Varrone A, Oikonen V, Forsberg A, Joutsa J, Takano A, Solin O, Haaparanta-Solin M, Nag S, Nakao R, Al-Tawil N, Wells LA, Rabiner EA, Valencia R, Schultze-Mosgau M, Thiele A, Vollmer S, Dyrks T, Lehmann L, Heinrich T, Hoffmann A, Nordberg A, Halldin C, Rinne JO. Positron emission tomography imaging of the 18-kDa translocator protein (TSPO) with [^{18}F]FEMPA in Alzheimer's disease patients and control subjects.
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Positron emission tomography imaging of the 18-kDa Translocator Protein (TSPO) with [^{18}F]FEMPA in Alzheimer´s disease patients and control subjects

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Abstract:	Imaging of the 18-kDa translocator protein (TSPO) is a potential tool for examining microglia activation and neuroinflammation in early Alzheimer´s disease (AD). [^{18}F]FEMPA is a novel high-affinity, second-generation, TSPO radioligand displaying suitable pharmacokinetic properties in pre-clinical studies. The aims of this study were to assess the quantification of the binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether higher [^{18}F]FEMPA binding in AD vs. controls could be detected in vivo. Methods. Ten AD patients (5M/5F, age 66.9±7.3 y, MMSE 25.5±2.5) and seven controls (3M/4F, age 63.7±7.2 y, MMSE 29.3±1.0) were studied using [^{18}F]FEMPA at Turku (n=13) and at Karolinska Institutet (n=4). The in vitro binding affinity for TSPO was assessed using PBR28 in a competition assay with [3H]PK11195 in 7 controls and 8 AD. Cortical and subcortical regions-of-interest were examined. Quantification was performed using two-tissue compartment model (2TCM) and Logan graphical analysis (GA). The outcome measure was the total distribution volume (VT). Repeated-measure analysis of variance was used to assess the effect of group or TSPO binding status on VT. Results. Five AD and 4 controls were high-affinity binders (HABs). Three AD and 3 controls were mixed-affinity binders. VT estimated with Logan GA correlated significantly with VT estimated with 2TCM in both controls (r=0.97) and AD patients (r=0.98) and was selected for the final analysis. In the medial temporal cortex, statistically significant higher VT (p=0.044) in AD vs. controls was found if the TSPO binding status was entered as covariate. If only HABs were included, statistically significant higher VT in AD patients vs. control subjects ($p<0.05$) was found in the medial and lateral temporal cortex, posterior cingulate, caudate, putamen, thalamus, and cerebellum. Conclusions. [^{18}F]FEMPA seems to be a suitable radioligand to detect increased TSPO binding in AD if the binding status is taken into account.
Response to Reviewers:	See Attachment for Reply to Reviewers.

We thank the Reviewers for the constructive and fruitful comments that helped improving the quality of the manuscript. Below is a point-by-point reply to the Reviewers´comments.

Reviewers' comments:

Reviewer #1: This is a well-designed and executed small trial of the TSPO PET tracer 18F-FEMPA in Alzheimer's disease subjects and controls. The trial was conducted at two PET centers examining 10 AD subjects and 7 controls characterized with regard to rs6971 polymorphism. A volume of interest analysis was utilized for assessing cortical and subcortical regions and data were modeled using a two tissue compartment model and a Logan graphical analysis with the primary outcome measure being total distribution volume (VT). A number of comments and questions for the authors follow:

1. The majority of the images were scanned at Turku on an ECAT EXACT HR+, while the remainder were imaged on the lower resolution ECAT EXACT HR PET tomograph. What efforts were made to standardize the reconstruction and post hoc processing of the PET data across the two sites? Were there phantoms or other analyses done to ensure the poolability of the quantitative data?

The following sentence has been included in the text to address this comment "A NEMA Jacszack phantom with spheres of different diameter and uniform background filled with 18F-radioactive solution at a ratio of ~4:1 was acquired at both centers under similar experimental conditions and using the standard reconstruction method at each centre. The difference of the recovery coefficient between the two PET systems was 9.4% for the spheres and 4.2 for the background, suggesting the possibility to pool the data from the two PET systems."
The table with the data is presented below for reviewer´s perusal. We added the above sentence in the manuscript to clarify the issue.

NEMA Jacszack phantom

Diameter (mm)	**9**	**12**	**15**	**17**	**25**	**32**	
Karolinska							
RCHot	45	48.4	53.4	61.3	64.2	63.3	
RCBkg	85.2	86.7	85.9	86.8	86.2	86.3	
Turku							
RCHot	40.6	48.1	57.8	64.1	74.5	76.9	
RCBkg	93.1	87.7	83.9	80.5	89.8	88.6	
% difference (Turku-KI)/Turku							Mean % difference
RCHot	10.8	0.6	7.7	4.4	13.8	17.7	9.2
RCBkg	8.5	1.1	2.3	7.8	4.0	2.6	4.4

RC = recovery coefficient

2. The distribution of focally increased radiotracer uptake occurring with neuroinflammation may not necessarily follow boundaries of typical VOIs derived from standardized templates. Was this the case in the present investigation? If so, did the authors consider a voxel-wise analysis as an alternative to volume of interest sampling? If not, a comment to that effect in the manuscript would be useful.

This is a good point of the reviewer. However, due to the small sample size and to prevent possible type I or II errors we decided not to perform voxel-based analysis.

3. Was there correlation between regional VT and any clinical measures?

In the text we included a new section "Additional considerations" that address this and other comments from both reviewers:

Additional considerations

The binding of [^{11}C]PBR28 to the TSPO has been shown to negatively correlate with the MMSE [19]. We examined the correlation of [^{18}F]FEMPA mean cortical (frontal, temporal, parietal, and occipital), limbic (medial temporal cortex and posterior cingulate) and sub-cortical (caudate, putamen and thalamus) V_T with MMSE and found a weak, non significant negative correlation (r between -0.37 and -0.41, *p*-value between 0.12 and 0.17) when combining data from controls and AD patients (data not shown), The lack of statistically significant correlation might be related to the limited sample size and further studies are needed to specifically examine the relationship between TSPO binding of [^{18}F]FEMPA and cognitive function in AD.

The analysis of the PET data was conducted using only conventional ROI-based approach. Voxel-based analysis could be useful to identify differences in small areas that can be underestimated by the use of large ROIs. In this study we did not apply voxel-based analysis because of the limited sample size of both groups and to avoid possible false-positive and negative results that can be associated with small samples.

The potential application of an ^{18}F-labelled tracer in the clinical setting could be aided by the use of a simplified acquisition protocol. However, in the case of [^{18}F]FEMPA, because of the lack of a reference region in the brain the arterial input function data is needed to estimate V_T. We did not observe differences in the parent fraction between AD patients and controls, suggesting that the observed differences in V_T are indeed reflecting differences in the brain distribution of the tracer. Such differences could be detected only by measuring the brain uptake as SUV. We did observe differences in SUV between the two groups, similar to differences in V_T (data not shown), which might suggest that SUV could be used as surrogate outcome measure. However, to validate SUV as potential outcome measure in the clinical setting, additional studies with [^{18}F]FEMPA in a larger group of AD patients and controls are needed.

4. What is the reason for the relatively poor discrimination between AD and control subjects who were mixed affinity binders, is this simply a function of signal to noise? Were the regions selected in figure 5 the "best" regions with regard to discrimination between the cohorts?

The worse discrimination between AD and control subjects in MABs can be due to the noise of the data as the reviewer noted but also on the small number of patients. The two regions presented in figure 5 were the most representative. However, all regions are presented in the Supplementary Figure.

5. The authors note a number of second generation TSPO PET radiopharmaceuticals are available. The discussion touches on some of the favorable characteristics of 18F-FEMPA, but would benefit from additional information comparing the relative merits of this TSPO agent with others like 11C-PBR28.

We have modified the text in the Introduction and included the following sentence to address this comment. "Based on these initial pre-clinical findings suggesting favorable kinetic properties of [^{18}F]FEMPA, it was decided to move forward with the characterization of the radioligand in human subjects. [^{18}F]FEMPA was considered to be a potential ^{18}F-labelled TSPO radioligand with similar properties (rapid wash-out from the brain and high target-to-background ratio) as the ^{11}C-labelled TSPO tracer PBR28. The aims of the present study were therefore to assess the quantification of the in vivo binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether in AD patients increased binding of [^{18}F]FEMPA to the TSPO could be demonstrated in vivo.

6. Based on these data, can the authors suggest a simplified acquisition protocol which might be suitable to clinical interrogation of neuroinflammation?

A simplified acquisition protocol cannot be easily suggested because of the need of arterial input function. Since there is no reference region in the brain for TSPO, the use of SUVratio values cannot be recommended. We did not find any obvious difference in the parent fraction, so in principle SUV data could be used, but this would require a larger number of patients and controls to confirm the findings and compare the differences between groups of V_T and SUV.

Reviewer #2: The purpose of this study was to assess the quantification of the binding of a novel high-affinity, second-generation, TSPO radioligand, [18F]FEMPA, in AD patients and controls and to investigate whether higher [18F]FEMPA binding in AD vs. controls could be detected in vivo.

The topic is relevant and by now few PET studies have explored TSPO imaging. None with this new ligand.

Some issues need to be addressed.

Page(P) 5 Line (L) 13 I would suggest some more recent review article like http://www.ncbi.nlm.nih.gov/pubmed/22315714

The reference has been changed following the recommendation of the reviewer.

P7 L49 AD was diagnosed according (NINCDS-ADRDA) and the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV). Did the patients satisfy also the updated criteria for probable Alzheimer's disease dementia with evidence of the Alzheimer's disease pathophysiological process (McKhann et al., 2011) or DSM V criteria? This is important because some cases of AD (AD2, AD3, AD9) have MMSE values of 28-29.

The patients with high MMSE had probable Alzheimer's disease dementia with evidence of the Alzheimer's disease pathophysiological process (McKhann et al., 2011). In particular, AD2 had positive biomarker evidence as presence of hippocampal atrophy and reduced beta-amyloid in CSF, and AD3 had hippocampal atrophy in MRI, reduced beta-amyloid and increased tau and phosphotau in CSF. AD8 was diagnosed according to criteria of NINCDS-

ADRDA and McKann et al.1984, neuropsychology, imaging and clinical data. AD9 was diagnosed according to the criteria of McKann et al. 2011 (pathological CSF, as well as neuropsychology data in agreement with AD).
We have included the following sentence in the text to address this comment: “In addition to these criteria, the diagnostic criteria defined by McKahn et al. that include imaging (1998) or CSF and in vivo biomarkers (2003) were used, particularly for those patients showing unimpaired global cognition (MMSE 28, 29 and 30).”

P11 L01 Why did the authors use RM-ANOVA to test the effect of the group (patients versus controls) and the TSPO binding status? The statistical design seems not to be repeated measures. A two-way ANOVA would probably be more appropriate.

As stated in the manuscript: “Repeated measure analysis of variance (RM-ANOVA) was applied to test the effect of the group (AD patients vs. controls) and TSPO binding status (MAB or HAB) on V_T. Brain region (VOI) was entered as within subject factor, the group as between subject factor and the TSPO binding status as covariate. RM-ANOVA was also applied only to the data from the HABs. In this case, no covariate was entered in the model. As post-hoc analysis, ANOVA was applied to test the differences in V_T between AD patients and controls in different brain regions.” So in principle, the statistical design was applied as a two-way ANOVA.

P13 L26 The description of the results of the RM-ANOVA seems rather to be the description of a two-way ANOVA.

See reply above.

P14 L02 This paragraph should be transposed into the 'Results' section maintaining here only the discussion of the results.

This has been done as suggested by the Reviewer.

P15 L36 The following part is too much speculative.

The reason why we included this part in the manuscript is to try to describe the results in a more comprehensive way and to try to estimate the binding potential of [^{18}F]FEMPA to provide the reader a means to compare the tracer with [^{11}C]PBR28. A similar approach has been used for [^{11}C]PBR28 by Kreisl et al. (Journal of Cerebral Blood Flow & Metabolism (2013) 33, 53–58;), providing an estimate of BP_{ND} for the HABs similar to the one of [^{18}F]FEMPA (see pages 56 and 57). Interestingly, the estimated BP_{ND} of [^{18}F]FEMPA we obtained for HABs and MABs was in agreement with the calculated BP_{ND} for [^{11}C]PBR28 recently reported by Owen et al. (Journal of Cerebral Blood Flow & Metabolism 2014; 34, 989–994) in a blocking study using the TSPO agonist XBD173. This sentence has been now included in the Discussion for clarification.

Positron emission tomography imaging of the 18-kDa Translocator Protein (TSPO) with [^{18}F]FEMPA in Alzheimer´s disease patients and control subjects

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Abstract

Imaging of the 18-kDa translocator protein (TSPO) is a potential tool for examining microglia activation and neuroinflammation in early Alzheimer´s disease (AD). [^{18}F]FEMPA is a novel high-affinity, second-generation, TSPO radioligand displaying suitable pharmacokinetic properties in pre-clinical studies. The aims of this study were to assess the quantification of the binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether higher [^{18}F]FEMPA binding in AD vs. controls could be detected *in vivo*.

Methods. Ten AD patients (5M/5F, age 66.9±7.3 y, MMSE 25.5±2.5) and seven controls (3M/4F, age 63.7±7.2 y, MMSE 29.3±1.0) were studied using [^{18}F]FEMPA at Turku (n=13) and at Karolinska Institutet (n=4). The *in vitro* binding affinity for TSPO was assessed using PBR28 in a competition assay with [^{3}H]PK11195 in 7 controls and 8 AD. Cortical and subcortical regions-of-interest were examined. Quantification was performed using two-tissue compartment model (2TCM) and Logan graphical analysis (GA). The outcome measure was the total distribution volume (V_T). Repeated-measure analysis of variance was used to assess the effect of group or TSPO binding status on V_T.

Results. Five AD and 4 controls were high-affinity binders (HABs). Three AD and 3 controls were mixed-affinity binders. V_T estimated with Logan GA correlated significantly with V_T estimated with 2TCM in both controls (r=0.97) and AD patients (r=0.98) and was selected for the final analysis. In the medial temporal cortex, statistically significant higher V_T (p=0.044) in AD vs. controls was found if the TSPO binding status was entered as covariate. If only HABs were included, statistically significant higher V_T in AD patients vs. control subjects (p<0.05) was found in the medial and lateral temporal cortex, posterior cingulate, caudate, putamen, thalamus, and cerebellum.

Conclusions. [^{18}F]FEMPA seems to be a suitable radioligand to detect increased TSPO binding in AD if the binding status is taken into account.

Key words. Neuroinflammation, microglia, translocator protein, dementia, Alzheimer

Introduction

Neuroinflammation is a pathological phenomenon characterized by microglia activation and reactive astrocytosis. Neuroinflammatory changes are observed in various neurodegenerative disorders including Alzheimer´disease (AD). Post-mortem studies in AD patients have shown that microglial activation is associated with the presence of amyloid plaques [1], suggesting a link between amyloid pathology and neuroinflammation. In vivo imaging of microglial activation can be a useful tool for early detection of neuroinflammation in AD. The 18-kD translocator protein (TSPO) is a mitochondrial protein [2, 3] expressed in macrophages [4], microglia cells [5] and reactive astrocytes [6] and is considered a marker of activated microglia and macrophages [7]. ((*R*)-1-(2-chlorophenyl)-*N*-^{11}C-methyl-*N*-(1-methylpropyl)-3-isoquinoline caboxamide ([^{11}C](*R*)-PK11195) was the first TSPO radioligand developed for imaging of activated microglia. The first evidence of increased TSPO binding in AD patients using [^{11}C](*R*)-PK11195 was reported by Cagnin et al. [8]. This finding was replicated in a group of 13 AD patients that were also examined with the amyloid radioligand [^{11}C]PIB [9]. A large overlap of TSPO binding signal was however observed between controls and patients with AD or with mild cognitive impairment (MCI), using [^{11}C](*R*)-PK11195 [10, 11]. It was suggested that either microglia activation in AD is a subtle phenomenon [11] or that [^{11}C](*R*)-PK11195 is not enough sensitive to detect in vivo increased microglia activation in AD [10].

Several TSPO radioligands with greater affinity than [^{11}C](*R*)-PK11195 have been developed [12] and some of them have been used for in vivo imaging of neuroinflammation. Increased TSPO binding in AD and MCI patients compared with controls has been found using the high-affinity radioligand *N*-(2,5-^{11}C-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)acetamide ([^{11}C]DAA1106) [13, 14]. When the ^{18}F-analog of DAA1106, *N*-

(5-fluoro-2-phenoxyphenyl)-*N*-(2-^{18}F-fluoroethyl-5-methoxybenzyl)acetamide ([^{18}F]FEDAA1106) was used, no statistically significant increase of TSPO binding in AD patients could be detected in comparison with controls [15]. A large variability of outcome measures of [^{18}F]FEDAA1106 among different subjects was observed [15].

A major source of variability in TSPO binding is known to be related to the presence of different binding affinity profiles. This property was first demonstrated and fully examined for the high-affinity TSPO radioligand (*N*-{[2-(methyloxy)phenyl]methyl}-*N*-[4-(phenyloxy)-3-pyridinyl]acetamide (PBR28) [16], but it was also shown for other second-generation TSPO ligands [17]. In the case of [^{11}C]PBR28 it has been demonstrated that the *rs6971* polymorphism of the TSPO gene is responsible for the presence of different binding affinity profiles [18]. Subjects can be high- mixed- and low-affinity binders (HABs, MABs, LABs) based on the homozygosity or heterozygosity for the polymorphism. Therefore, imaging of the TSPO using second-generation radioligands should take into account the binding status of the study participants, particularly when different groups of subjects are examined. Recently, increased TSPO binding in amyloid-positive AD patients has been demonstrated using ^{11}C-PBR28 and adjusting for TSPO genotype [19].

N-{2-[2-(^{18}F)fluoroethoxy]-5-methoxybenzyl}-N-[2-(4-methoxyphenoxy)pyridine-3-yl]acetamide ([^{18}F]FEMPA [CAS 1207345-42-3]) is an aryloxypyridylamide derivative that is less lipophilic than [^{18}F]FEDAA1106, and pre-clinical data in non-human primates showed a fast elimination from the brain and a better signal-to-noise ratio. Based on these initial pre-clinical findings suggesting favorable kinetic properties of [^{18}F]FEMPA, it was decided to move forward with the characterization of the radioligand in human subjects. [^{18}F]FEMPA was considered to be a potential ^{18}F-labelled TSPO radioligand with similar kinetic properties (rapid wash-out from the brain and high target-to-background ratio) as the ^{11}C-labelled TSPO

tracer PBR28. The aims of the present study were therefore to assess the quantification of the in vivo binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether in AD patients increased binding of [^{18}F]FEMPA to the TSPO could be demonstrated in vivo.

Materials and methods

Subjects

The study was conducted in line with the Helsinki Declaration and approved by FIMEA and the Swedish Medical Products Agency, the local Ethics Committee of the Southwest Hospital District of Finland and of the Stockholm region, and by the Radiation Safety Committee of the Turku Hospital and the Karolinska University Hospital. The study was registered at www.ClinicalTrials.gov (NCT01153607) and included a total of 24 participants. Seventeen of those participants were included in the present study, wheras 7 participants were included in a whole-body dosimetry study that will be reported separately.

Ten AD patients and 7 controls were studied at Turku PET Centre (13 subjects) and at Karolinska Institutet (4 subjects) (Table 1). All subjects gave written informed consent for participation in the study. AD patients were recruited from the University of Turku and from the Karolinska University Hospital, Huddinge. Controls were recruited by local advertisement and from a database at the Karolinska Trial Alliance in Stockholm. All subjects underwent careful clinical and neurological examinations, Mini-Mental State Examination (MMSE), and neuropsychological testing including assessment of memory function. Probable AD was diagnosed according to the clinical criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV). In addition to these criteria, the diagnostic criteria defined by McKahn et al. that include imaging (1998) or CSF and in vivo biomarkers (2003) were used, particularly for those patients showing unimpaired global cognition (MMSE 28, 29 and 30). The inclusion criterion was mild to moderate disease (MMSE score ≥20 and a Clinical Dementia Rating score of 1 or 2). Other forms of dementia (e.g. dementia with Lewy bodies)

had to be excluded. Patients were under stable treatment (at least 6 months before the study) with cholinesterase inhibitors. Additionally, neither AD patients nor controls were allowed to show signs of systemic autoimmune or inflammatory disease. Participants with other current treatments acting on the central nervous system (including anti-inflammatory treatments in pre-specified time frames) were also excluded in order to avoid interference with the in vivo binding of the radioligand.

PET experimental procedures

Details of radiolabelling procedures of [^{18}F]FEMPA are described in Supplementary Appendix 1. Specific radioactivity at time of injection was between 31 and 1343 GBq/μmol. The injected radioactivity was 251±16 MBq in control subjects and 251±10 MBq in AD patients. The injected mass was 0.68±0.97 (range 0.07-2.55) μg in control subjects and 0.67±1.16 (range 0.09-3.74) μg in AD patients. There were no significant adverse or clinically detectable pharmacologic effects in any of the 17 subjects. No significant changes in vital signs or the results of laboratory studies or electrocardiograms were observed.

PET measurements

PET measurements were performed with the ECAT EXACT HR+ (Turku PET Center) and the ECAT EXACT HR (Karolinska Institutet) systems in two PET sessions. The first PET session consisted of a 90-min dynamic acquisition with a series of frames of increasing duration (6x5 sec, 3x10 sec, 2x20 sec, 4x60 sec, 6x180 sec, 11x360 sec). The second PET session of 30 min was performed between 120 and 150 min after radioligand injection and consisted of 5 frames of 360 sec. A transmission scan of 5 min was acquired before each dynamic acquisition using three rotating ^{68}Ge sources. At Turku, images were reconstructed with filtered back projection, a 256x256 matrix, and a pixel size of 1.226x1.226 mm. At

Karolinska Institutet, images were reconstructed with filtered back projection, with a 2-mm Hanning filter, a zoom factor of 2.17, and a 128x128 matrix. Images were corrected for attenuation and scatter. A NEMA Jacszack phantom with spheres of different diameter and uniform background filled with ^{18}F-radioactive solution at a ratio of ~4:1 was acquired at both centers under similar experimental conditions and using the standard reconstruction method at each centre. The difference of the recovery coefficient between the two PET systems was 9.4% for the spheres and 4.2 for the background, suggesting the possibility to pool the data from the two PET systems.

Arterial blood sampling was performed using an automated blood sampling system (Allogg AB, Mariefred, Sweden) for the first 10 min and using manual samples thereafter. Samples for metabolite analysis (HLPC, Appendix 1) were taken at 2, 5, 10.5, 20, 30, 45, 60, 90, 120, and 150 min.

Magnetic resonance imaging

MRI was performed at Turku University using a Philips Gyroscan Intera 1.5 T Nova Dual scanner (Philips, Best, the Netherlands) and at the Karolinska Institutet using a 1.5-T GE Signa system (GE Healthcare, Milwaukee, WI). MRI scans consisted of a T2-weighted sequence for ruling out pathological changes and a 3-D T1-weighted spoiled gradient recalled (SPGR) sequence for both coregistration with PET and volume-of-interest (VOI) analysis. MRI scans were evaluated for white matter changes according to the Age-Related White Matter Changes (ARWMC) scale [20], and exclusion criteria were an ARWMC score of >1 in the basal ganglia and >2 in the subcortical white matter.

Image analysis

Image analysis was performed at Turku PET Centre. PET images were coregistered to the T1-weighted MRI using SPM2 (Wellcome Department of Imaging Neuroscience, London, UK). Volumes of interest (VOIs) were delineated using the software Imadeus 1.20 (Forima Inc, Turku, Finland). The following regions were defined: frontal cortex, parietal cortex, lateral and medial temporal cortex, occipital cortex, posterior cingulate cortex, caudate, putamen, thalamus, pons, cerebellum and the subcortical white matter.

TSPO binding status

The TSPO binding status was measured at Imanova Centre for Imaging Sciences from peripheral blood samples. In two AD patients (AD1 and AD2) the plasma was not available for the binding competition assay. The PBR28 binding status was measured using competition binding assay with ^{3}H-PK11195 on platelet membrane suspension (Supplementary Appendix 2). Data were analysed using GraphPad Prism 5.0 Software. One and two site binding models were compared using a sum-of-square *F*-test. In four subjects (CS6, CS7, AD8, and AD9), the binding status was less reliably measured because of low protein concentration in the samples.

Data analysis

A preliminary analysis showed that the first PET session was sufficient for quantification of [^{18}F]FEMPA binding. Therefore, only 90 min of data were used for the final analysis. The radioactivity concentration in the different brain regions was reported as standard uptake value (SUV) and calculated as SUV=kBq/cm^3 ÷ Bq injected / body weight (g). Two parameters were measured to assess the kinetic properties of [^{18}F]FEMPA: the time to peak uptake (t_{peak}) and the time when the brain radioactivity decreased to 50% of the peak ($t_{half\text{-}peak}$), both expressed in min. The quantification was performed using kinetic and Logan

graphical analysis (GA). Kinetic analysis was performed with nonlinear least square (NLS) fitting and two tissue compartment model (2TCM), with four parameters (K_1, K_1/k_2, k_3/k_4, k_4) and blood volume fitted for each region. The outcome measure was the total distribution volume (V_T). In one subject (AD2), arterial blood sampling was not successful, and this patient was excluded from further analyses. The variability of V_T estimated with 2TCM and Logan GA was calculated as the ratio between the SD over the mean for each brain region and expressed as percentage (coefficient of variance=COV%).

Statistical analysis

Regression analysis was used to assess the agreement between 2TCM and Logan GA in the estimation of V_T. F-test was used to compare the variability of V_T (%COV) estimated with 2TCM and Logan GA. Repeated measure analysis of variance (RM-ANOVA) was applied to test the effect of the group (AD patients vs. controls) and TSPO binding status (MAB or HAB) on V_T. Brain region (VOI) was entered as within subject factor, the group as between subject factor and the TSPO binding status as covariate. RM-ANOVA was also applied only to the data from the HABs. In this case, no covariate was entered in the model. As post-hoc analysis, ANOVA was applied to test the differences in V_T between AD patients and controls in different brain regions. Statistical significance was evaluated at $p<0.05$.

Results

TSPO binding status

Four controls were HABs and 3 were MABs, whereas 5 AD patients were HABs and 3 were MABs (Supplementary Appendix 2). No LABs were observed in either group. The K_i high for the HABs was 2.26±0.18 nM. The K_i high and low for the MABs were 1.93±0.75 nM and 189.8±14.4 nM, respectively.

Radiometabolite analysis

[^{18}F]FEMPA showed rapid metabolism in vivo with <20% of tracer present in plasma 20 min after injection and <10% after 90 min (Figure 1 and Supplementary Figure 1 and 2). There were no statistically significant differences in the parent fraction or in the fraction of metabolites between control subjects and AD patients and between MABs and HABs (Figure 1).

Kinetic properties of [^{18}F]FEMPA

Representative SUV images and mean time-activity curves of [^{18}F]FEMPA are presented in Figures 2 and 3. In each binding group there were no statistically significant differences between controls and AD patients in kinetic parameters based on SUV data (Supplementary Table 1). However, among the AD patients the $t_{half\text{-}peak}$ was significantly lower in MABs than in HABs (p=0.008), whereas only a trend was observed in the controls (p=0.15).

PET quantification

A preliminary comparison between one tissue compartment model and 2TCM showed that 2TCM provided a better fitting of the data by visual inspection and based on Akaike

Information Criteria, therefore only 2TCM was used in the final analysis of the data (Supplementary Figure 3, Supplementary Table 2 and 3). In HABs, V_T values were significantly higher (p<0.05) in AD patients compared with controls in parietal cortex, lateral and medial temporal cortex, posterior cingulate, thalamus and cerebellum.

Representative Logan plots of [^{18}F]FEMPA are presented in Figure 4. There was a statistically significant correlation between V_T estimated with 2TCM and with Logan GA in controls (r=0.97, p<0.001) and AD patients (r=0.98, p<0.001) across all regions and subjects, with values close to the line of identity (Supplementary Figure 4). The mean COV% of V_T estimated with Logan GA tended to be lower than the mean COV% of V_T estimated with 2TCM in AD patients (p=0.05, Supplementary Table 4). Logan GA was selected for the final analysis of the data, considering the high correlation of V_T between Logan GA and 2TCM and the slightly lower COV% of V_T estimated with Logan GA in AD patients.

RM-ANOVA using Logan V_T showed a significant effect of TSPO (F=17.3, p=0.001) and a significant region*TSPO binding status interaction (F=5.2, p=0.004). The group showed only a non-significant trend (F=3.7, p=0.077). No statistically significant region*group interaction was found. However, when only HABs were included in the analysis, a significant effect of group (F=9.2, p=0.02) was observed but no statistically significant region*group interaction was found. In all subjects, if the TSPO binding status was entered as covariate, a statistically significant difference between groups was found in the medial temporal cortex (Table 2). If only the HABs were included, statistically significant differences between groups were found in lateral and medial temporal cortex, posterior cingulate, caudate, putamen, thalamus and cerebellum (Table 2). In HABs, the V_T values (mean±SD) in these regions were on average 19.5±3.0% higher in AD patients as compared with controls, ranging from 15%

higher in the lateral temporal cortex to 24% in the thalamus (Table 2, Figure 5 and Supplementary Figure 5).

Discussion

This study was designed to examine the quantification of the binding to TSPO of the novel radioligand [^{18}F]FEMPA in controls and AD patients and to evaluate whether increased TSPO binding in AD could be demonstrated in vivo. The primary outcome measure in this study was V_T, estimated using kinetic and Logan GA and the metabolite corrected arterial input function, since no reference region for TSPO is present in the brain. Since a major source of variability in V_T for all second-generation TSPO radioligands is known to come from the *rs6971* polymorphism of the TSPO [18], the binding status of the subjects was evaluated using competition assay with ^{3}H-PK11195 and PBR28. In a separate work, the binding properties of FEMPA have been tested on human brain tissue samples, known to belong to different binder subtypes, and it was found that the ratio in affinity between LABs and HABs was approximately 12 (unpublished, data), thus ~4.6 times lower than PBR28.

The main finding of this study was that increased in vivo binding of [^{18}F]FEMPA to TSPO in AD patients could be demonstrated if the binding status of the subjects was taken into account and more specifically if only HABs were included. [^{18}F]FEMPA appeared to be a suitable radioligand for in vivo TSPO quantification, displaying good brain uptake, fast wash-out from the brain and relatively fast metabolism. V_T estimated using Logan GA was in very good agreement with V_T estimated using 2TCM and showed also lower variability in both controls and AD patients.

TSPO binding status

In this study the TSPO binding status was examined in a competition assay with ^{3}H-PK11195 and PBR28. It is known that this assay provides results in agreement with the analysis of the polymorphism of the TSPO gene [21]. The K_i high for the HABs was in good

agreement with the K_i value (3.10±5 nM) previously reported by Owen et al. [16]. The K_i high and low for the MABs were also in agreement with the K_i high and low values (4.0±2.4 and 313±76.8 nM) previously reported [16], although the K_i low for MABs was more in agreement with the K_i low previously reported for LABs (188±15.6 nM) [16]. Although in 4 subjects the protein concentration in the assay was low, leading to a reduced signal-to-noise ratio, the V_T for HABs was approximately 2.2 times higher than the V_T for MABs, in agreement with the ratio of V_T between HABs and MABs found across all subjects, which was approximately 1.5. This ratio is also in agreement with the ratio between HABs and MABs reported for ^{11}C-PBR28 [18].

Quantification of [^{18}F]FEMPA binding to TSPO

The fast kinetic properties of [^{18}F]FEMPA compared with its analog $^{[18F]}$FEDAA1106 represent a potential advantage for its clinical use. The kinetic analysis showed that the 2TCM was a suitable model for the quantification of [^{18}F]FEMPA and that V_T estimates obtained with Logan GA were in close agreement with the 2TCM. In this study, we only observed HABs and MABs according to the in vitro binding affinity data. We attempted to estimate the V_T for a LAB, based on the results of the MABs and HABs (Supplementary Appendix 3). The estimated $V_{T\,LAB}$ was 0.57±0.08 in controls and 0.74±0.28 in AD patients. Interestingly, this value is similar to the lowest V_T value found in the AD patient that was not analysed for the binding status and that most likely corresponds to a LAB. Assuming that the non-specific binding is similar in HABs, MABs and LABs, and that $V_{ND}<V_T^{LAB}$, the binding potential (BP_{ND}) calculated from the distribution volumes ($BP_{ND}=V_T/V_{ND}$-1) can be estimated to be at least ~2 in HABs and ~1 in MABs. Interestingly, the estimated BP_{ND} of [^{18}F]FEMPA we obtained for HABs and MABs was in agreement with the calculated BP_{ND} for [^{11}C]PBR28 recently reported by Owen et al. in a blocking study using the TSPO agonist XBD173 [22].

Increased TSPO binding in AD

We observed that in HABs the increase of [^{18}F]FEMPA binding to the TSPO was between 15% and 24%. These findings are in agreement with previous reports using either [^{11}C](*R*)-PK11195 in AD patients (approximately 20-35% increased in cortical binding as compared with controls) (*9*), [^{11}C]DAA1106 in MCI (26% increase) and AD patients (18% increase) (*13, 14*), or [^{11}C]PBR28 in AD patients (38% increase) (*19*). Considering the relatively small sample size of this study, statistically significant increased TSPO signal in early AD was detected only after controlling for the TSPO binding status, suggesting the potential of [^{18}F]FEMPA to detect microglia activation in AD.

Additional considerations

The binding of [^{11}C]PBR28 to the TSPO has been shown to correlate negatively with the MMSE [19]. We examined the correlation of [^{18}F]FEMPA mean cortical (frontal, temporal, parietal, and occipital), limbic (medial temporal cortex and posterior cingulate) and sub-cortical (caudate, putamen and thalamus) V_T with MMSE and found a weak, non significant negative correlation (r between -0.37 and -0.41, p-value between 0.12 and 0.17) when combining data from controls and AD patients (data not shown), The lack of statistically significant correlation might be related to the limited sample size and further studies are needed to specifically examine the relationship between TSPO binding of [^{18}F]FEMPA and cognitive function in AD.

The analysis of the PET data was conducted using only conventional ROI-based approach. Voxel-based analysis could be useful to identify differences in small areas that can be underestimated by the use of large ROIs. In this study we did not apply voxel-based

analysis because of the limited sample size of both groups and to avoid possible false-positive and negative results that can be associated with small samples.

The potential application of an ^{18}F-labelled tracer in the clinical setting could be aided by the use of a simplified acquisition protocol. However, in the case of [^{18}F]FEMPA, because of the lack of a reference region in the brain the arterial input function data is needed to estimate V_T. We did not observe differences in the parent fraction between AD patients and controls, suggesting that the observed differences in V_T are indeed reflecting differences in the brain distribution of the tracer. Such differences could be detected only by measuring the brain uptake as SUV. We did observe differences in SUV between the two groups, similar to differences in V_T (data not shown), which might suggest that SUV could be used as surrogate outcome measure. However, to validate SUV as potential outcome measure in the clinical setting, additional studies with [^{18}F]FEMPA in a larger group of AD patients and controls are needed.

Conclusions

[^{18}F]FEMPA seems to be a suitable radioligand for in vivo imaging and quantification of TSPO in early AD, provided that the TSPO binding status is determined or by including only HABs. Future studies are needed to confirm these findings in a larger cohort of AD patients.

Acknowledgments

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Karolinska Institutet PET Centre and the Karolinska University Hospital for technical support.

Table 1. Details of Controls and AD patients and their binding status.

Controls/AD patients	Centre	Gender	Age (y)	MMSE	Binding status	Treatment
CS1	Turku	M	66	28	HAB	n.a.
CS2	Turku	F	56	29	MAB	n.a.
CS3	Turku	M	55	30	MAB	n.a.
CS4	Turku	F	69	30	HAB	n.a.
CS5	Turku	F	71	28	HAB	n.a.
CS6	KI	M	58	30	MAB	n.a.
CS7	KI	F	71	30	HAB	n.a.
Mean±SD	64±7		29.3±1.0			
AD1	Turku	M	74	23	na	Rivastigmine 9.5mg QD
AD2*	Turku	F	56	29	na	Donepezil 10mg QD
AD3	Turku	M	69	28	HAB	Rivastigmine 9.5mg QD
AD4	Turku	M	55	25	MAB	Donepezil 10mg QD
AD5	Turku	F	67	26	HAB	Rivastigmine 9.5mg QD
AD6	Turku	F	76	24	HAB	Donepezil 10mg QD
AD7	Turku	F	67	22	MAB	Donepezil 5mg QD
AD8	KI	F	71	27	HAB	Donepezil 5 mg
AD9	KI	M	61	28	MAB	Galantamine 16 mg
AD10	Turku	M	73	23	HAB	Donepezil 10 mg QD
Mean±SD	67±7		25.5±2.5†			

*AD2: not analysed because only 10 min of blood data available

†= significantly different from Controls by two-tailed un-paired t-test, p=0.002

HAB = High Affinity Binder (9), MAB = Mixed Affinity Binder (6), LAB = Low Affinity Binder (0).

Table 2. V_T values of Logan GA in Controls and AD patients (mean ± SD) with results of ANOVA. The analysis was conducted on all subjects (HABs and MABs) and on the HAB subjects only (4 Controls, 5 AD patients). In the ANOVA conducted on all subjects, the TSPO binding status was included as covariate.

	V_T, all subjects		**Group**	**V_T, only HABs**		**Group**
Region	Controls	AD patients	*F* (*p* value)	Controls	AD patients	*F* (*p* value)
Frontal cx	1.59±0.40	1.68±0.63	1.8 (0.202)	1.85±0.12	2.12±0.23	4.4 (0.075)
Parietal cx	1.40±0.32	1.61±0.52	4.6 (0.052)	1.61±0.17	1.86±0.16	5.2 (0.056)
Lat Temp cx	1.50±0.35	1.61±0.55	2.8 (0.122)	1.72±0.10	1.98±0.17	7.0 (0.033)
Med Temp cx	1.64±0.48	1.88±0.69	5.1 (0.044)	1.95±0.12	2.36±0.18	15.2 (0.006)
Occip cx	1.46±0.35	1.60±0.52	2.3 (0.154)	1.65±0.28	1.82±0.23	1.0 (0.353)
Post Cingulate	1.62±0.44	1.86±0.67	4.1 (0.066)	1.91±0.10	2.30±0.24	9.1 (0.020)
Caudate	1.25±0.33	1.35±0.49	1.9 (0.193)	1.45±0.05	1.68±0.19	6.0 (0.045)
Putamen	1.46±0.44	1.67±0.64	3.6 (0.080)	1.73±0.10	2.10±0.23	8.9 (0.020)
Thalamus	1.64±0.48	1.89±0.75	4.1 (0.064)	1.94±0.09	2.40±0.29	9.1 (0.019)
Pons	1.70±0.50	1.92±0.80	2.8 (0.121)	2.00±0.15	2.42±0.35	5.0 (0.060)
Cerebellum	1.43±0.35	1.61±0.60	4.2 (0.064)	1.67±0.06	2.00±0.24	6.9 (0.034)
White matter	1.44±0.44	1.61±0.66	2.4 (0.146)	1.70±0.14	2.03±0.33	3.3 (0.111)

Figure Legend

Figure 1. Mean plasma parent fraction in mixed-affinity binder (MAB) and high-affinity binder (HAB) in controls and AD patients. The error bars represent 1 SD.

Figure 2. Representative SUV images of mixed-affinity binders (MAB) and high-affinity binders (HAB). Transaxial slices at the level of the basal ganglia from two control subjects and two AD patients are shown. Frames between 5 and 30 min (top row) and between 60 and 90 min (bottom row) were averaged.

Figure 3. Mean TACs from mixed-affinity binder (MAB) and high-affinity binder (HAB) Controls and AD patients in thalamus and medial temporal cortex. Error bars represent 1 SD.

Figure 4. Representative Logan plots of thalamus and medial temporal cortex from mixed-affinity binders (MAB) and high-affinity binders (HAB). Data are from the same control subjects and AD patients displayed in Figure 2.

Figure 5. Scatter plot of V_T values in control subjects and AD patients (HABs and MABs) for thalamus and medial temporal cortex. The AD patient displayed with the open square was not analysed for TSPO binding.

Conflict of interest

Ray Valencia, Marcus Schultze-Mosgau, Andrea Thiele, Sonja Vollmer, Thomas Dyrks, Lutz Lehman, Tobias Heinrich, Anja Hoffmann were employed by Bayer Healthcare, Berlin, Germany at the time of the conduction of the study.

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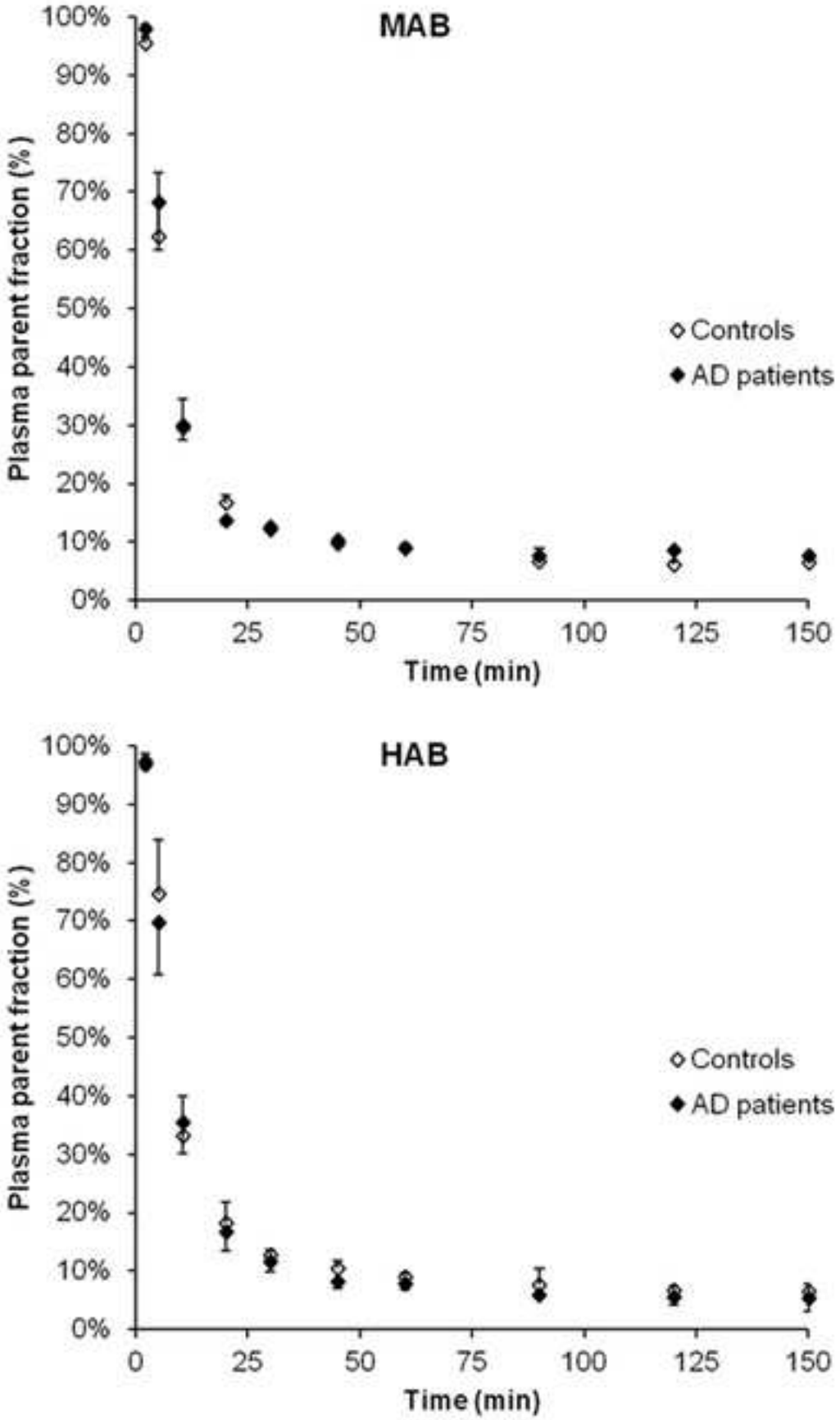

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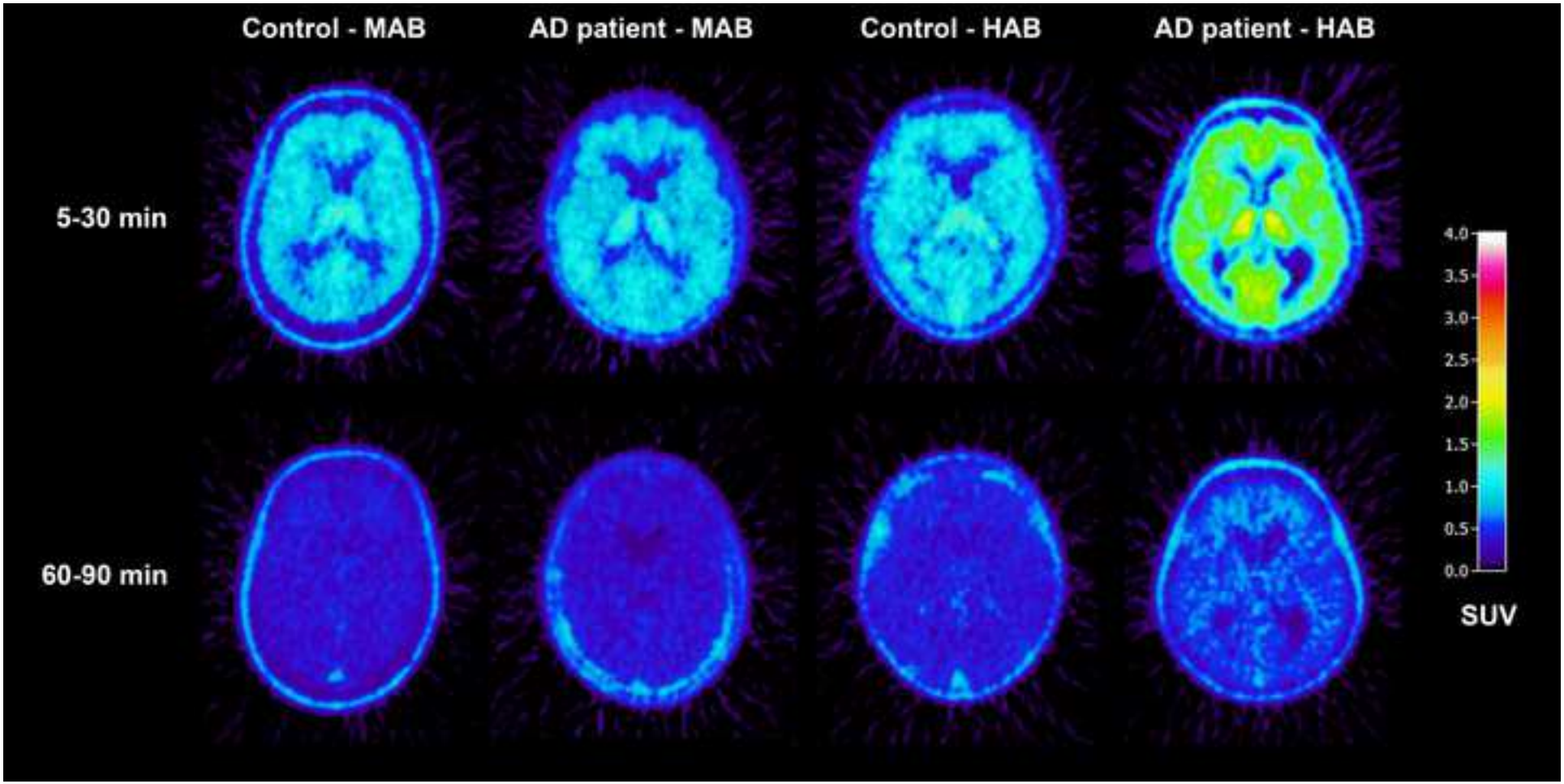

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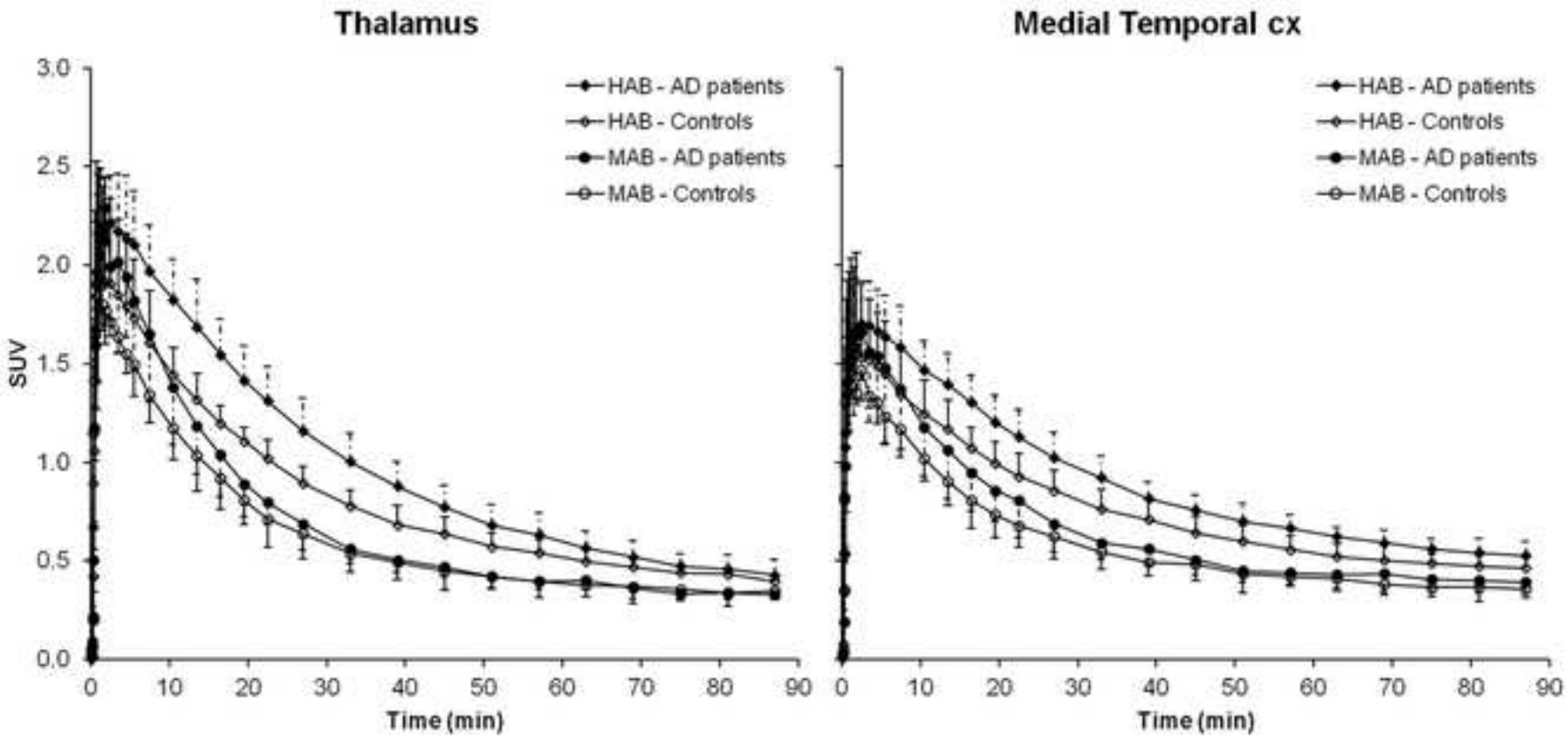

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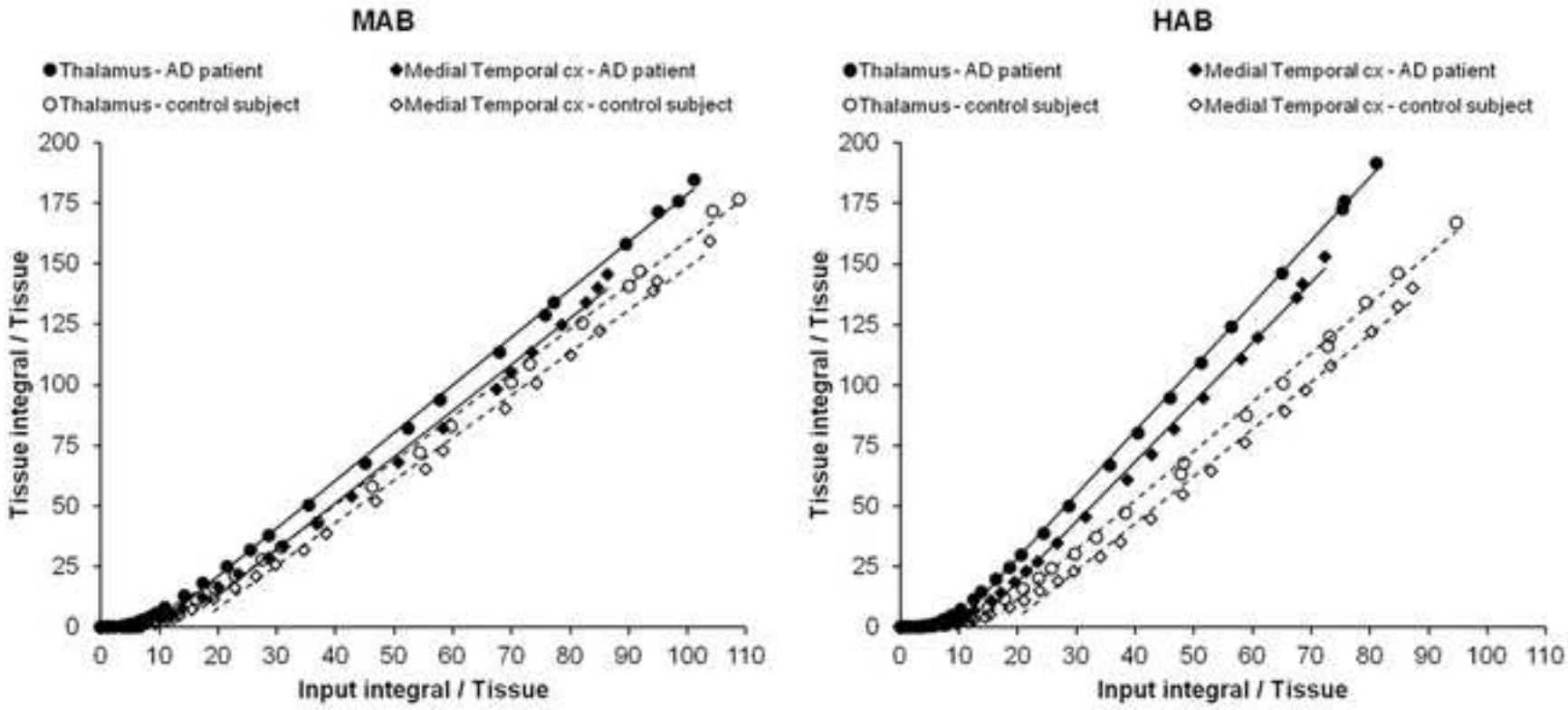

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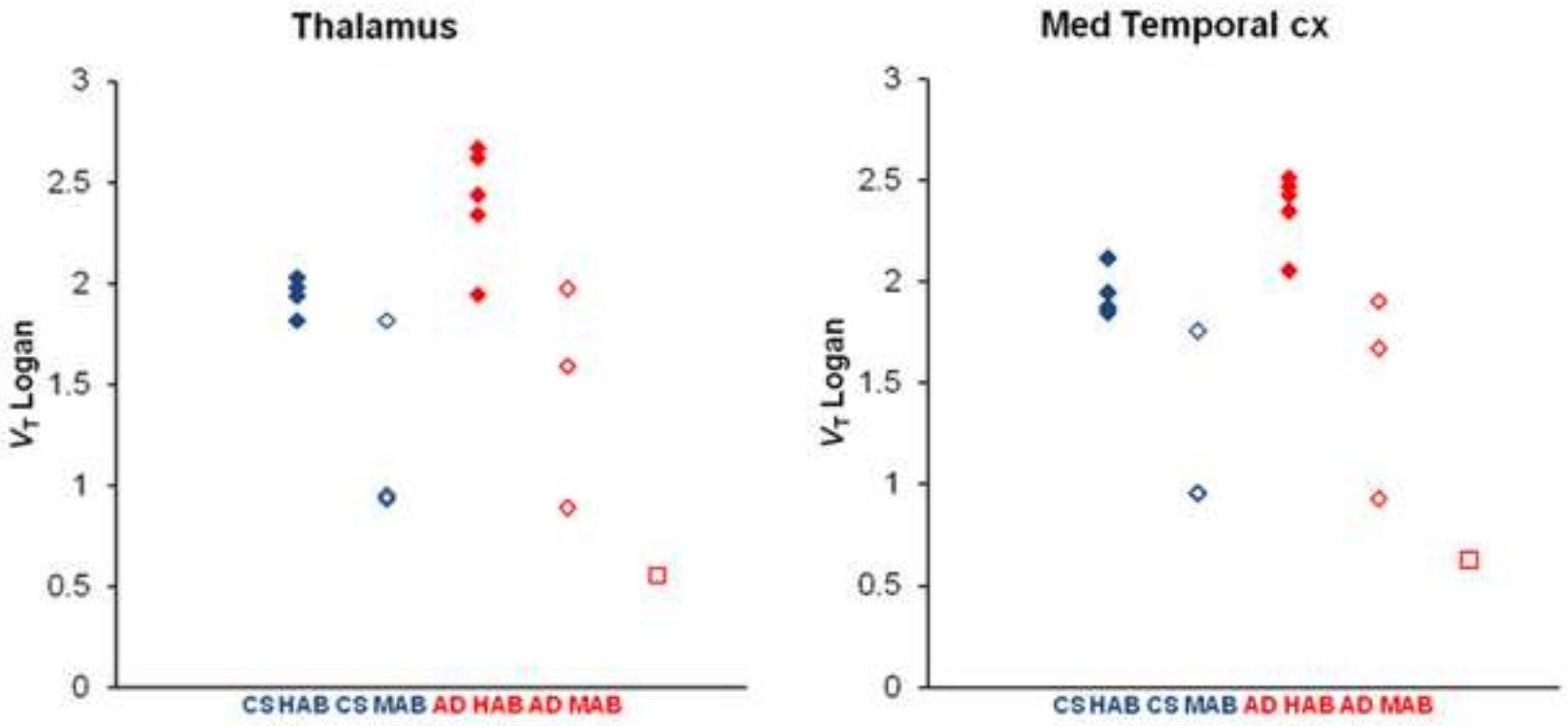

Positron emission tomography imaging of the 18-kDa Translocator Protein (TSPO) with [^{18}F]FEMPA in Alzheimer´s disease patients and control subjects

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Abstract

Imaging of the 18-kDa translocator protein (TSPO) is a potential tool for examining microglia activation and neuroinflammation in early Alzheimer´s disease (AD). [^{18}F]FEMPA is a novel high-affinity, second-generation, TSPO radioligand displaying suitable pharmacokinetic properties in pre-clinical studies. The aims of this study were to assess the quantification of the binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether higher [^{18}F]FEMPA binding in AD vs. controls could be detected *in vivo*.

Methods. Ten AD patients (5M/5F, age 66.9±7.3 y, MMSE 25.5±2.5) and seven controls (3M/4F, age 63.7±7.2 y, MMSE 29.3±1.0) were studied using [^{18}F]FEMPA at Turku (n=13) and at Karolinska Institutet (n=4). The *in vitro* binding affinity for TSPO was assessed using PBR28 in a competition assay with [^{3}H]PK11195 in 7 controls and 8 AD. Cortical and subcortical regions-of-interest were examined. Quantification was performed using two-tissue compartment model (2TCM) and Logan graphical analysis (GA). The outcome measure was the total distribution volume (V_T). Repeated-measure analysis of variance was used to assess the effect of group or TSPO binding status on V_T.

Results. Five AD and 4 controls were high-affinity binders (HABs). Three AD and 3 controls were mixed-affinity binders. V_T estimated with Logan GA correlated significantly with V_T estimated with 2TCM in both controls (r=0.97) and AD patients (r=0.98) and was selected for the final analysis. In the medial temporal cortex, statistically significant higher V_T (p=0.044) in AD vs. controls was found if the TSPO binding status was entered as covariate. If only HABs were included, statistically significant higher V_T in AD patients vs. control subjects (p<0.05) was found in the medial and lateral temporal cortex, posterior cingulate, caudate, putamen, thalamus, and cerebellum.

Conclusions. [^{18}F]FEMPA seems to be a suitable radioligand to detect increased TSPO binding in AD if the binding status is taken into account.

Key words. Neuroinflammation, microglia, translocator protein, dementia, Alzheimer

Introduction

Neuroinflammation is a pathological phenomenon characterized by microglia activation and reactive astrocytosis. Neuroinflammatory changes are observed in various neurodegenerative disorders including Alzheimer´disease (AD). Post-mortem studies in AD patients have shown that microglial activation is associated with the presence of amyloid plaques [1]~~[1]~~, suggesting a link between amyloid pathology and neuroinflammation. In vivo imaging of microglial activation can be a useful tool for early detection of neuroinflammation in AD. The 18-kD translocator protein (TSPO) is a mitochondrial protein [2, 3]~~[2, 3]~~ expressed in macrophages [4]~~[4]~~, microglia cells [5]~~[5]~~ and reactive astrocytes [6]~~[6]~~ and is considered a marker of activated microglia and macrophages [7]~~[7]~~. ((*R*)-1-(2-chlorophenyl)-*N*-^{11}C-methyl-*N*-(1-methylpropyl)-3-isoquinoline caboxamide ([^{11}C](*R*)-PK11195) was the first TSPO radioligand developed for imaging of activated microglia. The first evidence of increased TSPO binding in AD patients using [^{11}C](*R*)-PK11195 was reported by Cagnin et al. [8]~~[8]~~. This finding was replicated in a group of 13 AD patients that were also examined with the amyloid radioligand [^{11}C]PIB [9]~~[9]~~. A large overlap of TSPO binding signal was however observed between controls and patients with AD or with mild cognitive impairment (MCI), using [^{11}C](*R*)-PK11195 [10, 11]~~[10, 11]~~. It was suggested that either microglia activation in AD is a subtle phenomenon [11]~~[11]~~ or that [^{11}C](*R*)-PK11195 is not enough sensitive to detect in vivo increased microglia activation in AD [10]~~[10]~~.

Several TSPO radioligands with greater affinity than [^{11}C](*R*)-PK11195 have been developed [12]~~[12]~~ and some of them have been used for in vivo imaging of neuroinflammation. Increased TSPO binding in AD and MCI patients compared with controls has been found using the high-affinity radioligand *N*-(2,5-^{11}C-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)acetamide ([^{11}C]DAA1106) [13, 14]~~[13, 14]~~. When the ^{18}F-analog of

DAA1106, *N*-(5-fluoro-2-phenoxyphenyl)-*N*-(2-^{18}F-fluoroethyl-5-methoxybenzyl)acetamide ([^{18}F]FEDAA1106) was used, no statistically significant increase of TSPO binding in AD patients could be detected in comparison with controls [15]~~[15]~~. A large variability of outcome measures of [^{18}F]FEDAA1106 among different subjects was observed [15]~~[15]~~.

A major source of variability in TSPO binding is known to be related to the presence of different binding affinity profiles. This property was first demonstrated and fully examined for the high-affinity TSPO radioligand (*N*-{[2-(methyloxy)phenyl]methyl}-*N*-[4-(phenyloxy)-3-pyridinyl]acetamide (PBR28) [16]~~[16]~~, but it was also shown for other second-generation TSPO ligands [17]~~[17]~~. In the case of [^{11}C]PBR28 it has been demonstrated that the *rs6971* polymorphism of the TSPO gene is responsible for the presence of different binding affinity profiles [18]~~[18]~~. Subjects can be high- mixed- and low-affinity binders (HABs, MABs, LABs) based on the homozygosity or heterozygosity for the polymorphism. Therefore, imaging of the TSPO using second-generation radioligands should take into account the binding status of the study participants, particularly when different groups of subjects are examined. Recently, increased TSPO binding in amyloid-positive AD patients has been demonstrated using ^{11}C-PBR28 and adjusting for TSPO genotype [19]~~[19]~~.

N-{2-[2-(^{18}F)fluoroethoxy]-5-methoxybenzyl}-N-[2-(4-methoxyphenoxy)pyridine-3-yl]acetamide ([^{18}F]FEMPA [CAS 1207345-42-3]) is an aryloxypyridylamide derivative that is less lipophilic than [^{18}F]FEDAA1106, and pre-clinical data in non-human primates showed a fast elimination from the brain and a better signal-to-noise ratio. Based on these initial pre-clinical findings suggesting favorable kinetic properties of [^{18}F]FEMPA, it was decided to move forward with the characterization of the radioligand in human subjects. [^{18}F]FEMPA was considered to be a potential ^{18}F-labelled TSPO radioligand with similar kinetic properties (rapid wash-out from the brain and high target-to-background ratio) as the ^{11}C-labelled TSPO

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tracer PBR28. The aims of the present study were therefore to assess the quantification of the in vivo binding of [^{18}F]FEMPA to TSPO in AD patients and controls and to investigate whether in AD patients increased binding of [^{18}F]FEMPA to the TSPO could be demonstrated in vivo.

Materials and methods

Subjects

The study was conducted in line with the Helsinki Declaration and approved by FIMEA and the Swedish Medical Products Agency, the local Ethics Committee of the Southwest Hospital District of Finland and of the Stockholm region, and by the Radiation Safety Committee of the Turku Hospital and the Karolinska University Hospital. The study was registered at www.ClinicalTrials.gov (NCT01153607) and included a total of 24 participants. Seventeen of those participants were included in the present study, wheras 7 participants were included in a whole-body dosimetry study that will be reported separately.

Field Code Changed

Ten AD patients and 7 controls were studied at Turku PET Centre (13 subjects) and at Karolinska Institutet (4 subjects) (Table 1). All subjects gave written informed consent for participation in the study. AD patients were recruited from the University of Turku and from the Karolinska University Hospital, Huddinge. Controls were recruited by local advertisement and from a database at the Karolinska Trial Alliance in Stockholm. All subjects underwent careful clinical and neurological examinations, Mini-Mental State Examination (MMSE), and neuropsychological testing including assessment of memory function. Probable AD was diagnosed according to the clinical criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV). In addition to these criteria, the diagnostic criteria defined by McKahn et al. that include imaging (1998) or CSF and in vivo biomarkers (2003) were used, particularly for those patients showing unimpaired global cognition (MMSE 28, 29 and 30). The inclusion criterion was mild to moderate disease (MMSE score ≥20 and a Clinical Dementia Rating score of 1 or 2). Other forms of dementia (e.g. dementia with Lewy bodies)

had to be excluded. Patients were under stable treatment (at least 6 months before the study) with cholinesterase inhibitors. Additionally, neither AD patients nor controls were allowed to show signs of systemic autoimmune or inflammatory disease. Participants with other current treatments acting on the central nervous system (including anti-inflammatory treatments in pre-specified time frames) were also excluded in order to avoid interference with the in vivo binding of the radioligand.

PET experimental procedures

Details of radiolabelling procedures of [^{18}F]FEMPA are described in Supplementary Appendix 1. Specific radioactivity at time of injection was between 31 and 1343 GBq/μmol. The injected radioactivity was 251±16 MBq in control subjects and 251±10 MBq in AD patients. The injected mass was 0.68±0.97 (range 0.07-2.55) μg in control subjects and 0.67±1.16 (range 0.09-3.74) μg in AD patients. There were no significant adverse or clinically detectable pharmacologic effects in any of the 17 subjects. No significant changes in vital signs or the results of laboratory studies or electrocardiograms were observed.

PET measurements

PET measurements were performed with the ECAT EXACT HR+ (Turku PET Center) and the ECAT EXACT HR (Karolinska Institutet) systems in two PET sessions. The first PET session consisted of a 90-min dynamic acquisition with a series of frames of increasing duration (6x5 sec, 3x10 sec, 2x20 sec, 4x60 sec, 6x180 sec, 11x360 sec). The second PET session of 30 min was performed between 120 and 150 min after radioligand injection and consisted of 5 frames of 360 sec. A transmission scan of 5 min was acquired before each dynamic acquisition using three rotating ^{68}Ge sources. At Turku, images were reconstructed with filtered back projection, a 256x256 matrix, and a pixel size of 1.226x1.226 mm. At

Karolinska Institutet, images were reconstructed with filtered back projection, with a 2-mm Hanning filter, a zoom factor of 2.17, and a 128x128 matrix. Images were corrected for attenuation and scatter. A NEMA Jacszack phantom with spheres of different diameter and uniform background filled with ^{18}F-radioactive solution at a ratio of ~4:1 was acquired at both centers under similar experimental conditions and using the standard reconstruction method at each centre. The difference of the recovery coefficient between the two PET systems was 9.4% for the spheres and 4.2 for the background, suggesting the possibility to pool the data from the two PET systems.

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Arterial blood sampling was performed using an automated blood sampling system (Allogg AB, Mariefred, Sweden) for the first 10 min and using manual samples thereafter. Samples for metabolite analysis (HLPC, Appendix 1) were taken at 2, 5, 10.5, 20, 30, 45, 60, 90, 120, and 150 min.

Magnetic resonance imaging

MRI was performed at Turku University using a Philips Gyroscan Intera 1.5 T Nova Dual scanner (Philips, Best, the Netherlands) and at the Karolinska Institutet using a 1.5-T GE Signa system (GE Healthcare, Milwaukee, WI). MRI scans consisted of a T2-weighted sequence for ruling out pathological changes and a 3-D T1-weighted spoiled gradient recalled (SPGR) sequence for both coregistration with PET and volume-of-interest (VOI) analysis. MRI scans were evaluated for white matter changes according to the Age-Related White Matter Changes (ARWMC) scale [20]~~[20]~~, and exclusion criteria were an ARWMC score of >1 in the basal ganglia and >2 in the subcortical white matter.

Image analysis

Image analysis was performed at Turku PET Centre. PET images were coregistered to the T1-weighted MRI using SPM2 (Wellcome Department of Imaging Neuroscience, London, UK). Volumes of interest (VOIs) were delineated using the software Imadeus 1.20 (Forima Inc, Turku, Finland). The following regions were defined: frontal cortex, parietal cortex, lateral and medial temporal cortex, occipital cortex, posterior cingulate cortex, caudate, putamen, thalamus, pons, cerebellum and the subcortical white matter.

TSPO binding status

The TSPO binding status was measured at Imanova Centre for Imaging Sciences from peripheral blood samples. In two AD patients (AD1 and AD2) the plasma was not available for the binding competition assay. The PBR28 binding status was measured using competition binding assay with ^{3}H-PK11195 on platelet membrane suspension (Supplementary Appendix 2). Data were analysed using GraphPad Prism 5.0 Software. One and two site binding models were compared using a sum-of-square *F*-test. In four subjects (CS6, CS7, AD8, and AD9), the binding status was less reliably measured because of low protein concentration in the samples.

Data analysis

A preliminary analysis showed that the first PET session was sufficient for quantification of [^{18}F]FEMPA binding. Therefore, only 90 min of data were used for the final analysis. The radioactivity concentration in the different brain regions was reported as standard uptake value (SUV) and calculated as SUV=kBq/cm^3 ÷ Bq injected / body weight (g). Two parameters were measured to assess the kinetic properties of [^{18}F]FEMPA: the time to peak uptake (t_{peak}) and the time when the brain radioactivity decreased to 50% of the peak ($t_{half\text{-}peak}$), both expressed in min. The quantification was performed using kinetic and Logan graphical analysis (GA). Kinetic analysis was performed with nonlinear least square (NLS)

fitting and two tissue compartment model (2TCM), with four parameters (K_1, K_1/k_2, k_3/k_4, k_4) and blood volume fitted for each region. The outcome measure was the total distribution volume (V_T). In one subject (AD2), arterial blood sampling was not successful, and this patient was excluded from further analyses. The variability of V_T estimated with 2TCM and Logan GA was calculated as the ratio between the SD over the mean for each brain region and expressed as percentage (coefficient of variance=COV%).

Statistical analysis

Regression analysis was used to assess the agreement between 2TCM and Logan GA in the estimation of V_T. F-test was used to compare the variability of V_T (%COV) estimated with 2TCM and Logan GA. Repeated measure analysis of variance (RM-ANOVA) was applied to test the effect of the group (AD patients vs. controls) and TSPO binding status (MAB or HAB) on V_T. Brain region (VOI) was entered as within subject factor, the group as between subject factor and the TSPO binding status as covariate. RM-ANOVA was also applied only to the data from the HABs. In this case, no covariate was entered in the model. As post-hoc analysis, ANOVA was applied to test the differences in V_T between AD patients and controls in different brain regions. Statistical significance was evaluated at $p<0.05$.

Results

TSPO binding status

Four controls were HABs and 3 were MABs, whereas 5 AD patients were HABs and 3 were MABs (Supplementary Appendix 2). No LABs were observed in either group. The K_i high for the HABs was 2.26±0.18 nM. The K_i high and low for the MABs were 1.93±0.75 nM and 189.8±14.4 nM, respectively.

Radiometabolite analysis

[^{18}F]FEMPA showed rapid metabolism in vivo with <20% of tracer present in plasma 20 min after injection and <10% after 90 min (Figure 1 and Supplementary Figure 1 and 2). There were no statistically significant differences in the parent fraction or in the fraction of metabolites between control subjects and AD patients and between MABs and HABs (Figure 1).

Kinetic properties of [^{18}F]FEMPA

Representative SUV images and mean time-activity curves of [^{18}F]FEMPA are presented in Figures 2 and 3. In each binding group there were no statistically significant differences between controls and AD patients in kinetic parameters based on SUV data (Supplementary Table 1). However, among the AD patients the $t_{half\text{-}peak}$ was significantly lower in MABs than in HABs (p=0.008), whereas only a trend was observed in the controls (p=0.15).

PET quantification

A preliminary comparison between one tissue compartment model and 2TCM showed that 2TCM provided a better fitting of the data by visual inspection and based on Akaike

Information Criteria, therefore only 2TCM was used in the final analysis of the data (Supplementary Figure 3, Supplementary Table 2 and 3). In HABs, V_T values were significantly higher (p<0.05) in AD patients compared with controls in parietal cortex, lateral and medial temporal cortex, posterior cingulate, thalamus and cerebellum.

Representative Logan plots of [^{18}F]FEMPA are presented in Figure 4. There was a statistically significant correlation between V_T estimated with 2TCM and with Logan GA in controls (r=0.97, p<0.001) and AD patients (r=0.98, p<0.001) across all regions and subjects, with values close to the line of identity (Supplementary Figure 4). The mean COV% of V_T estimated with Logan GA tended to be lower than the mean COV% of V_T estimated with 2TCM in AD patients (p=0.05, Supplementary Table 4). Logan GA was selected for the final analysis of the data, considering the high correlation of V_T between Logan GA and 2TCM and the slightly lower COV% of V_T estimated with Logan GA in AD patients.

RM-ANOVA using Logan V_T showed a significant effect of TSPO (F=17.3, p=0.001) and a significant region*TSPO binding status interaction (F=5.2, p=0.004). The group showed only a non-significant trend (F=3.7, p=0.077). No statistically significant region*group interaction was found. However, when only HABs were included in the analysis, a significant effect of group (F=9.2, p=0.02) was observed but no statistically significant region*group interaction was found. In all subjects, if the TSPO binding status was entered as covariate, a statistically significant difference between groups was found in the medial temporal cortex (Table 2). If only the HABs were included, statistically significant differences between groups were found in lateral and medial temporal cortex, posterior cingulate, caudate, putamen, thalamus and cerebellum (Table 2). In HABs, the V_T values (mean±SD) in these regions were on average 19.5±3.0% higher in AD patients as compared with controls, ranging from 15%

higher in the lateral temporal cortex to 24% in the thalamus (Table 2, Figure 5 and Supplementary Figure 5).

Discussion

This study was designed to examine the quantification of the binding to TSPO of the novel radioligand [^{18}F]FEMPA in controls and AD patients and to evaluate whether increased TSPO binding in AD could be demonstrated in vivo. The primary outcome measure in this study was V_T, estimated using kinetic and Logan GA and the metabolite corrected arterial input function, since no reference region for TSPO is present in the brain. Since a major source of variability in V_T for all second-generation TSPO radioligands is known to come from the *rs6971* polymorphism of the TSPO [18]~~[18]~~, the binding status of the subjects was evaluated using competition assay with ^{3}H-PK11195 and PBR28. In a separate work, the binding properties of FEMPA have been tested on human brain tissue samples, known to belong to different binder subtypes, and it was found that the ratio in affinity between LABs and HABs was approximately 12 (unpublished, data), thus ~4.6 times lower than PBR28.

The main finding of this study was that increased in vivo binding of [^{18}F]FEMPA to TSPO in AD patients could be demonstrated if the binding status of the subjects was taken into account and more specifically if only HABs were included. [^{18}F]FEMPA appeared to be a suitable radioligand for in vivo TSPO quantification, displaying good brain uptake, fast wash-out from the brain and relatively fast metabolism. V_T estimated using Logan GA was in very good agreement with V_T estimated using 2TCM and showed also lower variability in both controls and AD patients.

TSPO binding status

In this study the TSPO binding status was examined in a competition assay with ^{3}H-PK11195 and PBR28. It is known that this assay provides results in agreement with the analysis of the polymorphism of the TSPO gene [21]~~[21]~~. The K_i high for the HABs ~~(2.26±0.18 nM)~~ was in good agreement with the K_i value (3.10±5 nM) ~~(3.4±0.5 nM)~~ previously reported by Owen et al. [16]~~[16]~~. The K_i high and low for the MABs ~~(1.93±0.75 nM and 189.8±14.4 nM)~~ were also in agreement with the K_i high and low values (4.0±2.4 and 313±76.8 nM) previously reported [16]~~[16]~~, although the K_i low for MABs was more in agreement with the K_i low previously reported for LABs (188±15.6 nM) [16]~~[16]~~. Although in 4 subjects the protein concentration in the assay was low, leading to a reduced signal-to-noise ratio, the V_T for HABs was approximately 2.2 times higher than the V_T for MABs, in agreement with the ratio of V_T between HABs and MABs found across all subjects, which was approximately 1.5. This ratio is also in agreement with the ratio between HABs and MABs reported for ^{11}C-PBR28 [18]~~[18]~~.

Quantification of [^{18}F]FEMPA binding to TSPO

The fast kinetic properties of [^{18}F]FEMPA compared with its analog $^{[18F]}$FEDAA1106 represent a potential advantage for its clinical use. The kinetic analysis showed that the 2TCM was a suitable model for the quantification of [^{18}F]FEMPA and that V_T estimates obtained with Logan GA were in close agreement with the 2TCM. In this study, we only observed HABs and MABs according to the in vitro binding affinity data. We attempted to estimate the V_T for a LAB, based on the results of the MABs and HABs (Supplementary Appendix 3). The estimated $V_{T\,LAB}$ was 0.57±0.08 in controls and 0.74±0.28 in AD patients. Interestingly, this value is similar to the lowest V_T value found in the AD patient that was not analysed for the binding status and that most likely corresponds to a LAB. Assuming that the non-specific binding is similar in HABs, MABs and LABs, and that $V_{ND}<V_T^{LAB}$, the binding potential

(BP_{ND}) calculated from the distribution volumes ($BP_{ND}=V_T/V_{ND}-1$) can be estimated to be at least ~2 in HABs and ~1 in MABs. Interestingly, the estimated BP_{ND} of [^{18}F]FEMPA we obtained for HABs and MABs was in agreement with the calculated BP_{ND} for [^{11}C]PBR28 recently reported by Owen et al. in a blocking study using the TSPO agonist XBD173 [22].

Increased TSPO binding in AD

We observed that in HABs the increase of [^{18}F]FEMPA binding to the TSPO was between 15% and 24%. These findings are in agreement with previous reports using either [^{11}C](*R*)-PK11195 in AD patients (approximately 20-35% increased in cortical binding as compared with controls) (*9*), [^{11}C]DAA1106 in MCI (26% increase) and AD patients (18% increase) (*13*, *14*), or [^{11}C]PBR28 in AD patients (38% increase) (*19*). Considering the relatively small sample size of this study, statistically significant increased TSPO signal in early AD was detected only after controlling for the TSPO binding status, suggesting the potential of [^{18}F]FEMPA to detect microglia activation in AD.

Additional considerations

The binding of [^{11}C]PBR28 to the TSPO has been shown to correlate negatively with the MMSE [19]. We examined the correlation of [^{18}F]FEMPA mean cortical (frontal, temporal, parietal, and occipital), limbic (medial temporal cortex and posterior cingulate) and sub-cortical (caudate, putamen and thalamus) V_T with MMSE and found a weak, non significant negative correlation (r between -0.37 and -0.41, p-value between 0.12 and 0.17) when combining data from controls and AD patients (data not shown). The lack of statistically significant correlation might be related to the limited sample size and further studies are needed to specifically examine the relationship between TSPO binding of [^{18}F]FEMPA and cognitive function in AD.

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The analysis of the PET data was conducted using only conventional ROI-based approach. Voxel-based analysis could be useful to identify differences in small areas that can be underestimated by the use of large ROIs. In this study we did not apply voxel-based analysis because of the limited sample size of both groups and to avoid possible false-positive and negative results that can be associated with small samples.

The potential application of an ^{18}F-labelled tracer in the clinical setting could be aided by the use of a simplified acquisition protocol. However, in the case of [^{18}F]FEMPA, because of the lack of a reference region in the brain the arterial input function data is needed to estimate V_T. We did not observe differences in the parent fraction between AD patients and controls, suggesting that the observed differences in V_T are indeed reflecting differences in the brain distribution of the tracer. Such differences could be detected only by measuring the brain uptake as SUV. We did observe differences in SUV between the two groups, similar to differences in V_T (data not shown), which might suggest that SUV could be used as surrogate outcome measure. However, to validate SUV as potential outcome measure in the clinical setting, additional studies with [^{18}F]FEMPA in a larger group of AD patients and controls are needed.

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Conclusions

[^{18}F]FEMPA seems to be a suitable radioligand for in vivo imaging and quantification of TSPO in early AD, provided that the TSPO binding status is determined or by including only HABs. Future studies are needed to confirm these findings in a larger cohort of AD patients.

Acknowledgments

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Framework Programme (FP7/2007-2013) under grant agreement n° HEALTH-F2-2011-278850 (INMIND). The compound [^{18}F]FEMPA is now part of the portfolio of the Piramal Imaging GmbH, Berlin, Germany. The authors thank the staff of the Turku PET Centre, the Karolinska Institutet PET Centre and the Karolinska University Hospital for technical support.

Table 1. Details of Controls and AD patients and their binding status.

Controls/AD patients	Centre	Gender	Age (y)	MMSE	Binding status	Treatment
CS1	Turku	M	66	28	HAB	n.a.
CS2	Turku	F	56	29	MAB	n.a.
CS3	Turku	M	55	30	MAB	n.a.
CS4	Turku	F	69	30	HAB	n.a.
CS5	Turku	F	71	28	HAB	n.a.
CS6	KI	M	58	30	MAB	n.a.
CS7	KI	F	71	30	HAB	n.a.
Mean±SD	64±7		29.3±1.0			
AD1	Turku	M	74	23	na	Rivastigmine 9.5mg QD
AD2*	Turku	F	56	29	na	Donepezil 10mg QD
AD3	Turku	M	69	28	HAB	Rivastigmine 9.5mg QD
AD4	Turku	M	55	25	MAB	Donepezil 10mg QD
AD5	Turku	F	67	26	HAB	Rivastigmine 9.5mg QD
AD6	Turku	F	76	24	HAB	Donepezil 10mg QD
AD7	Turku	F	67	22	MAB	Donepezil 5mg QD
AD8	KI	F	71	27	HAB	Donepezil 5 mg
AD9	KI	M	61	28	MAB	Galantamine 16 mg
AD10	Turku	M	73	23	HAB	Donepezil 10 mg QD
Mean±SD	67±7		25.5±2.5†			

*AD2: not analysed because only 10 min of blood data available

†= significantly different from Controls by two-tailed un-paired t-test, p=0.002

HAB = High Affinity Binder (9), MAB = Mixed Affinity Binder (6), LAB = Low Affinity Binder (0).

Table 2. V_T values of Logan GA in Controls and AD patients (mean ± SD) with results of ANOVA. The analysis was conducted on all subjects (HABs and MABs) and on the HAB subjects only (4 Controls, 5 AD patients). In the ANOVA conducted on all subjects, the TSPO binding status was included as covariate.

	V_T, all subjects		**Group**	**V_T, only HABs**		**Group**
Region	Controls	AD patients	*F* (*p* value)	Controls	AD patients	*F* (*p* value)
Frontal cx	1.59±0.40	1.68±0.63	1.8 (0.202)	1.85±0.12	2.12±0.23	4.4 (0.075)
Parietal cx	1.40±0.32	1.61±0.52	4.6 (0.052)	1.61±0.17	1.86±0.16	5.2 (0.056)
Lat Temp cx	1.50±0.35	1.61±0.55	2.8 (0.122)	1.72±0.10	1.98±0.17	7.0 (0.033)
Med Temp cx	1.64±0.48	1.88±0.69	5.1 (0.044)	1.95±0.12	2.36±0.18	15.2 (0.006)
Occip cx	1.46±0.35	1.60±0.52	2.3 (0.154)	1.65±0.28	1.82±0.23	1.0 (0.353)
Post Cingulate	1.62±0.44	1.86±0.67	4.1 (0.066)	1.91±0.10	2.30±0.24	9.1 (0.020)
Caudate	1.25±0.33	1.35±0.49	1.9 (0.193)	1.45±0.05	1.68±0.19	6.0 (0.045)
Putamen	1.46±0.44	1.67±0.64	3.6 (0.080)	1.73±0.10	2.10±0.23	8.9 (0.020)
Thalamus	1.64±0.48	1.89±0.75	4.1 (0.064)	1.94±0.09	2.40±0.29	9.1 (0.019)
Pons	1.70±0.50	1.92±0.80	2.8 (0.121)	2.00±0.15	2.42±0.35	5.0 (0.060)
Cerebellum	1.43±0.35	1.61±0.60	4.2 (0.064)	1.67±0.06	2.00±0.24	6.9 (0.034)
White matter	1.44±0.44	1.61±0.66	2.4 (0.146)	1.70±0.14	2.03±0.33	3.3 (0.111)

Figure Legend

Figure 1. Mean plasma parent fraction in mixed-affinity binder (MAB) and high-affinity binder (HAB) in controls and AD patients. The error bars represent 1 SD.

Figure 2. Representative SUV images of mixed-affinity binders (MAB) and high-affinity binders (HAB). Transaxial slices at the level of the basal ganglia from two control subjects and two AD patients are shown. Frames between 5 and 30 min (top row) and between 60 and 90 min (bottom row) were averaged.

Figure 3. Mean TACs from mixed-affinity binder (MAB) and high-affinity binder (HAB) Controls and AD patients in thalamus and medial temporal cortex. Error bars represent 1 SD.

Figure 4. Representative Logan plots of thalamus and medial temporal cortex from mixed-affinity binders (MAB) and high-affinity binders (HAB). Data are from the same control subjects and AD patients displayed in Figure 2.

Figure 5. Scatter plot of V_T values in control subjects and AD patients (HABs and MABs) for thalamus and medial temporal cortex. The AD patient displayed with the open square was not analysed for TSPO binding.

Conflict of interest

Ray Valencia, Marcus Schultze-Mosgau, Andrea Thiele, Sonja Vollmer, Thomas Dyrks, Lutz Lehman, Tobias Heinrich, Anja Hoffmann were employed by Bayer Healthcare, Berlin, Germany at the time of the conduction of the study.

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