Summary and discussion

Main findings

The general objective of the work described in this thesis was to develop methods for quantitative *in vivo* assessment of brain inflammation in Alzheimer's disease (AD). This is important as it may provide further insight in the role of inflammation in the pathophysiology underlying AD. In addition, it may serve as a (surrogate) marker for disease modifying agents that target brain inflammation. Two approaches were used: studies in blood and cerebrospinal fluid (CSF), and imaging studies using positron emission tomography (PET). In blood and CSF, the inflammatory markers C-reactive protein (CRP), interleukin-6 (IL-6), α1-antichymotrypsin (ACT) and serum amyloid P (SAP) were related to clinical stages of AD. Using PET, the focus was on regional *in vivo* assessment of activated microglia in cerebral tissue of patients with prodromal AD (i.e. mild cognitive impairment (MCI)) and AD dementia using *(R)*-[^{11}C]PK11195, a highly selective ligand for the peripheral type benzodiazepine receptor (PBR), recently renamed as translocator protein 18 kDa (TSPO), which, in the brain, is upregulated in activated microglia cells.[1]

In the first part of this thesis the presence of inflammatory markers in serum and CSF of MCI and AD patients was examined. In the study described in **chapter 2** higher CRP levels were found in both CSF and blood of MCI cases compared to AD patients even after adjustment for age, ApoE ε4 genotype and cardiovascular diseases. This difference remained present in MCI patients with a low-risk Aβ42/tau biomarker profile for AD after adjustment for ApoE ε4, cardiovascular diseases, white matter lesions and brain infarcts. CSF IL-6 levels were also significantly higher in MCI patients with a low-risk CSF profile. No differences were found for ACT between MCI and AD patients. Next, in **chapter 3**, it was investigated whether serum amyloid P (SAP) levels in CSF or serum reflect involvement of SAP at various stages of AD. Results showed that SAP levels in CSF seem to mirror accumulation of SAP in amyloid deposits in different stages of AD. Lower levels of SAP in CSF were associated with a twofold higher risk of progression to dementia, predominantly

AD. Although a strong correlation between serum and CSF concentrations of SAP was found, serum levels of SAP did not discriminate between groups.

The second part of this thesis is dedicated to *in vivo* assessment of activated microglia, which are known to be associated with inflammation of the brain. Building on previous studies on the development of quantitative methods for measuring activated microglia using (*R*)-[^{11}C]PK11195 and PET, first several methodological aspects of assessing (*R*)-[^{11}C]PK11195 binding at the voxel level were evaluated. In **chapter 4**, the performance of various parametric methods was compared with the results of full compartmental analysis, which is considered to be the gold standard. Logan graphical analysis[2] was found to be a robust and fast method for obtaining parametric volume of distribution (V_d , currently also abbreviated as V_T) images of (*R*)-[^{11}C]PK11195. In addition, parametric reference tissue methods for measuring binding potential (BP_{ND}) were evaluated using cerebellum as reference region. In the end, one of the Ichise linearizations (ICH1),[3] one of the basis function methods (RPM1)[4], and Logan graphical analysis with reference tissue input (Reference Logan)[5] provided reasonably accurate and precise estimates of BP_{ND} at normal flow rates, as compared with results of the simplified reference tissue model (SRTM).[6] In addition, effects of blood volume and blood flow on the accuracy of reference tissue parametric methods were evaluated. Simulations suggested that RPM1 is the best method in case of reduced flow rates, as Reference Logan and ICH1 showed more bias than RPM1 under these conditions. In **chapter 5** performance of these plasma input (Logan)[2] and reference tissue parametric (RPM)[4] methods on the outcome of statistical parametric mapping (SPM) analyses of (*R*)-[^{11}C]PK11195 binding in young and elderly subjects was evaluated. Results showed that parametric BP images without proportional scaling provided the most sensitive framework for determining differences in (*R*)-[^{11}C]PK11195 binding between younger and elderly subjects. V_d images could only demonstrate differences in (*R*)-[^{11}C]PK11195 binding when analysed with proportional scaling due to intersubject variation in K_1/k_2 (rate constants of forward and reverse transport across the blood-brain barrier) and/or global (whole brain) inter-subject differences in (*R*)-[^{11}C]PK11195 binding.

Finally, (*R*)-[^{11}C]PK11195 BP_{ND} was evaluated in healthy aging as well as in prodromal AD and AD dementia patients. In healthy control subjects and AD patients definition of a reference region can be difficult, as it requires delineation of an unknown region without microglial activation. Therefore, RPM1 in combination with supervised cluster analysis was applied to extract the reference tissue input function.[7,8] In **chapter 6** increased BP_{ND} of (*R*)-[^{11}C]PK11195 was found in healthy aging in frontal lobe, anterior and posterior cingulate cortex, medial inferior temporal lobe, insula, hippocampus, entorhinal cortex, thalamus, parietal and occipital lobes, and cerebellum. **Chapter 7** provides BP_{ND} of *(R)*-[^{11}C]PK11195 in prodromal AD and AD dementia patients. Small clusters of increased *(R)*-[^{11}C]PK11195 binding were found in occipital lobe in AD dementia patients using a voxel based analysis. Region of interest analysis, however, showed no differences between diagnostic groups, with large overlap between subject groups. No correlations were found between cognitive tests at baseline and *(R)*-[^{11}C]PK11195 BP_{ND} in any of the brain regions and there were no differences in BP_{ND} between prodromal AD patients who remained clinically stable and those who progressed clinically to dementia.

General Discussion

Inflammatory proteins in Alzheimer's disease

Epidemiological, pathological and animal studies support the hypothesis that inflammatory mechanisms are involved in the pathogenesis of AD. Indeed, activated microglia cells and inflammatory cytokines, such as IL-6, are elevated in AD.[9] Immunopathological studies have shown that amyloid plaques are co-localized with a variety of inflammation related proteins, and clusters of activated microglia and reactive astrocytes.[10-12] Interestingly, the presence of microglial activation in AD patients with low Braak scores suggests that inflammation is an early event in AD pathophysiology.[13,14] The question remains, however, whether this inflammatory response is beneficial or detrimental.

Population studies suggest that higher serum levels of inflammatory markers, such as CRP, IL-6 and ACT can predict cognitive decline or dementia.[15-18] In chapter 2.1, significantly higher CRP levels in both CSF and serum, and higher levels of IL-6 in CSF of MCI were found compared to AD patients. This supports the hypothesis that inflammation occurs early in the pathological cascade of AD. However, levels of CSF proteins should be interpreted cautiously, as levels of inflammatory proteins measured in CSF can be the result of local production, but also of diffusion across the blood-CSF barrier. Diffusion of proteins from blood into CSF is a non-linear process, strongly dependent on molecular size. No differences in albumin quotient (i.e. CSF/serum concentrations of albumin) between MCI and AD patients were found, suggesting that blood-CSF barrier permeability is not different between the patient groups studied (chapter 2.1).[19] A correction for interindividual variation in blood-CSF barrier integrity was performed using an index of inflammatory markers[20] and albumin. No differences in inflammatory indexes between AD and MCI patients, corrected for albumin, were found, suggesting that blood-CSF barrier permeability is not different between the two patient groups. For CRP a relatively low index was found suggesting that most CRP in CSF originated from diffusion across the blood-CSF barrier, although some contribution by local production cannot be excluded. The (relatively) high ACT and IL-6 indexes suggest that these factors are (at least in part) produced locally, rather than being transported across the blood-CSF barrier. Particularly IL-6 levels, which were slightly higher in CSF than in plasma, are indicative for local intracerebral production.

The amyloid associated protein SAP is normally produced at low levels in the brain. SAP synthesis, however, is upregulated in AD brain.[21] In chapter 2.2 lower SAP levels were found in CSF of MCI patients who converted to AD. Hypothetically, these lower levels of SAP might be a consequence of Aβ-SAP binding in the brain, as it has been shown that SAP accumulates in Aβ plaques and binds to newly formed amyloid fibrils.[22-24] These low levels of SAP in MCI further support the hypothesis that neuropathological changes occur long before patients fulfil clinical diagnostic criteria for AD.

In vivo imaging of microglial activation

At present, the isoquinoline (*R*)-[^{11}C]PK11195 is the best characterized and most widely used radioligand for *in vivo* quantification of activated microglia. It binds to the peripheral benzodiazepine receptor binding site, which was recently renamed to translocator protein 18 kDa (TSPO).[25] TSPO is a hydrophobic protein, primary located in the outer mitochondrial membrane. Usually, low levels of TSPO are present in the brain and they are limited to glial cells (astrocytes and microglia).[26] However, in response to neuronal damage a dramatic increase in TSPO levels can be found.[27] Quantification of TSPO as a measure of microglial activation has been performed in several neurodegenerative diseases, such as AD, Parkinson's disease and multiple sclerosis, but also in traumatic brain injury.[28-33] Although astrocytes also show high levels of binding of PK11195 *in vitro*, focally increased PK11195 binding primarily appears to be related to locally activated microglia. This is supported by *in vivo* studies using both microglial and astrocytic markers, which have demonstrated that the distribution pattern of increased PK11195 binding matches that of activated microglia rather than that of reactive astrocytes.[34,35]

Microglial cells are related to cells of the mononuclear lineage and are activated at a very early stage in response to brain injury, in which a rapid transformation of microglia from a resting to an activated state takes place.[36,37] They proliferate and migrate to the sites of brain injury[38] and release a variety of growth factors and inflammatory cytokines.[9] They also upregulate different surface receptors, which are thought to regulate microglial activation. Normally, microglial activation subsides and cells reset to their resting state. However, the changes over time in AD are not clear, and further longitudinal studies are needed to elucidate the time course of activation.

Microglial activation in aging

Despite several studies on microglial activation in neurodegenerative diseases, relatively little is known about microglial activation in normal aging. In normal brain there are generally low levels of TSPO expression. In chapter 4.1, increased *(R)*-[^{11}C]PK11195 BP_{ND} was found

throughout several cortical and subcortical brain regions, namely frontal cortex, anterior and posterior cingulate cortex, parietal and occipital cortex, thalamus and cerebellum. Age related increases were also found in various temporal regions: medial inferior temporal lobe, insula, hippocampus and entorhinal cortex. These results indicate that activated microglia appear in several temporal lobe structures during healthy aging, which is in agreement with post mortem studies.[39,40] Interestingly, regional cerebral atrophy with aging has been described previously and includes prominent grey matter loss in frontal areas, but also in anterior cingulate cortex, temporal, parietal and subcortical areas, as well as in insula, which is consistent with increased binding of *(R)*-[^{11}C]PK11195 in these areas.[41,42] The clinical relevance of these findings, however, is not clear, Recently, it was hypothesized that microglia are subject to cell senescence (dysfunction), which implies that progressive microglia activation precedes neurodegeneration. In that case, neurodegenerative changes in AD may be the result of age related diminution of neuroprotective functions of microglia. In other words, when the supporting function of microglia cells falls away, neurons are more prone to neurodegenerative changes.[43]

Microglial activation in AD

In chapter 4.2, small clusters of increased *(R)*-[^{11}C]PK11195 binding were found in occcipital lobe in AD dementia. Cagnin et al. were the first to report increased *(R)*-[^{11}C]PK11195 binding in AD.[29] They also reported that increased *(R)*-[^{11}C]PK11195 binding precedes grey matter loss as seen on MRI, indicating that microglial activation is an early phenomenon in the course of AD. Recently, Okello et al. demonstrated increased *(R)*-[^{11}C]PK11195 binding in the frontal cortex in [^{11}C]PIB positive MCI patients, suggesting that microglial activation is mainly associated with amyloid plaque deposition.[44] This has also been demonstrated with immunohistochemical studies.[45] In addition, it has been shown that microglia are attracted to the site of amyloid plaque formation.[46] Despite increased *(R)*-[^{11}C]PK11195 binding in frontal cortex mentioned above, no correlation between amyloid depositions, as measured with [^{11}C]PIB, and *(R)*-[^{11}C]PK11195 binding, could be demonstrated in MCI.[44]

This thesis (chapter 4.2) shows subtle increased *(R)*-[^{11}C]PK11195 binding in AD dementia patients, indicating that microglial activation is present in AD. Based on post mortem studies demonstrating that activation of microglia precedes neurodegenerative changes in the neocortex[10,47] one might expect increased *(R)*-[^{11}C]PK11195 BP_{ND} in prodromal AD, occurring before structural changes can be seen. However, no increased *(R)*-[^{11}C]PK11195 binding was found in prodromal AD patients.

Likewise, differences in BP_{ND} between prodromal AD patients who progress clinically to dementia and those who remain clinically stable might be expected as well. However, *(R)*-[^{11}C]PK11195 BP_{ND} was not associated with clinical progression and there were no correlations between cognitive function at baseline and *(R)*-[^{11}C]PK11195 BP_{ND} in any of the brain regions (chapter 4.2), which is consistent with previous findings.[44]

The function of microglia cells in AD remains a matter of debate. Whether activation states are beneficial or harmful is still not known. *In vitro* data demonstrate that activated microglia can cause neuronal damage and therefore may play a role in disease progression.[48] In contrast, there is also increasing evidence that presence of microglia might be protective by mediating clearance of Aβ.[49] It has been hypothesized that microglia cells function differently at different time points in the progression of AD. In early stages of AD they clear Aβ depositions, whereas in advanced stages they become dysfunctional and unable to clear all of the Aβ.[49]

The notion that activated microglia and other inflammatory mediators may contribute to the pathogenesis of AD resulted in treatment with anti-inflammatory drugs in order to reduce inflammatory reactions. Several epidemiological studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) may exert neuroprotective effects in AD.[50-54,54-56] This has also been suggested by several retrospective case-control studies reporting a protective effect of NSAIDs in AD.[57,58] Unfortunately, randomized clinical trials studying beneficial effects of NSAIDs in AD have been disappointing.[59-61] A possible explanation might be the long term use of NSAIDs in epidemiologic studies, which can not be achieved in randomized clinical trials. Other important factors might be the timing of anti-inflammatory

treatment or their mode of action (classical NSAIDs versus newer agents with a more selective action against COX-2). Interestingly, there is preliminary evidence that celecoxib, a COX-2 inhibitor, has *in vivo* anti-inflammatory properties, that could be detected using (*R*)-[^{11}C]PK11195 and PET.[62] At present, no clinical studies have been performed to investigate the effects of COX-2 inhibitors in AD patients.

Methodological considerations

The human *(R)*-[^{11}C]PK11195 PET studies, described in this thesis, were all analysed using validated tracer kinetic models with both plasma and reference tissue input functions. In chapter 3, results of various parametric methods were compared with those of full compartmental analysis, which is considered to be the gold standard. For all parametric reference tissue methods cerebellum was used as reference region and RPM1[4] was shown to be the most accurate parametric method for analysing *(R)*-[^{11}C]PK11195 data. The choice of a reference region can, however, be difficult in healthy controls and AD patients, as it requires delineation of a region devoid of microglial activation. To circumvent this problem, a cluster analysis technique can be employed to extract a reference tissue input function. Recently, Turkheimer et al. demonstrated that this supervised cluster analysis method provides a robust and reproducible quantitative assessment of *(R)*-[^{11}C]PK11195 binding in the human brain.[7] This method may provide some practical advantages over the use of a plasma input curve as no arterial sampling is required, making studies less invasive and laborious. Therefore, more recently, this new supervised cluster analysis method was further optimised. [63] Kinetic analyses were performed using both simplified reference tissue method (SRTM) with non-linear regression and RPM1. RPM1, with and without blood volume correction, showed similar results compared to SRTM.[63] Therefore, in chapter 4, this new supervised cluster analysis method was used for extracting the reference tissue input function followed with RPM1 analysis to obtain quantitative parametric images of binding.

Another important methodological issue in PET imaging is the effect of subject motion. Patient motion is a limiting factor, which may result in under- or overestimation of

measured tracer binding. Although in the present studies data were analysed without applying motion corrections, the effects on the final results will be small, as data from patients who moved substantially were not included in the final analysis.

Furthermore, quantification of PET studies may be affected by partial volume effects, resulting from the limited spatial resolution. To limit these partial volume effects, a PVC-OSEM reconstruction algorithm was used in the clinical studies described in chapter 4. This algorithm uses the scanner's point spread function to significantly enhance the spatial resolution of reconstructed scans. This method was recently validated by Mourik et al. for the HR+ scanner,[64] the scanner used in the present studies, resulting in an improvement in spatial resolution from approximately 4.1 – 7.8 mm FWHM to 2.3 – 3.4 mm FWHM.[64,65] Nevertheless, some partial volume effects will remain. Consequently, it is likely that observed BP_{ND} values still underestimate both extent and magnitude of microglial activation and it might be hypothesized that in relatively small regions surrounded by cerebrospinal fluid a disproportionate underestimation of the true signal occurs.

New microglia ligands

Although *in vivo* imaging of microglial activation using (*R*)-[^{11}C]PK11195 has provided important information in a variety of neurological disorders, the relatively high level of non-specific binding and associated low signal-to-noise ratio makes quantification of the signal difficult. Therefore, the development of new tracers with more favourable binding profiles is being pursued by many radiopharmaceutical research groups worldwide. Recently, a number of novel TSPO receptor ligands have been synthesized. [^{11}C]DAA1106 (N-(2,5-dimethoxybenzyl)-N-(5-fluoro-2-phenoxyphenyl)-acetamide) is a ligand that binds selectively and with higher affinity to the TSPO receptor than (*R*)-[^{11}C]PK11195.[66] In a recent study, Yasuno et al. demonstrated increased [^{11}C]DAA1106 binding in various regions in a relative small number of subjects with AD.[67] Data were thoroughly analysed using a metabolite corrected plasma input function, although no partial volume correction was applied. These first results in humans are encouraging, but further development and validation are highly

needed. $[^{11}C]$CLINME (2-[6-chloro-2-(4-iodophenyl)-imidazo[1,2-*α]*pyridine-3-yl]-N-ethyl-N-methyl-acetamide) is another promising radioligand. In a rat model of local acute neuroinflammation it was demonstrated *in vivo* that $[^{11}C]$CLINME has a good specific to non-specific binding ratio.[68] The pyrazolopyrimidine, N,N-diethyl-2-[2-(4-methoxyphenyl)-5,7-dimethyl-pyrazolo[1,5-*α*]pyrimidin-3-yl]-acetamide ($[^{11}C]$DPA-713), with a lower lipophilicity and higher selectivity, also has potential as a potent ligand for microglial activation studies, showing higher affinity and better signal-to-noise ratio than (*R*)-$[^{11}C]$PK11195.[69] Labelling with $[^{18}F]$ would make it an attractive ligand for widespread distribution and more options for clinical use. Therefore, an ^{18}F-labeled derivative of DPA-713, $[^{18}F]$DPA-714, has been synthesized and evaluated in rodents and normal male baboon.[70-72] It was shown to have suitable *in vivo* properties with rapid brain uptake and high specificity for the TSPO receptor,[70] although specific binding was much lower than that of $[^{11}C]$DPA-713.[71] $[^{11}C]$PBR28 (N-(2-methoxybenzyl)-N-(4-phenoxypyridin3-yl)acetamide is another ligand with lower liphophilicity than (*R*)-$[^{11}C]$PK11195 and $[^{11}C]$DAA1106, which has also favourable characteristics. First results were promising as high specific binding in monkey brain was found.[73] However, it appeared that 10-14 percent of human subjects show no binding to $[^{11}C]$-PBR28 due to a 10-fold lower affinity for PBR28 than in so-called binders.[74,75] Subsequent quantitative analyses revealed three binding affinity patterns in human subjects, i.e. high-, median- and low-affinity binding patterns. Substantial differences were found between high-affinity, medium-affinity and low-affinity binders for the TSPO ligands PBR28, PBR06, DAA1106, DPA713 and PBR111.[76] For (*R*)-$[^{11}C]$PK11195 no differences in selectivity were found, indicating that the expected BP_{ND} will be the same for the three groups. This makes (*R*)-$[^{11}C]$PK11195 still the tracer of choice for *in-vivo* assessment of activated microglia. Current research focuses on the development of a novel series of N1-methyl-(2-phenylindol-3-yl)glyoxylamides, amongst others.[77] First results are promising, showing high affinity for all types of binders. Clearly, further studies are needed to establish whether these ligands are suitable for imaging of microglia activation *in vivo* in human subjects.

Recommendations for future research

This thesis shows that inflammatory markers in CSF as well as *in vivo* quantification of (*R*)-[^{11}C]PK11195 as a measure of microglial activation reflect the involvement of inflammation in AD. The combination of CSF markers and *in vivo* imaging of (*R*)-[^{11}C]PK11195 binding was studied, aiming to better understand the neurobiological basis of the disease. At present, there is no ideal PET ligand for *in vivo* quantification of microglial activation. Despite its limitations, (*R*)-[^{11}C]PK11195 still provides a useful tool to study neuroinflammation *in vivo*. Ultimately, the development of a TSPO ligand with a high specific signal could facilitate further exploration of the mechanisms involved in the pathogenesis of AD and may allow selection of patients eligible for treatment directed against inflammation, even at the stage of the earliest clinical manifestations. Furthermore, it might facilitate monitoring disease progression and evaluate individualised therapy with an anti-inflammatory agent.

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