TSPO imaging in stroke: from animal models to human subjects

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Short title: TSPO imaging in stroke

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Abstract

Stroke is a major health problem in developed countries and neuroinflammation has emerged over the last 2 decades as major contributor to the pathophysiological processes of brain damage following stroke. PET imaging of the translocator 18kDa protein (TSPO) provides a unique non-invasive point of access to neuroinflammatory processes and more specifically microglial and astrocytic reaction after stroke in both animal models and patients. Here we are reviewing both the experimental and clinical literature about *in vivo* TSPO PET and SPECT imaging in stroke. The studies in animal models of stroke reviewed here highlight a slightly faster time-course for TSPO expression in permanent vs. temporary stroke and a stronger activation in the infarct core in temporary stroke vs. a stronger activation in peri-infarct areas in permanent stroke. Altogether these findings suggest that areas where neuroinflammatory events occur post-stroke are at higher risk of secondary damage. The time-course of TSPO expression is slower in humans versus animal models of stroke. In human studies the TSPO expression in the peri-infarct areas peaks 3-4 weeks after stroke and increased TSPO expression is demonstrated for months after the stroke in remote areas both ipsilesional to pyramidal tracts damage and in the contralesional hemisphere. Further clinical studies are warranted to address the role of TSPO and neuroinflammation in functional recovery and reorganisation after stroke and the possible therapeutic implications. TSPO imaging appears to be a valid biomarker for demonstrating the dynamic process of neuroinflammation in stroke. But it is also clear that as the processes of microglial activation are increasingly complex, the need for new biomarkers and tracers targeting other aspect of glial reaction are needed to further investigate neuroinflammatory processes in patients.

Introduction

After being identified and initially named peripheral benzodiazepine receptors (PBR)[1,2] or peripheral benzodiazepine binding site (PBBS) and later renamed TSPO[3], the potential of PBR/TSPO as biomarkers of brain damage after stroke was rapidly identified[4,5] and taken forward in *in vivo* PET imaging[6] and exploited in subsequent studies. After being initially identified as general marker of brain damage, it quickly became clear that expression of TSPO was associated with glial reaction and/or macrophages infiltration[4,5,7]. Later on Stephenson *et al.* [8] demonstrated that activated microglial cells were the main source of parenchymal TSPO. Overall these studies paved the way to the use of TSPO as biomarker of neuroinflammation, and more specifically microglial activation and macrophages infiltration. In the early *in vivo* PET imaging studies, the tracer used for TSPO PET imaging was the [^{11}C] labelled version of one of the ligand initially used for the characterisation of the PBR/TSPO: PK11195[9]. As detailed below, the first *in vivo* post-stroke PET studies performed in the early 1990's were focusing on the characterisation of the time-course of TSPO expression to establish its potential use as biomarker of ischemic damage. In these early studies and until Shah *et al.*[10] showed that the R enantiomer of PK11195 had greater affinity for TSPO in 1994, a racemic of R/S-PK11195 was used. With the emergence, in the late 1990's, of neuroinflammation as a major contributor in stroke damage[11,12], TSPO PET imaging gained a renewed interest. Between the first study in a stroke model in baboons by Sette *et al.* [13] in 1993 and the next *in vivo* PET study in experimental stroke in 2007[14], clinical studies in stroke patients took place and are discussed in the clinical part of this review and several *ex vivo* experimental studies [8,15-19] using [^{3}H]PK11195 were performed confirming the more rapid and transient time course of glial activation in rodents than in primates. With the emergence of preclinical PET scanner dedicated to small animals and the regained interest

for neuroinflammation, TSPO imaging also saw a major effort my many groups to develop new TSPO tracers with improved characteristics when compared to [^{11}C]-(R)-PK11195, namely lower non-specific binding leading to an improved signal-to-noise ratio. Many of these experimental studies used various models of excitotoxicity as a quick and easy way of inducing microglial activation in animals to screen new TSPO tracers (for review see [20]). Following the development of series of new tracers, those were then tested in models more relevant from a clinical point-of-view than crude excitotoxic lesion, including stroke models; and from the mid 2000's stroke models were then coupled with TSPO imaging in order to *i)* better understand the time-course of microglial activation and *ii)* test new TSPO PET radiotracer in a clinically relevant model.

In vivo TSPO PET imaging in experimental stroke

In order to select references for this review, the following search was performed in PubMed: (omega* OR TSPO OR PBR OR PK11195) AND (PET OR positron) AND (brain OR cereb*) AND (stroke OR ischem* OR ischaem*) AND (rat OR mouse OR mice OR animal* OR experimental OR pig OR gerbil OR rabbit OR guinea OR primate OR dog OR cat) AND (English[lang]). This search returned 53 references, of which 27 are reviewed in the sections below. Were excluded all references using other models than stroke lesions, purely *in vitro* work (binding or autoradiographic studies) or clinical work, the latter being reviewed in a dedicated paragraph of this manuscript.

TSPO as biomarker of neuroinflammation in experimental stroke

Experimental models of stroke

Various model of experimental stroke are widely used in stroke research inducing either focal or global ischemia in the brain. Only models of focal ischemia have been used in

conjunction with TSPO PET imaging, therefore only those models are briefly discussed below, for more details on the subject and pros and cons of each model, extensive reviews on the subject are available[21-24]. Whether focal ischemia is obtained by occlusion of a major vessel such as the middle cerebral artery (MCA) or injection of micro-emboli, these models can grossly be categorised in transient and permanent which results in a different pattern of brain lesion from a morphological and time-course point of views. Injection of micro-emboli at the bifurcation between the MCA and the circle of Willis or electrocoagulation of the MCA result in permanent stroke inducing brain damages which evolve faster and are primarily hypoxic, rather than inflammatory, when compared with transient ischemia. In transient ischemia (induced by temporary occlusion through the use of a thrombin clot or a filament, by ligature of the MCA), the hypoxic early stages of the ischemia are followed by secondary damages induced by oxidative stress and inflammatory components including gliosis and infiltration of immune cells in the parenchyma. In the following paragraph we will review and compare the results of experimental stroke studies using transient and permanent models of stroke.

Transient ischemia

In a rather complex first *in vivo* PET study by Sette *et al.*[13], baboons were scanned 1 to 4 times at various time-points (1 to 91 days) after transient middle cerebral artery occlusion (MCAO) with ^{15}O-labeled $C0_2$, 0_2, and CO to map CBF and blood-volume-corrected $CMRO_2$, respectively, and with [^{11}C]PK11195 for TSPO expression and [^{11}C]flumazenil for central benzodiazepine receptor expression as marker of neuronal cell death. As each animal was not scanned at each time-point, the data points represent a trend over a population rather than a longitudinal follow-up of the TSPO expression; nevertheless these results suggest a gradual

increase in TSPO expression in infarcted and peri-infarcted areas between day 10 and day 30, before returning to baseline thereafter. Interestingly, this study showed significant increase in [^{11}C]PK11195 uptake together with decrease in [^{11}C]flumazenil binding but importantly neither of those tracer uptake were related to change in CBF or $CMRO_2$, hence demonstrating that *i)* these alterations were not just reflecting changes in perfusion and *ii)* were respectively reflecting induction of a glial response and neuronal loss in the infarct but also in areas not directly affected by the hypoperfusion. It is only much later, in the late 1990's, that new TSPO PET imaging studies will take place due to the emergence of microPET system dedicated to small animals. In such a study, Imaizumi *et al.*[14] measured [^{11}C]PBR28 uptake following bolus or bolus-infusion protocols in rats 4 and 7 days post-MCAO (60min intra-luminal MCAO) and showed increased [^{11}C]PBR28 specific binding in the peri-infarct and to less extent in the core of the infarct, a result confirmed by *in vitro* [^{3}H]PK11195 autoradiography. However, the study is underpowered (n=2 for bolus injection and n=1 for bolus-infusion protocol) and it is unclear from the methods at which time-points each protocol was performed, hence no conclusion can be made whether there was significant increase in TSPO expression between day 4 and day 7 post-MCAO. Therefore this study provides little information about the time-course of TSPO expression after stroke but demonstrates the feasibility of PET imaging in rodent after stroke. In a more comprehensive study, Rojas *et al.*[25] described more precisely the time course of TSPO expression following experimental stroke (60min intra-luminal MCAO) showing no significant increase in [^{11}C]-(R)-PK11195 uptake until 4 days post-MCAO with a further increase 7 days post-MCAO, results which were confirmed by [^{3}H]PK11195 autoradiography, TSPO PCR and immunohistochemistry analysis. In this study the authors also showed that CD11b (Ox42) (a cell surface protein expressed by activated microglia and infiltrated macrophages) positive cells were mostly responsible for TSPO expression although

in the peri-infarct region some activated astrocytes also over-expressed TSPO. Interestingly, the authors noticed that the expression by microglia/macrophages and astrocytes was heterogeneous, in term of localisation and time-course. From day 4, TSPO expression was found in amoeboid microglia/macrophages located in the core of the infarct and in the peri-infarct region although these cells were less abundant in the peri-infarct region supporting the observation of lower [^{11}C]-(R)-PK11195 uptake or TSPO immunostaining in this region. In the peri-infarct region the predominant cell type is reactive astrocytes of which some were found positive for TSPO immunostaining. Overall, these observations demonstrate a differential expression of TSPO depending on the cells type and the phenotype and level of activation or of these cells. The relation between cell types, level of activation and over-expression of TSPO is still unclear, but it however seems to be associated with phagocytic activity (erythrophagocytosis in this case) which is consistent with the amoeboid phenotype of the microglia/macrophages found in the ischemic core. In two consecutive studies Martin *et al.*[26,27] used one of the new TSPO tracer: [^{18}F]DPA-714 to thoroughly investigate TSPO expression after experimental transient ischemia in rats (2h intra-luminal MCAO). These authors followed TSPO expression between 1 and 30 days post-MCAO and demonstrated that TSPO expression increased significantly from day 4 and peaked at day 11 post-MCAO and gradually decreased thereafter but remained significantly higher than baseline up to 21 days post-MCAO[28], results that were confirmed by autoradiography. Interestingly, they demonstrated by immunohistochemistry that between day 4 and day 11, microglia/macrophages were the main cell populations responsible for TSPO over-expression and that thereafter the number of TSPO-positive decreased while the number of TSPO-positive astrocytes gradually increased. The expression of TSPO by microglial cells was already well established and later confirmed by Hughes *et al.* [29] showing a significant correlation

between the localisation and amplitude of [^{11}C]-(R)-PK11195 uptake and Ox42 (CD11b) immunostaining 14 days after 45min distal MCAO. Similarly, the possible expression of TSPO by astrocytes[28] was reported in fewer publication[25,7] but was later more specifically confirmed by others[30]. In a more recent study using a new model of remote temporary MCAO[31], Toth *et al.* [32] demonstrated using [^{11}C]PBR28 that TSPO expression peaked in the infarct core slightly earlier (day 4 post-ischemia) in their model than the previous reports, although there was no evidence of a significant difference in TSPO level between day 4 and 7 post-stroke, with again expression of TSPO in microglia (peak at day 4) and then slightly later on in astrocytes (peak at day 7). Altogether these publications describe a common time-course and pattern of microglial cell activation and TSPO expression (Figure 1). Despite the studies by Rojas *et al.*[25] and Toth *et al.*[32] indicating that TSPO expression is concomitant or precedes an increase in phagocytic activity, the precise function of TSPO in microglia and astrocytes and the reason for this shift in expression remain to be fully understood. This question of the relation between TSPO expression and function of glial cells has become quite essential if TSPO PET imaging is going to be used as read-out for therapeutic efficacy of drugs that modulate microglia activity or neuroinflammation in stroke and other neurodegenerative diseases. For example, Martin *et al.*[26] demonstrated that minocycline (an antibiotic which is able to reduce microglial proliferation) was able to decrease TSPO expression measured by [^{18}F]DPA-714 PET but not infarct size. This finding is partly in contradiction with a more recent study[33] which showed no effect of minocycline on [^{11}C]-(R)-PK11195 uptake nor infarct size, although it must be noted that this study used a model of permanent stroke by microembolism rather than a temporary MCAO model[26]. These two studies suggest that the overall inhibition of microglial proliferation might not be the best strategy when compared to other approaches targeting specific cytokines[34-40], at least in the context of

stroke. However, the study by Martin *et al.*[28], together with the clinical study by Dodel *et al.*[41], still support the use of TSPO PET imaging as a valid biomarker of microglial proliferation and/or activation for therapeutic read-out. The study of Wang *et al.*[42] illustrates this point further as they used [^{18}F]DPA-714 PET imaging to assess the anti-inflammatory effects of the drug AMD3100 (a specific antagonist of the chemokine receptor 4 (CXCR4)) following 60min of intraluminal MCAO in mice. Despite the challenges of microPET imaging in mice, the authors confirmed in mice a similar time-course of TSPO expression observed in rats. However, one can note that the amplitude of the changes measured in mice are lower than those observed in rats (ipsi- to contralateral ratio <2 in mice and 4.6 in rats[43]), the explanation for this, outside possible difference between rat and mouse model, are likely to be the consequence of partial volume effect affecting more severely mouse than rat data quantification. Nevertheless, these authors were able to show a decrease in TSPO expression at 3 days post-stroke in the AMD3100-treated group. Overall, these studies using inflammatory modulators demonstrate that TSPO PET imaging is a suitable tool to monitor anti-inflammatory therapies.

Permanent ischemia

In a model of permanent stroke by microembolism (injection of macrospheres of 315-355µm in diameter in the internal carotid artery), Schroeter *et al.*[44] used a combination of [^{18}F]FDG uptake modelling and [^{11}C]-(R)-PK11195 imaging to determine three region of interest (ROI): infarct core (decreased cerebral blood flow (CBF) and metabolism), infarct margins (decreased CBF but unchanged metabolism) and peri-infarct (increased metabolism and increased [^{11}C]-(R)-PK11195 uptake). Whether the increased metabolism in the peri-infarct zone was purely due to increased metabolic demand from neurons or a combination

of neuronal activity and glial proliferation remains unclear, however together with the presence of neuroinflammation it is likely to indicate an increased risk to secondary inflammatory damage for the brain tissue in this area. Fukumoto *et al.*[45,46] further investigated, in two multi-tracer studies, using [^{11}C]-(R)-PK11195 and [^{11}C]flumazenil for central benzodiazepine receptor (CBR) and [^{18}F]FDG for glucose metabolism[45] or [^{18}F]BMS-747158-02 (Mitochondrial Complex-1 ligand)[46], the time-course and localisation of TSPO expression after permanent stroke (photothrombotic model). These authors found that [^{11}C]-(R)-PK11195 uptake matched in localisation the hypermetabolism detected in the peri-infarcted area by [^{18}F]FDG, whereas a decrease in [^{18}F]flumazenil[45] and [^{18}F]BMS-747158-02 [46] uptake could be detected in the infarct core. In term of time-course, they showed that TSPO expression was low 1 day post-stroke[46] and increases progressively to peak around day 7 post-ischemia and decrease thereafter (day 14)[45]. In another study, Walberer *et al.*[47] pushed further the time-course assessment of neuroinflammation and TSPO expression by imaging rats at 2, 7, 14, 21, 42 days and 7 months after photothrombotic stroke. They confirmed the known pattern of preferential expression of TSPO in the peri-infarcted region and lower in the infarct core increasing from day 2 up to day 7, decreasing thereafter. They however demonstrated that neuroinflammation as detected by [^{11}C]-(R)-PK11195 PET and *ex vivo* measurement, appeared at later time-point (7 months post-stroke) in the ventrolateral part of the thalamus, an area remote of the infarct and in which perfusion and [^{18}F]FDG uptake appeared normal. These findings are in agreement with *ex vivo* data reporting retrograde degeneration of thalamic nuclei several months after permanent stroke in rats[48,49]. In a follow-up study using the macrosphere model, the same group demonstrated overlap between over-expression of TSPO and infiltration of Ultrasmall SuperParamagnetic Iron Oxide (USPIO)-loaded macrophages (to image phagocytic activity)

mostly in the infarct margins whereas the peri-infarct zone and sites of secondary injury (ventrolateral thalamus) were only positive for [^{11}C]-(R)-PK11195 at day 7 post-stroke. By following animals at 28 and 56 days post-stroke, their study suggest that areas positive for USPIO-loaded macrophages alone or USPIO-loaded macrophages and [^{11}C]-(R)-PK11195 at 7 days post-stroke were not viable at later time-point whereas areas positive only for [^{11}C]-(R)-PK11195 remains viable.

Following the general consensus from all the studies described above, it is interesting to note that the preferential localisation of the TSPO over-expression in the peri-infarct area reported by Imaizumi *et al.*[14] after transient ischemia matches the results of the studies using permanent stroke rather than transient stroke. This suggests, in the absence of perfusion data in the study of Imaizumi *et al.*[14], that reperfusion might have been poor or inexistent leading to *i)* a different profile of microglial activation (i.e. higher in the rim of the infarct rather than the core) and *ii)* prevented tracer diffusion in the core of the infarct, hence explaining the higher uptake the peri-infarct area observed by Imaizumi *et al.*[14].

Validation of new TSPO PET radiotracers in stroke models

In other studies, stroke models were used not so much to investigate neuroinflammatory processes but to test or validate new TSPO radiotracers. As mention before, this field did regain much interest over the last decade due to the renewed interest for neuroinflammation in stroke and various neurodegenerative diseases and the availability of dedicated animal PET scanners. In such a study, Yui *et al.*[50] tested two new compounds, [^{18}F]-FEAC and [^{18}F]-FEDAC, in rat post-MCAO. The authors showed that the uptake ratios between ipsilateral and contralateral sides for [^{18}F]-FEAC and [^{18}F]-FEDAC were respectively 3.03 and 2.76 twenty minutes after injection, and the BP_{ND} values were 1.70±0.19 for [^{18}F]-

FEAC and 1.37±0.06 for [^{18}F]-FEDAC. In 2 related studies, the same authors investigated another compound, [^{11}C]DAC[51,52], and demonstrated the importance of using high specific activity (SA) in small animals in order to get the best signal-to-noise ratio, as illustrated by the differences in ipsi- to contralateral ratio ranging from 4.62±0.15 for high SA, 2.94±0.43 for middle SA, and 2.87±0.63 for low SA. Although, in all these studies, the authors did not compare these 2 tracers with [^{11}C]-(R)-PK11195, the ipsi- to contralateral ratio obtained with [^{18}F]-FEAC and [^{18}F]-FEDAC are similar or even lower to those obtained with [^{11}C]-(R)-PK11195 in other studies, whereas those obtained with [^{11}C]DAC at high SA are better than those typically obtained with [^{11}C]-(R)-PK11195 [25,53,54]. It must be noted however than these studies[25,53,54], the SA of [^{11}C]-(R)-PK11195 was not as high as those used by Yui *et al.*[51,52] for [^{11}C]DAC. In their studies, Yui *et al.*[51,52] also confirmed the preferential localisation of the highest TSPO tracer uptake after temporary stroke in the core of the infarct and the time course of TSPO expression with progressive increase from day 4 to 7 post-stroke in rats. This issue of high/low SA should been given some consideration most particularly when using mice as the amount (i.e. in nmol) of tracer can more easily reach high level of receptor occupancy in mice due to the small size of the animals as suggested for other tracers[55,56]. One must however note that both sensitivity (~8-10% efficiency) and resolution (~1-1.8mm) of preclinical PET scanners have dramatically when compared with those reported (~4% and 3mm) by Hume *et al.*[56]. In a similar study, Lartey *et al.*[57] evaluated the potential of [^{18}F]PBR06 in mice following 30min of intraluminal MCAO and found that [^{18}F]PBR06 could specifically image TSPO in stroke although the amplitude of the difference between ipsi- and contralateral ROI was much smaller than observed in rats with other tracers, including [^{11}C]-(R)-PK11195. This could obviously be due to difference in the sensitivity of the tracer but other factor such as partial volume effect due to the small size of

the mouse brain are probably also having a significant impact on the results. One of the major issue of the studies afore mentioned is that they assess new tracer without comparing them with the reference tracer [^{11}C]-(R)-PK11195 which they are trying to supersede. While many tracer validation studies have done comparisons with [^{11}C]-(R)-PK11195 using mostly excite-toxic lesion models (for review see [20,58]), in more recent studies it appears that direct comparisons (i.e. animals scanned twice, once with [^{11}C]-(R)-PK11195 and once with the tracer to be tested) is essential when using more clinically relevant, but more variable, models such as stroke. For example and as described above, Martin *et al.*[28] provided evidence that [^{18}F]DPA-714 was a suitable tracer for TSPO imaging in stroke (see paragraph above); they did not however provided direct evidence of its superiority *vs* [^{11}C]-(R)-PK11195. We later showed[53] that [^{18}F]DPA-714 provided a better contrast (ipsi- to contralateral ratio) than [^{11}C]-(R)-PK11195 only when comparing animals that had been scanned with both tracer sequentially, whereas we were able to obtain a similar results using the less variable model of intrastriatal injection of AMPA and unpaired scans[59]. Taking into account our previous experience[53], we used this more robust study design to later evaluate [^{18}F]GE-180[54]. Overall, both [^{18}F]DPA-714 and [^{18}F]GE-180 provided better contrast than [^{11}C]-(R)-PK11195, with the most interesting feature being a significantly reduced non-specific (contralateral) uptake when compared to [^{11}C]-(R)-PK11195, supporting both tracers as strong candidate for TSPO imaging. Although not comparing their new tracer, [^{11}C]MBMP, in the same animals than [^{11}C]-(R)-PK11195, Tiwari *et al.*[60] were able to show a significant improvement in ipsi-to contralateral ratio of [^{11}C]MBMP uptake when compared with [^{11}C]-(R)-PK11195 of similar amplitude (1.5 fold improvement) to those obtained with [^{18}F]DPA-714[53] and [^{18}F]GE-180[54] using the same model of stroke. In a more recent publication[61], the same group tested a [^{18}F]-labelled derivative of MBMP, [^{18}F]FEBMP, in the same intraluminal stroke model

(30min) but did not perform a direct comparison with [^{11}C]-(R)-PK11195 and only compared their results with their previous study[60]. They found that the BP_{ND} of [^{18}F]FEBMP was significantly higher than that of [^{11}C]MBMP and [^{11}C]-(R)-PK11195 previously reported[60] (BP_{ND}: [^{18}F]FEBMP: 2.72±0.27, [^{11}C]MBMP: 2.03±0.24, [^{11}C]-(R)-PK11195 : 1.59±0.33). More interestingly, and although performed only in 4 subjects by *ex vivo* autoradiography, they showed that [^{18}F]FEBMP is as [^{11}C]-(R)-PK11195 insensitive to the TSPO polymorphism in term of binding. This issue of high, mixed and low affinity binders[62-64] has slowed down and complicated dramatically the implementation in clinical settings of new TSPO tracers. The [^{18}F]FEBMP binding data in human subject needs confirmation in a larger study as well as in an *in vivo* PET study in patients, but if confirmed [^{18}F]FEBMP may have great potential as TSPO tracer.

TSPO imaging in human subjects

Material

Eleven studies have been published from 1992-2014 presenting human TSPO data in 65 patients after ischemic stroke[65-75]. In two separate studies[74,73] there was an overlap in the patient material with two subsequently published articles by the two research groups[72,71]. The mean age ± SD of patients was 64 ± 16 years (range 19-88 years). The studies included 24 females and 35 males. Gender was not reported in one study[68]. The vascular territories involved were the middle cerebral artery (MCA) territory (57 patients), the posterior cerebral artery (PCA) territory (6 patients)[68,75,72,66], and the anterior cerebral artery (ACA) territory (1 patient)[72]. One study reported an ischemic stroke involving the brain stem[68]. Assessments for clinical stroke severity were not consistently reported. In the study by Gerhard et al.[66] neurological signs were rated 1-3 days after stroke in five patients

to the Scandinavian Stroke Scale (SSS) (median 34, mean 32.7, sd 8.4, range 19-36, no impairment is 58/58). Thus, the stroke severity was moderate. In the study by Price et al. [69] neurological signs were rated on admission in four patients to the National Institutes of Health Stroke Scale Score (NIHSS score) (median 12, mean 13, sd 4.3, range 9-19, no impairment 0/42). Thus, the stroke severity was minor to moderate. In the studies by Radlinska et al. [70] and Thiel et al. [71] NIHSS test scores (median 2, mean 2.5, SD 1.9, range 0-7) and limb motor function (arm subsection of Rivermead Motor Function Test (RMFT)) (median 13, mean 10.7, sd 4.9, range 1-15, no impairment 15/15) "in the acute post-stroke phase" were reported. Thus, the overall stroke severity was minor but arm function ranging from no impairment to severely impaired. Two studies included a total of six patients with transient ischemic attacks as control subjects[70,71]. The time since debut of symptoms to PET scan is not reported. Two studies included a total of five healthy controls[69,75].

Longitudinal studies were performed in 25 patients: three patients were studied three times and 22 patients were studied two times[65,66,68,69,71,75]. Thus, a total of 93 scans were conducted using positron emission tomography (PET, n = 85) and single photon emission computed tomography (SPECT, n = 8). Patients were scanned from two days to 2 years after stroke. Forty-five patients were scanned with [^{11}C]-(R)-PK11195[65-71,73], 9 patients with [^{11}C]vinpocetine[72] (4 of these patients were also examined with PET and[^{11}C]-(R)-PK11195[73]), 9 patients were scanned with [^{18}F]DPA-714[74] and 6 patients were scanned with [^{123}I]CLINDE[75]. In three articles the main purpose was to explore the suitability of newly developed second generation tracers for TSPO imaging: [^{11}C]Vinopocetine[73], [^{18}F]DPA-714[74] and [^{123}I]CLINDE[75].

Methodological considerations

Quantification of binding parameters was done very differently. In the study by Ramsay et al. [65] the [^{11}C]-(R)-PK11195 outcome parameter was calculated as the tissue radioactivity from 40-60 min post-injection relative to the integral (0-50 min) of radioactivity in metabolite-corrected plasma. In the study by Gerhard et al. [66] the [^{11}C]-(R)-PK11195 outcome parameter was calculated as the tissue radioactivity from 30-40 min relative to the corresponding area of the same size on the contralateral, unaffected side of the brain. Radlinska et al. [70] and Thiel et al. [71] used a similar approach but the tissue radioactivity from 20-60 min was preferred. In the [^{18}F]DPA-714 paper by Ribeiro et al. [74] both approaches was used with a contralateral hemispheric region and a ipsilateral cerebellar region to define the reference region. In the papers by Gerhard et al. [68] and Price et al. [69] parametric images of regional [^{11}C]-(R)-PK11195 binding was generated using a simplified reference tissue model[76] using the cerebellar hemisphere ipsilateral to the stroke to define the reference region. Pappata et al. [67] used a similar approach but a white matter region in the contralateral hemisphere to define the reference region. In the [^{11}C]vinpocetine studies by Gulyás et al. [72] the percentage standardized uptake values % (%SUV) was used as main outcome parameter to recognize the difficulties in using cerebellum as a reference region in [^{11}C]-(R)-PK11195 studies. %SUV is calculated as the radioactivity in the target relative to the total injected radioactivity per body weight x 100%. In the paper by Feng et al. [75] 2-tissue compartment modeling with metabolite corrected arterial plasma was used.

In the study by Price et al. [69] significant differences between [^{11}C]-(R)-PK11195 binding potential with and without cerebral blood volume correction were not observed in patients between 2 and 30 days after stroke. Ribeiro et al. [74] described an increased uptake

of [^{18}F]DPA-714 beyond areas of MRI gadolinium enhancement in patients 1-2 weeks after stroke. Modeling of the impact of blood-brain-barrier (BBB) disruption on TSPO binding has been described in a longitudinal study in patients with traumatic brain injury[^{11}C]-(R)-PK11195-PET scanned 7-10 days, 1 and 6 months after trauma[77]. Analysis using plasma input model showed an increased variability of K_1/k_2 across the brain in patients 7-10 days after trauma compared to healthy volunteers. This variation was interpreted as representing disruption of the BBB but comparisons to MRI with gadolinium were not done. In studies on TSPO binding in patients with glioblastoma multiforme only a limited overlap between areas of increased TSPO binding and areas with extravasation of gadolinium was demonstrated and K_1 was not significantly different[75,78]. Several studies describe the hematogeous uptake of macrophages (supermagnetic iron oxide particles) as a marker of cellular neuroinflammation to be independent of BBB disruption in patients 1-2 week after stroke[79,80].

The binding affinity of second generation TSPO tracers to TSPO is significantly affected by the rs6971 polymorphism[81,82]. Genotyping was performed in the [^{123}I]CLINDE study [75] but not done in the [^{18}F]DPA-714 study[74] and the [^{11}C]vinpocetine studies [72,73].

Volumes of interest (VOIs) were mostly delineated in the co-registred MR and PET/SPECT images. The ischemic core VOI, assumed to represent not-viable tissue after stroke, was delineated in T1-weighted images. The peri-infarct zone VOI was defined as the high-binding region in the PET/SPECT scan ipsilateral to the stroke minus the overlap with the ischemic core VOI. Remote VOIs was mirrored to the contralateral hemisphere and VOIs were delineated in the brainstem, cerebellum and pyramidal tracts (PT).

Results

Since binding data are quantified very differently and quantitative regional data not consistently reported it is not possible to lump data together for overall analyses. Several studies have reported regional TSPO changes in the first week after stroke[66,68,72,69,70,65,71]. Very subtle increases in TSPO binding are present already 2-3 days after stroke and located in the periphery of the MR stroke volume [69,68,70,71]. Within the first week the volume of increased tracer binding increases substantially and reaches a maximum in the ischemic core zone in the second week[69]. In the peri-infarct zone tracer binding increases in the second week[66,68] and is highest in the third and fourth week[65,69]. The absence of longitudinal studies in single subjects that compare tracer binding in the third and fourth week with tracer binding in the second month after stroke impede a more precise determination of peak binding in the peri-infarct zone. The TSPO binding in the peri-infarct zone was clearly present but reduced 150 days after stroke compared to 28 after stroke (figure 2)[68]. In the large study by Thiel et al. [71] uptake ratios in the infarcts was significantly reduced from week 1-3 to 6 months after stroke. Six months after stroke uptake ratios in the infarct were not significantly different from controls (patients with transient ischemic attacks). The range of the uptake ratios six months after stroke appears larger than in controls indicating more variability in patients with possible clinical significance. In the study by Feng et al. [75] TSPO binding normalized in the peri-infarct zone in two patients 187 and 252 days compared to the scans in the fourth week after stroke.

Increases in TSPO binding in areas remote to the immediate surroundings of the infarct have been demonstrated in several studies[67-71,75]. Pappata et al. [67] was the first to describe TSPO binding in thalamus ipsilateral and in some subjects contralateral to the

lesion between 2 and 24 months after stroke. However, interpretations to be made from the study are impeded by the absence of longitudinal studies and/or a control material. Between 3-14 days Gerhard et al. [68] did not see remote effects. Gerhard et al. [68] demonstrated the different time-course of changes in TSPO binding in thalamus and pons ipsilateral and contralateral to the lesion. An increased TSPO binding in the thalamus and pons was present ipsilateral to the infarct zone 28 days after stroke and spreading to the contralateral thalamus and pons in the same patient 150 days after stroke (figure 2). These remote changes in TSPO signal are hypothesized to represent Wallerian degeneration along pathways connecting different anatomical areas[68]. Radlinska et al. [70] tested this hypothesis by dividing stroke patients into two groups with (n = 11) and without (n = 5) subcortical infarcts affecting the pyramidal tract (PT) as verified by MRI. Patients were examined with PET between 2-20 days after stroke. Uptake ratios were not significantly different in the infarct comparing the PT group and the non-PT group. Uptake ratios at the level of pons, midbrain and internal capsule were only increased in the PT group. No changes were detected retrograde to the stroke in the oval center. In the study by Thiel et al. [71] two additional patients were studied compared to the Radlinska et al. [70] study and follow-up data on 16 patients 6 months after the stroke were added (figure 3). The authors reported that significantly increased uptake ratios in remote areas persisted in the PT group at follow-up and correlated with initial PT damage as measured by Diffusion Tensor Imaging (DTI) in the same tract portion. The uptake ratios in the infarct at the first PET-scan correlated with anterograde PT damage at follow-up. The existence of increased TSPO binding in the contralesional cortex has been reported in two studies within the first month following stroke[69] and 287 and 252 days after stroke[75]. A note of caution concerning the use of uptake ratios and reference regions for TSPO

quantification is necessary. It may be a source of bias if TSPO binding is increased in remote areas including contralesional hemisphere and cerebellum.

The studies by Radlinska et al. [70] and Thiel et al. [71] are the only studies reasonably dimensioned to test TSPO binding in relation to clinical outcome. The authors found no significant correlation between RMFT scores (arm function subsection) and infarct size or TSPO uptake in the infarct, pons or midbrain ratio 2-20 days after stroke but a significant negative correlation between RMFT and uptake ratio in the internal capsule[70]. When controlling for damage to PT (fractional anisotropy (FA)) the authors demonstrated a significant positive partial correlation between initial (2-20 days after stroke) remote TSPO binding and RMFT six months after stroke and a trend toward a negative partial correlation between initial TSPO binding in the infarct and RMFT[71]. This indicates that initial TSPO binding in the infarct influence outcome negatively while initial TSPO binding in remote areas influence outcome positively.

Conclusion and future perspectives

Since 1992 TSPO imaging in stroke has been reported concomitantly in animal models and in human subjects. The most consistent finding across animal models and clinical studies has been the demonstration of temporal changes in TSPO expression in infarcted areas following stroke. TSPO expression is very subtle in the first 2-4 days after stroke and increases for 7-14 days in animals and at least 3-4 weeks in humans. In humans TSPO binding in the peri-infarcted areas is subject to individual variation but appear to normalize in most subjects approximately 6 months after stroke. A differential pattern of localisation of the TSPO expression between permanent and transient ischemia has been noticed in animal models of stroke with higher TSPO expression in margins or peri-infarct areas after permanent ischemia

whereas the highest TSPO expression are found in the core of the infarct after transient ischemia. From pathophysiological point of view, this observation is in agreement with the different dynamic of events that will take place after permanent vs transient ischemia. Without reperfusion in the core of the infarct following permanent occlusion, the main areas where neuroinflammation will and can take place are the margins and the peri-infarct zones. Whereas in transient ischemia, the reperfusion will drive increased oxidative stress, activation and proliferation of the resident microglia and to some extent, depending on the severity of the stroke, infiltration of macrophages and neutrophils[83,11,28,26]. Increased TSPO binding in areas remote to the immediate surroundings of the infarct appears weeks and months after stroke in patients most consistently reported in relation to the ipsilateral pyramidal tract suggested to reflect Wallerian degeneration but also in contralesional regions in the thalamus, brainstem and cortices. Similarly, in animal TSPO expression is detected in areas remote of the infarct (mostly the ventrolateral part of the thalamus) several months after permanent ischemia, this is thought to be due to retrograde degeneration of thalamo-cortical connections.

What are the functional implications of the spatial and temporal variation in TSPO expression after stroke? Is an increased TSPO expression in the peri-infarct zone or remote areas at a given time point after stroke detrimental or beneficial to stroke recovery? So far only two human studies based upon the same patient material addressed questions of clinical significance and failed to demonstrate robust correlations between TSPO binding in the first weeks after stroke and motor function at baseline and at follow-up after 6 months. Only when controlling for permanent tract damage (based upon DTI studies at follow-up) using partial correlations a significant positive correlation between initial brain stem TSPO binding and

follow-up motor function was demonstrated and a trend toward a negative correlation between initial TSPO binding in the cortical infarction area and motor outcome at 6 months.

TSPO imaging studies clearly demonstrate that TSPO expression regionally in the brain after stroke is a highly dynamic process with spatial and temporal characteristics that varies between patients. Indeed, more clinical studies addressing the functional implications of TSPO imaging in stroke is warranted. Future TSPO imaging studies are likely to benefit from the new TSPO tracers with higher target to background ratios and should aim at performing quantification of TSPO binding using validated quantitative methods and for second generation TSPO tracers implement information about genotype in the data analysis. Combining TSPO imaging studies in stroke with task-based functional MRI and DTI tractography may provide new insights into the relation between microglial activation and the functional organisation of the residual systems and may facilitate our understanding of how microglia is involved in the change of structure and function after stroke. From a clinical point of view it is of great interest whether TSPO imaging at a given time point after stroke may help in predicting the capacity of an individual for further functional recovery after stroke. Inherent to this approach is testing the effect of drugs targeting neuroinflammatory processes including microglial and astrocytic reaction after stroke and testing the effect of different therapeutic approaches ranging from regional brain stimulation (like repetitive transcranial magnetic stimulation) to conventional physiotherapy. From an experimental point of view, TSPO imaging combined with other modalities such as MRI, multiple PET tracers, DTI allow a better understanding of neuroinflammatory processes occurring post-stroke through longitudinal studies in animal, hence increasing the power of the statistical analysis while decreasing the number of animal needed. Only by understanding better the

pathophysiology of stroke and the role of neuroinflammation in stroke, we will be able to design therapeutic strategies aiming at inhibiting the deleterious events of neuroinflammation while supporting repair and remodelling also done by glial cells. From that perspective, it also appears that *i)* we need to better understand what TSPO functions in glial cells are and what the expression of TSPO means in term of phenotype of glial cells, and that *ii)* we will also need new biomarkers and tracers to be able to image non-invasively in animal and in patients other events that TSPO imaging does not give us access to. To some extent, progress are being made with imaging of astrogliosis with monoamine oxidase B (MAO-B) tracer [^{11}C]-deuterium-L-deprenyl[84,85] and cannabinoid type 2 (CB2) receptors[86], although results with the CB2 receptors post-stroke have been disappointing[87]. Several molecules (cytokines and chemokines receptors, adhesion molecules expressed at endothelial level, receptor specific to the immune response or activation of glial cells such as P2X7 receptor, toll-like receptors, etc.) are all potential candidates, but whether developing tracers for them is doable or whether they will prove difficult remains to be determine and is certainly a work under progress.

Compliance with Ethics Guidelines

Hervé Boutin and Lars H. Pinborg declare no conflicts of interest. The article contains data from studies with human and animal subjects performed by the authors of this article. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. All institutional and national guidelines for the care and use of laboratory animals were followed.

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Figure legends:

Figure 1: schematic representation of the time-course of TSPO expression by activated microglia/infiltrated macrophages and astrocytes after transient (**A**) and permanent brain ischemia (**B**) (adapted from [25,32,27](A) and [45,47](**B**), highlighting the slightly earlier and shorter expression pattern following permanent ischemia when compared with transient ischemia. It must be noted that in transient ischemia the highest values of TSPO expression are found in the core of the infarct whereas they are found in the peri-infarcted area after permanent ischemia. Values were estimated from the data available in each publications either in the text or graphs, normalised to the peak value, and averaged across all studies. Mean±1SD values illustrate the variability at each time-point across studies.

Figure 2: [11C](R)-PK11195 PET and MRI scans for a patient at different time points: (a–h) show transverse (a–d) and coronal (e–h) MRI and PET sections through the brain of a patient who was scanned 28 and 150 days after the ischemic stroke. (a) and (e) show the T1-weighted MRI after 28 days and (b) and (f) the [11C](R)-PK11195 BP map co-registered to the MRI. In (c) and (g), the same transverse and coronal planes with the [11C](R)-PK11195 BP map co- registered to the MRI are shown after 150 days. At 28 days, the [11C](R)-PK11195 PET and T1-weighted MRI hypointensity cover a similar area while after 150 days the PET lesion has expanded to the ipsi- and contralateral thalamus. (d) and (h) demonstrate the evolving atrophy in this patient 150 days after the ischemic stroke as seen by MR subtraction imaging. The image colors are calibrated for BP values from 0 (dark blue) to one (red); values N1 appear white. Extracerebral binding has been masked. Reprinted from Gerhard *et al.* [68] with permission from Elsevier.

Figure 3: (A) Microglial activation in a patient with small subcortical infarct and good recovery. Initial activated microglia in infarct decreased over 6 months (white arrows), whereas microglial activation in brain stem persisted (red arrows). DTI showed decreased FA primarily in infarct (blue arrows) and less along tract at level of cerebral peduncles (yellow arrows). (B) Patient with complete transection of PT and poor recovery. Microglial activity in infarct decreased but still persisted after 6 mo (white arrows), as did activity in brain stem (red arrows). FA decreased in area of infarct (blue arrows) and along tract in cerebral peduncle (yellow arrows). Microglial activity in patient in whom PT was not affected (C) decreased over 6 mo (white arrows). No tracer uptake at level of brain stem was observed, and FA along tracts was not decreased. This research was originally published in JNM by Thiel *et al.*[71]. Copyrights by the Society of Nuclear Medicine and Molecular Imaging, Inc.

Author contribution

Hervé Boutin: Literature search and review, manuscript writing and editing.

Lars H. Pinborg: Literature search and review, manuscript writing and editing.

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Figure 1

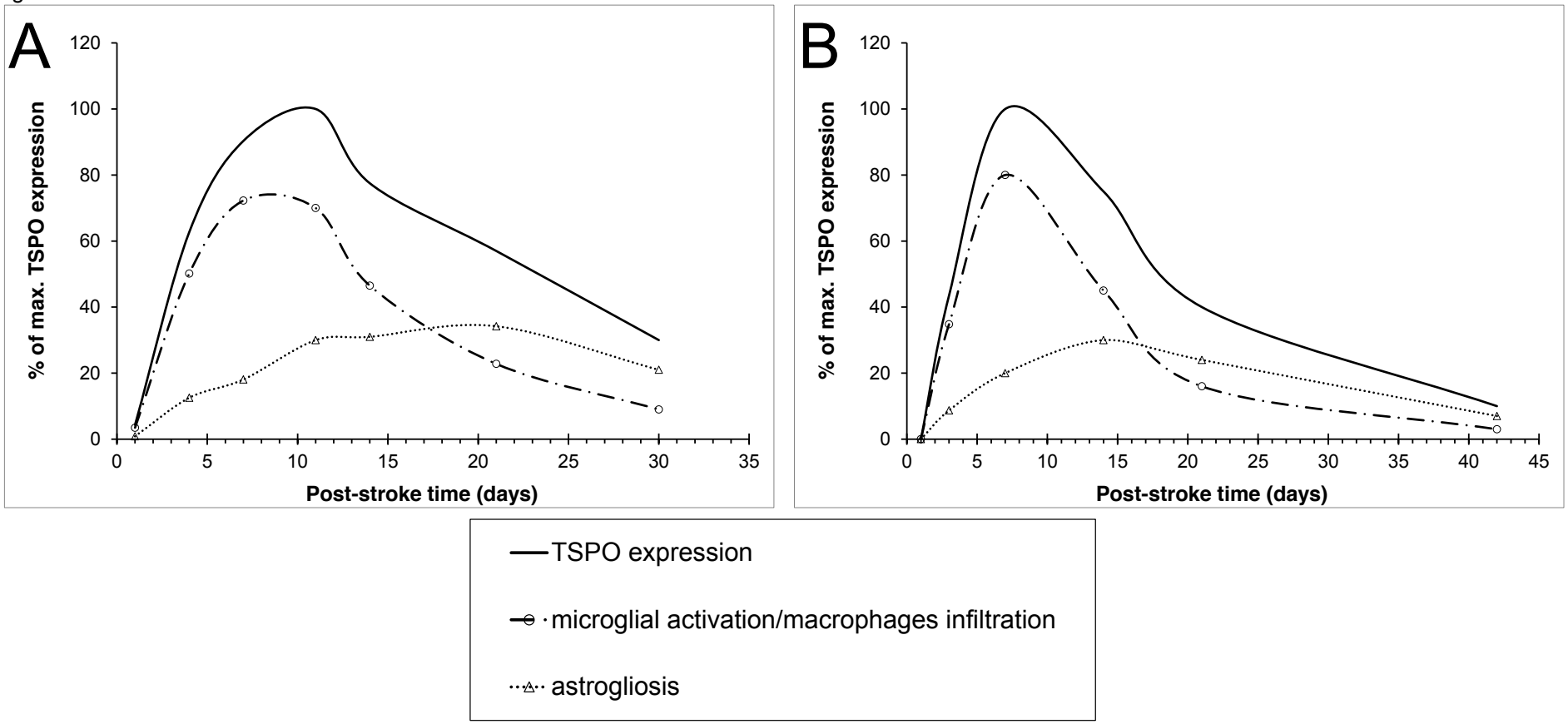
A
B
% of max. TSPO expression
Post-stroke time (days)
TSPO expression
microglial activation/macrophages infiltration
astrogliosis

Figure 2
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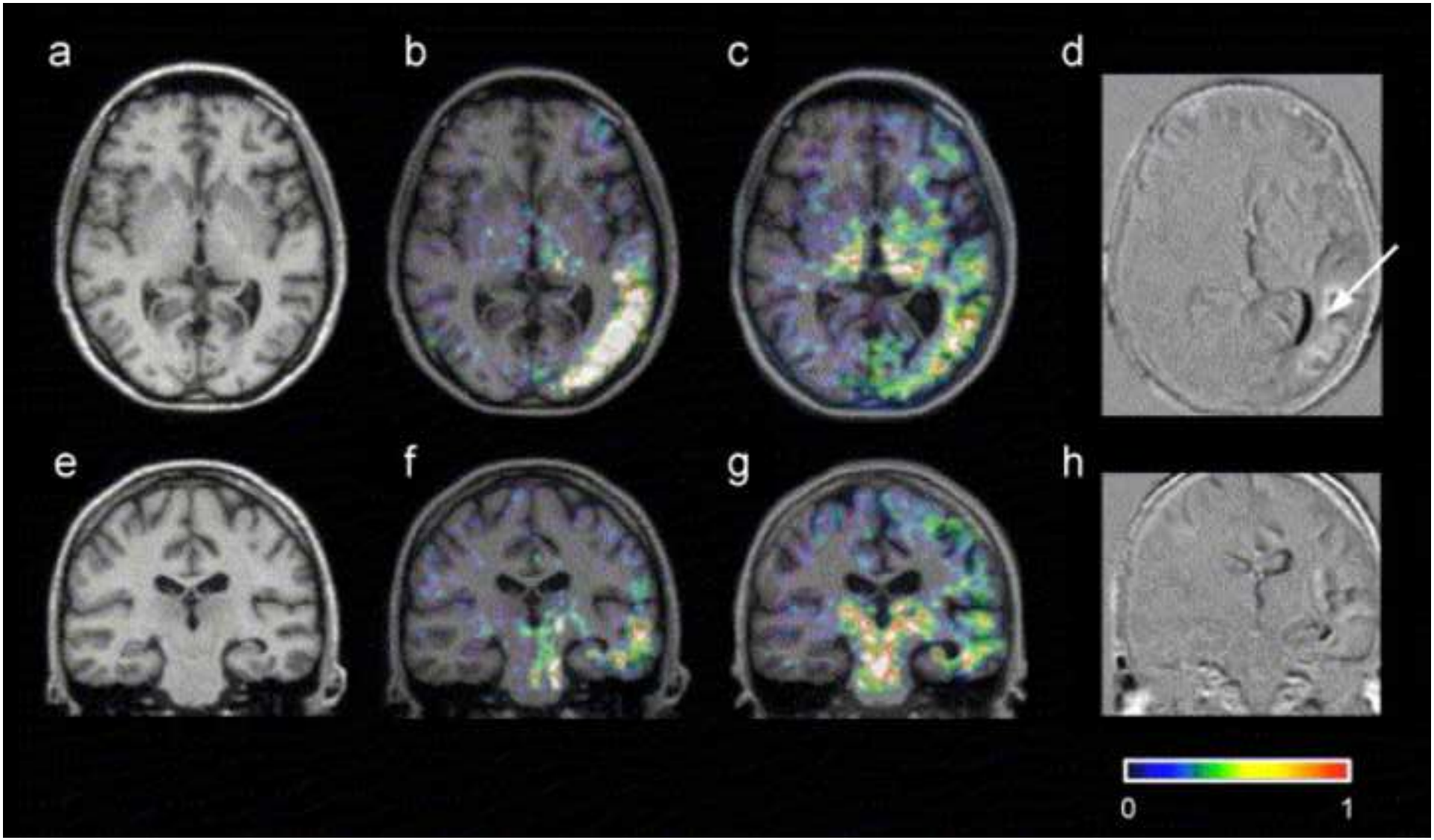

Figure 3
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