risicofactoren zoals hypertensie, apoE4 en beroerte te onderzoeken tijdens de zeer vroege ontwikkeling van neurodegeneratieve processen binnen AD. De vasculaire risicofactoren voor AD worden bediscussieerd in hoofdstuk 1. Een ander doel van dit proefschrift was om te ontrafelen of vasculaire risicofactoren, zoals hypertensie of een herseninfarct, de potentie hebben om het verloop van neurodegeneratieve processen in AD te versnel len. Daarnaast is deze thesis bedoeld om te onderzoeken of bloeddrukverlagende middelen en specifieke multinutriënt diëten als preventieve of therapeutische middelen tegen vasculaire risicofactoren en AD symptomen in het vroege stadium een positief effect kunnen hebben.

Om de vasculaire oorsprong van neurodegeneratieve processen in AD te kunnen ontrafelen, hebben wij in hoofdstuk 2 onderzoek gedaan naar de relatie tussen de systolische bloeddruk (SBP), cerebrale doorbloeding (CBF), vasoreactiviteit, hersenstructuur en -functie in 16-18 maanden oude AD-muismodellen (AβPP_swe/PS1_ΔE9 (AβPP/PS1)). Deze oude AβPP/PS1 muizen hadden een verhoogd SBP gekoppeld aan een verlaagd CBF. Met behulp van geavanceerde MRI-technieken werd de achteruitgang van de functionele en structurele connectiviteit in het brein
onthuld in deze AD muismodellen. Daarbij hadden deze muizen een verminderde cognitie, verhoogde bewegingsactiviteit en angst-gerelateerd gedrag. Post mortem analyses toonden ook aan dat de neuroinflammatie was verhoogd, terwijl de synaptogenese en de neurogenese in deze AβPP/PS1 muizen was gedaald. Ook werden afwijkende concentraties van vetzuren en sterolen in het hersenweefsel van de AβPP/PS1 muizen gevonden, waardoor men kan concluderen dat deze transgene muizen een slecht aangepast cerebrale vetzuur metaboli sme hebben.

Onze bevindingen suggereren een verband tussen verhoogde SBP, verminderde cerebrale hemodynamiek en connectiviteit in een AD muismodel gedurende het verouderingsproces. Dit zorgt uiteindelijk voor afwijkingen in gedrag en cognitie. Deze resultaten weerspiegelen de complexe klinische symptomatiek in de preklinische fase van AD en daarom stellen wij voor dat dit AD muismodel kan worden gebruikt voor het onderzoek naar strategieën voor de preventie en behandeling in vroegtijdige AD patiënten. Bovendien draagt deze studie bij aan het ontwikkelen van meer en betere therapeutisch en diagnostiek voor het zeer vroege stadium van AD. Hypertensie is namelijk goed te behandelen al wanneer een mogelijke kans biedt ter preventie van AD. Verhoogde bloedwaarden van angiotensine II (AngII) zijn een belangrijke oorzaak van hypertensie. AngII heeft schadelijke effecten op de endotheelfunctie van bloedvaten en CBF, beide kunnen bijdragen tot de ontwikkeling van AD. AngII remmende middelen zijn daardoor potentiële kandidaten om AD risicofactoren bij hypertensieve patiënten te verlagen. Daarom hebben wij in hoofdstuk 3 het effect onderzocht van 2 maanden geïnduceerde hypertensie en daaropvolgend het effect van antihypertensiva op de SBP en CBF in 10 maanden oude wild-type (WT) C57BL/6J en AβPP/PS1 muizen. Hypertensie werd geïnduceerd met behulp van AngII-infusie via onderhuidse osmotische micropompen en als antihypertensiva werden dan wel eprosartan mesylaat (EM, 0,35 mg / kg) of hydrochloorthiazide (HCT, 7,5 mg / kg) gebruikt na 1 maand van geïnduceerde hypertensie. SBP werd twee keer per maand gemeten via staart manchet plethysmografie. CBF werd gemeten met MRI met een “arteriële spin labeling”, bij deze methode wordt het bloed zelf als endogene tracer gebruikt. Chronische AngII-infusie veroorzaakte een verhoging van SBP zowel in AβPP/PS1 als in WT-muizen. Deze verhoging van SBP ging gepaard met een CBF afname in de hippocampus en thalamus in de AβPP/PS1 muizen. De SBP in de AβPP/PS1 muizen was hoger onder zowel hypertensieve en normotensieve condities in de WT muizen. Antihypertensiva waren niet effectief in het reduceren van AngII-geïnduceerde hoge bloeddruk tot normale waarden in AβPP/PS1 muizen, terwijl deze wel effectief de bloeddruk in WT-muizen verlaagden. Uit deze resultaten kunnen wij concluderen dat AngII-geïnduceerde verhoogde bloeddruk kan leiden tot een vermindering in CBF gecombineerd met een verlaagde gevoeligheid voor een bloeddrukverlagende behandeling in een transgeen AD-muismodel. Onze
bevindingen suggereren een verband tussen hypertensie op middelbare leeftijd en een verminderde CBF in een AD muismodel, vergelijkbaar met het al in AD patiënten gevonden verband. Zoals al in hoofdstuk 2 geconcludeerd, konden wij met deze studie bevestigen, dat dit translationele muismodel kan worden gebruikt om mogelijke preventie en behandelingsstrategieën voor AD te onderzoeken. In hoofdstuk 4 hebben wij ons verder verder diep in het effect van 2 maanden AngII-geïnduceerde hypertensie op SBP in WT en AβPP/PS1 muizen. Bovendien onderzochten wij ook het effect van de behandeling met de AngII-receptor blokker, EM, 1 maand na geïnduceerde hypertensie in WT en AβPP/PS1 muizen. In dit hoofdstuk zijn wij nu op EM als bloeddrukverlagend middel gefocust. Dit omdat wij in het onderzoek beschreven in hoofdstuk 3 hebben gevonden dat EM het minst effectief is als bloeddrukverlager in AβPP/PS1 muizen. AβPP/PS1 muizen hadden ook in deze studie een hogere SBP dan WT muizen. Na één maand onbehandelde hypertensie herstelde de behandeling met EM de SBP in alle muizen. Daarbij was de functionele connectiviteit verlaagd in hypertensieve AβPP/PS1 en WT muizen. Verder lieten 12 maanden oude AβPP/PS1 muizen ook weer een verlaagde CBF zien. De hypertensieve AD muizen waren slechter in het ruimtelijke leren in de Morris water maze (MWM). Bij deze gedragstest moesten de muizen aan de hand van visuele orientatiepunten een verstopt platform vinden in het ondoorzichtige water. AngII-geïnduceerde hypertensie verergerde in AD muizen de AD-achtige symptomen de CBF, functionele connectiviteit, en cognitie, en ook in gezonde WT muizen leed deze vorm van hypertensie tot een verminderde connectiviteit.
Het was helaas niet mogelijk om aan te tonen dat door de AngII-geïnduceerde hypertensie de Aβ productie en neuroinflammatoire processen konden worden verbeterd. Onze bevindingen suggereren een verband tussen hoge bloeddruk op middelbare leeftijd, verminderde cerebrale hemodynamiek en connectiviteit bij dit muismodel voor AD. Daarbij verbeterde de behandeling met EM de CBF en ook de cerebrale connectiviteit.
Onderzoek bevestigt dat levensstijl, zoals o.a. voeding, een belangrijke rol speelt bij het ontstaan en de progressie van AD. Het specifiek samengestelde dieet, Fortasyn, heeft een positief effect op de cognitieve gebreken in een AD muismodel. In de studie uit hoofdstuk 5 analyseerden we zoekstrategieën en ruimtelijk geheugen en inzicht van de 11 maanden oude AβPP/PS1 muizen in de MWM. Verder bestudeerden wij ook wat de invloed is van 9 maanden lang toedienen van het Fortasyn dieet versus een controle dieet op het ontwikkelen van zoekstrategieën in deze cognitieve gedragstest. Tijdens de vierde dag van de MWM, gebruikten AβPP/PS1 muizen op controle dieet vaker een willekeurige, niet-ruimtelijke zoekstrategie dan hun WT nestgenoten. AD muizen op Fortasyn dieet zwommen op een vaste afstand van de rand op zoek naar het platform, wat een compensatie strategie is. Dit gedrag toonden de AD muizen op het
Fortasyn dieet meer dan hun niet-transgene nestgenoten. Bij het verwijderen van het platform hadden de \( \text{A}\beta\text{PP}/\text{PS1} \) muizen geen duidelijke voorkeur voor het voormalige platform. Zowel transgene als niet-transgene muizen op het Fortasyn dieet zwommen eerder naar de voormalige positie van het platform dan hun nestgenoten op controle dieet. Samenvattend, het Fortasyn dieet verbeterde in \( \text{A}\beta\text{PP}/\text{PS1} \) muizen het zoekgedrag en -strategie en de effectiviteit om de verborgen platform in het MWM te vinden.

Zoals al in de inleiding benoemd, is apoE4 een belangrijke genetische (vasculaire) risicofactor voor AD. In de studie beschreven in hoofdstuk 6 veronderstellen wij dat het Fortasyn dieet ook in apoE4 muizen pathologische veranderingen in AD kan tegengaan, zoals o.a. cerebrovasculaire schade. Daarnaast evalueerden we het dieet effect op de CBF, functionele connectiviteit, grijze en witte stof integriteit en veranderingen en postsynaptische dichtheid in verouderde apoE4 muizen. 10-12 maanden oude apoE4 muizen toonden nog geen pathologische verschillen in vergelijking met WT muizen. Daarentegen, 16-18 maanden oude apoE4 muizen hadden verminderde CBF en toonden een verhoogd verlies van synapsen tegenover WT muizen. Met name het Fortasyn dieet was in staat de corticale CBF en het aantal synapsen te verhogen, en ook de witte stof integriteit en de functionele connectiviteit te verbeteren in zowel verouderde apoE4 en WT muizen. Met deze studie tonen wij aan dat beschermingsmechanismen op het gebied van vascularisatie en synaptogenese door het Fortasyn dieet in gang worden gezet en zelfs worden versterkt onafhankelijk van het apoE4 genotype. Met behulp van deze multidisciplinaire, translationele en geavanceerde MR neuroimaging aanpak beschreven in hoofdstuk 6, konden wij het positieve effect van een specifiek samengestelde dieet, zoals Fortasyn, in een muismodel van een vasculaire risicofactor, apoE4 bediscussiëren.

Occlusie van de middelste hersenslagader (MCAo) is een van de meest voorkomende oorzaken van ischemische stroke bij mensen. Cerebrale ischemie leidt tot hersenbeschadigingen bestaande uit een onomkeerbaar beschadigde kerngebied (core) en een ischemisch grensgebied (penumbra). De penumbra bestaat uit hersenweefsel, dat nog te herstellen is. In de studie beschreven in hoofdstuk 7 onderzochten wij met behulp van een muismodel met een tijdelijke (30 min) occlusie van de middelste cerebrale slagader (tMCAo) het effect van Fortasyn als therapeutische aanpak op de neuroinflammatie en de verlaagde structurele en functionele connectiviteit, CBF en motoriek na een experimenteel geïnduceerd herseninfarct. In mannelijke volwassen C57BL/6J muizen werd de rechter middelste cerebrale slagader tijdelijk voor 30 min afgebonden met behulp van het intraluminale filament. Direct na het experimenteel geïnduceerde herseninfarct werden de muizen onderverdeeld in twee diëet groepen. De ene helft van de muizen kreeg het specifiek samengestelde Fortasyn dieet en de andere
groep muizen kreeg het controle dieet. Op verschillende tijdstippen na de tMCAo werden verschillende gedragstesten, MRI en PET scans uitgevoerd om het effect van de specifiek samengestelde Fortasyn dieet op de neuroinflammatoire respons, verlies van de cerebrale connectiviteit en de hieraan gekoppelde verslechtering van de motorische functie te onderzoeken na een geïnduceerd herseninfarct. Muizen op het specifieke samengestelde Fortasyn dieet toonden een verminderde neuroinflammatie, verbeterde functionele en structurele connectiviteit, hadden een verhoogde CBF, en lieten ook een verbeterde motorische functie na een experimentele herseninfarct zien. Uit deze bevindingen blijkt, dat deze specifiek samengestelde Fortasyn dieet als therapeutisch aanpak gunstige effecten op het structurele en functionele herstel na een herseninfarct heeft.

**Slotopmerkingen**

Het eerste doel van dit proefschrift was het ontrafelen van de effecten van vasculaire risicofactoren, zoals hoge bloeddruk, apoE4 en herseninfract op de neurodegeneratieve processen in de vroege fase van AD met behulp van verschillende muismodellen. In AD muizen is hypertensie reeds op jonge leeftijd gekoppeld aan cerebrovasculaire stoornissen leidend tot problemen van structurele en functionele connectiviteit vergezeld van een verminderde cognitie. Met name oudere AD muizen ontwikkelden een verhoogde SBP gelijktijdig met een aantal pathologische AD symptomen, zoals verslechtering van de cerebrale hemodynamiek resulterend in een verminderde cognitie, structurele en functionele connectiviteit, verhoogde bewegingsactiviteit, en angst-gerelateerd gedrag zonder AngII-geïnduceerde hypertensie. Opmerkelijk was dat ook in oudere transgene apoE4 muizen een verminderde cerebrovasculaire circulatie, verlaagde corticale post-synaptische dichtheid, verstoord witte en grijze stof integriteit, en een verlaagde functionele connectiviteit gedetecteerd werd. Kortom, de betrokkenheid van (vasculaire) risicofactoren, die genetisch en/ of experimenteel geïnduceerd waren in onze muismodellen, in neurodegeneratieve processen in de vroege fase van AD is aangetoond. Alle in dit proefschrift gebruikte muismodellen zijn diermodellen voor de asymptomatische fase van AD, middels hun (vasculaire) risicofactoren die het verloop van neurodegeneratieve processen in AD kunnen ontwikkelen en versnellen. Een ander doel van dit proefschrift was de mogelijke capaciteit van antihypertensiva en specifieke multi-nutriënt diëten als preventieve aanpak of behandeling tegen AD-symptomen en vasculaire risicofactoren van AD te onderzoeken. Naast hun bloedverlagende effect, toonden we aan dat antihypertensiva zoals EM een gunstig effect kunnen hebben op pathologische processen van AD, zoals het tegengaan van de verminderde CBF, en de verminderde structurele en functionele connectiviteit. Daarom is het belangrijk om effectievere bloeddrukverlagende behandelingen voor AD
patiënten te ontwikkelen en het bewustzijn van hoge bloeddruk als risicofactor voor AD te vergroten. Verder hebben we aangetoond dat een multimodale aanpak, waaronder geavanceerde MRI technieken, geschikt is voor het detecteren van veranderingen in hersenstructuur en functie door een dieet. Fortasyn gaf positieve effecten als preventieve aanpak, maar ook als therapeutische aanpak in pathologische veranderingen voor (vasculaire) risicofactoren voor AD-muismodellen. Toekomstig onderzoek kan zich richten op de verbetering van de compositie van multinutriënten diëten, die onder andere voedingscomponenten van het mediterrane dieet bevatten, om de klinische uitkomst na een beroerte te verbeteren en daarmee de ontwikkeling van dementie na een beroerte kan tegen gaan. Alle experimenten werden uitgevoerd in relatief jonge dieren. Daarom kunnen toekomstige studies zich beter richten op het effect van vasculaire risicofactoren en diëten in oudere muizen (>12 maanden leeftijd). In overeenstemming hiermee, hebben Toth en zijn collega’s aangetoond dat hypertensie bij oudere muizen een verminderde cerebrovasculaire autoregulatie, cerebromicrovasculaire schade en neuroinflammatie [254] kan veroorzaken. Ook in onze studie met een occlusie van de MCAo beschreven in hoofdstuk 7 hebben wij gebruik gemaakt van relatief jonge dieren om onze specifiek samengestelde diëet te testen tegen pathologische veranderingen veroorzaakt door een geïnduceerd herseninfarct. Bovendien werden er seksverschillen voor apoE4 [511, 639], hypertensie [640], en ook voor herseninfarct [641] gevonden. Daarom is het van het grootste belang om seksverschillen met betrekking tot elk van deze risicofactoren van AD te onderzoeken door het bestuderen van oudere vrouwelijke muizen. Vasculaire risicofactoren voor AD hebben een aantal negatieve effecten op de zeer vroege ontwikkeling van neurodegeneratieve processen, die betrokken zijn in AD. Maar door de behandeling van deze risicofactoren kunnen we de incidentie van AD mogelijk verlagen. Onze experimentele resultaten en ook epidemiologische data hebben aangetoond dat behandeling met alleen bloeddrukverlagende middelen of zogenaamde “single-component” diëten niet toereikend zijn om volledig onderliggende processen van pathologische processen tijdens de vroege fase van dementie te neutraliseren. Daarom moet toekomstig onderzoek zich richten op preventieve/ therapeutische behandelingen met behulp van gepersonaliseerde multinutriënt diëten gericht op (neuro)pathologische processen zoals neuroinflammatie, verlaagde CBF, cognitieve stoornissen, en andere klinische symptomen van dementie. Toekomstig onderzoek zou moeten focussen op een preventieve aanpak bestaand uit een mix van cognitieve en fysieke inspanning [642, 643], gecombineerd met een gepersonaliseerde multinutriënt dieet tijdens de vroege fase van AD. Deze preventieve behandelingen zullen moeten worden afgestemd voor elke individuele dementie patiënt om zo een optimale persoonlijke preventieve therapie te ontwikkelen en op die manier elk individuele genetische make-up te dekken.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ApoE4</td>
<td>Apolipoprotein E ε4 allele</td>
</tr>
<tr>
<td>ARA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>ASL</td>
<td>Arterial spin labeling</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin II receptor type 1</td>
</tr>
<tr>
<td>AT2</td>
<td>Angiotensin II receptor type 2</td>
</tr>
<tr>
<td>AT-NPD1</td>
<td>Aspirin-triggered NPD1</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>ABPP</td>
<td>Amyloid-β precursor protein</td>
</tr>
<tr>
<td>ABPP/PS1</td>
<td>$\text{ABPP}<em>{\text{swc}}/\text{PS1}</em>{dE9}$</td>
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<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>C21</td>
<td>Compound 21</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu ammonis 1</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu ammonis 2</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu ammonis 3</td>
</tr>
<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CMI</td>
<td>Cortical microinfarct</td>
</tr>
<tr>
<td>Cpu</td>
<td>Caudate putamen</td>
</tr>
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<td>CVD</td>
<td>Cerebrovascular disease</td>
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<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
</tr>
<tr>
<td>EIMI</td>
<td>European Institute for Molecular Imaging</td>
</tr>
<tr>
<td>EM</td>
<td>Eprosartan mesylate</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Echo planar imaging</td>
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<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FA</td>
<td>Fractional anisotropy</td>
</tr>
<tr>
<td>FAIR</td>
<td>Flow-sensitive alternating inversion recovery</td>
</tr>
<tr>
<td>FC</td>
<td>Functional connectivity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GC-MS-SIM</td>
<td>Gas-chromatography-mass-spectrometry-selected-ion-monitoring</td>
</tr>
<tr>
<td>GE</td>
<td>Gradient echo</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose transporter type 1</td>
</tr>
<tr>
<td>GM</td>
<td>Grey matter</td>
</tr>
<tr>
<td>GP</td>
<td>Globus Pallidus</td>
</tr>
<tr>
<td>HCT</td>
<td>Hydrochlorothiazide</td>
</tr>
<tr>
<td>IBA-1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>IML</td>
<td>Inner molecular layer</td>
</tr>
<tr>
<td>LC-n3-FA</td>
<td>Long-chain omega-3 polyunsaturated fatty acids</td>
</tr>
<tr>
<td>MANOVA</td>
<td>Multivariate ANOVA</td>
</tr>
<tr>
<td>MCAo</td>
<td>Occlusion of the middle cerebral artery</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MD</td>
<td>Mean diffusivity</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini-Mental State Examination</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NE</td>
<td>North-East</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NPD1</td>
<td>Neuroprotectin 1</td>
</tr>
<tr>
<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>OML</td>
<td>Outer molecular layer</td>
</tr>
<tr>
<td>ORT</td>
<td>Object recognition test</td>
</tr>
<tr>
<td>OSCAR</td>
<td>Observational study on cognitive function and systolic blood pressure reduction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Ppi</td>
<td>Prepulse inhibition</td>
</tr>
<tr>
<td>PRIME</td>
<td>Preclinical Imaging Centre</td>
</tr>
<tr>
<td>PROGRESS</td>
<td>Perindopril Protection Against Recurrent Stroke Study</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>postsynaptic density-95</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>ROI</td>
<td>Regions of interest</td>
</tr>
<tr>
<td>rsfMRI</td>
<td>Resting state fMRI</td>
</tr>
<tr>
<td>Sal</td>
<td>Saline</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SHEP</td>
<td>Systolic Hypertension in the Elderly Program</td>
</tr>
<tr>
<td>SHSRP</td>
<td>Stroke-prone spontaneously hypertensive rats</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SLFF</td>
<td>Spontaneous low-frequency fluctuations</td>
</tr>
<tr>
<td>SL</td>
<td>Stratum lucidum</td>
</tr>
<tr>
<td>SPM</td>
<td>Statistical Parametric Mapping</td>
</tr>
<tr>
<td>SR</td>
<td>Stratum radiatum</td>
</tr>
<tr>
<td>SYN</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>Syst-Eur</td>
<td>Systolic Hypertension in Europe</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TI</td>
<td>Inversion time</td>
</tr>
<tr>
<td>tMCAo</td>
<td>Transient occlusion of the middle cerebral artery</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>TSPO</td>
<td>Translocator protein</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine monophosphate</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular dementia</td>
</tr>
<tr>
<td>VCI</td>
<td>Vascular cognitive impairment</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of interest</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
<tr>
<td>WMH</td>
<td>White matter hyperintensities</td>
</tr>
<tr>
<td>WML</td>
<td>White matter lesions</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
References

Appendices


Appendices


Appendices


223


Appendices


Appendices


Appendices


Appendices


Appendices
Appendices


236


Appendices


Acknowledgments

Nu eindelijk is na het afronden van alle experimenten, analyses en submitten van artikelen mijn proefschrift klaar. Maar dit had ik niet zonder hulp kunnen doen en daarom wil ik iedereen bedanken, die mij heeft geholpen deze mijlpaal te bereiken.

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habt mir auch immer gut zugesprochen. Ihr seid in vielen Aspekten meine größten Vorbilder!
Curriculum vitae

Maximilian Wiesmann was born on May 29th in 1988 in Bocholt, Germany. After receiving his high school diploma (Abitur) in 2007, in first instance he was interested in studying veterinary medicine. But his fascination in biology helped him to choose for a Bachelor of Science (B.Sc.) in (medical) biology at the Radboud university Nijmegen. During his bachelor internship under the supervision of Professor Tamás Kozicz (Department of cellular animal physiology, Radboud university Nijmegen) he investigated the impact of chronic stress on the activity of urocortin 1 (Ucn1) neurons in the Edinger-Westphal-nucleus, which is involved in chronic stress and depression. His interest in translational research and the brain stimulated him to start a research Master of Science (M.Sc.) in medical biology at the Radboud university Nijmegen. During his first master internship (department of Anatomy, Radboud university medical center) he investigated the effects of docosahexaenoic acid (DHA) combination diets compared to control diet on spatial learning and memory, and cerebral hemodynamics in 10-12 month old male Alzheimer’s disease (AD) model mice versus their wild-type (WT) littermates. Combining the topics of his Bachelor and Master internship he addressed the question whether the mRNA levels of Ucn1 are decreased in the Edinger-Westphal-nucleus of AD patients due to the neuronal cell loss leading to altered mood and cognitive problems in AD patients. Finally, he accomplished his master degree in medical biology in 2007 with cum laude. Directly after his M.Sc. he applied for a PhD position at the Radboud university medical center under the supervision of Dr. Amanda Kiliaan, Dr. Jurgen Claassen, and Professor Arend Heerschap. Being a cooperation between the three departments Anatomy, Geriatric Medicine, and Radiology and Nuclear Medicine, the objective of his PhD project, which is presented in this thesis, was to investigate the underlying pathological processes of major vascular risk factors and their accelerating effect during the very early development of neurodegenerative processes in AD using several mice models. Another aim of this PhD-project was to study the possible capacity of antihypertensives and specific multi-nutrient diets to serve as preventive or treatment against AD-like symptoms and vascular risk factors for AD. During this PhD-project he supervised several excellent bachelor and master students with their internship projects and theses. Additionally, he presented his research at multiple international conferences in oral/ poster sessions. As of June 2016, Maximilian works in the research group of Dr. Amanda Kiliaan as a postdoctoral research. Here, he elucidates the impact of stroke on the pathology of AD in male and female mice via a longitudinal approach. Furthermore, in collaboration with the departments Neurology (Professor Frank-Erik de Leeuw) and Anatomy (Dr. Amanda Kiliaan) he analyzes the role of small vessel disease in the development of white matter lesions in human post mortem brains using both high-field (7T) and ultrahigh-field MR systems (11.7T).
Appendices

List of publications


10. **Wiesmann M**, Timmer NM, Zinnhardt B, Eligehausen S, Reinhardt D, Jacobs AH, Kiliaan AJ. Does a multi-nutrient diet have similar beneficial effects on stroke in female mice as it has in male mice? In preparation.


Radboud Alzheimer Centrum Series


10. Timmer, N.M. (2011). The interaction of heparan sulfate proteoglycans with the amyloid b protein


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