King's College London

King's Research Portal

DOI:
10.3389/fneur.2020.541377
10.3389/fneur.2020.541377

Document Version
Peer reviewed version

Link to publication record in King's Research Portal

Citation for published version (APA):
Wright, P., Veronese, M., Mazibuko, N., Turkheimer, F. E., Rabiner, E. A., Ballard, C. G., Williams, S. C. R., Hari Narayanan, A. K., Osrah, B., Williams, R., Marques, T. R., Howes, O. D., Roncaroli, F., & O'Sullivan, M. J. (2020). Patterns of Mitochondrial TSPO Binding in Cerebral Small Vessel Disease: An in vivo PET Study With Neuropathological Comparison. *Frontiers in Neurology*, *11*, 541377. Article 541377. https://doi.org/10.3389/fneur.2020.541377, https://doi.org/10.3389/fneur.2020.541377

Citing this paper
Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights
Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

•Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
•You may not further distribute the material or use it for any profit-making activity or commercial gain
•You may freely distribute the URL identifying the publication in the Research Portal

Take down policy
If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 14. Jan. 2025

frontiers

Patterns of mitochondrial TSPO binding in cerebral small vessel disease: an *in vivo* PET study with neuropathological comparison

Paul Wright[1*], Mattia Veronese[1*], Ndabezinhle Mazibuko[1], Federico E Turkheimer[1], Eugenii A Rabiner[1,2], Clive G Ballard[3], Steven CR Williams[1], Avinash Kumar Hari Narayanan[4], Bahiya Osrah[4], Ricky Williams[4], Tiago R Marques[5], Oliver Howes[5], Federico Roncaroli[4#], Michael J O'Sullivan[1,6,7#†]

[1]Department of Neuroimaging, IoPPN, King's College London, London, UK

[2]Invicro, London, UK

[3]University of Exeter Medical School, Exeter, UK

[4]Division of Neuroscience and Experimental Psychology, Faculty of Biology, Medicine & Health, University of Manchester and Manchester Centre for Clinical Neuroscience, Salford Royal Foundation Trust, UK

[5]Department of Psychosis studies, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK

[6]University of Queensland Centre for Clinical Research, Brisbane, Australia

[7]The Royal Brisbane and Women's Hospital, Herston, Australia

** Share first authorship; # Share senior authorship*

[†] **Correspondence:**
Michael J O'Sullivan
m.osullivan1@uq.edu.au

Keywords: Immunohistochemistry, Microglia, PET, TSPO, Small Vessel Disease

Abstract

Small vessel disease (SVD) can be associated with cognitive impairment in older age and be implicated in vascular dementia. Post-mortem studies show proliferation of activated microglia in the affected white matter. However, the role of inflammation in SVD pathogenesis is incompletely understood and better biomarkers are needed. We hypothesized that expression of the 18kDa translocator protein (TSPO), a marker of microglial activation, would be higher in SVD. Positron emission tomography (PET) was performed with the second-generation TSPO ligand [11C]PBR28 in 11 participants with SVD. TSPO binding was evaluated by a two-tissue compartment model, with and without a vascular binding component, in white matter hyperintensities (WMH) and normal-appearing white matter (NAWM). In post-mortem tissue, in a separate cohort of individuals with SVD, immunohistochemistry was performed for TSPO and a pan-microglial marker Iba1. Kinetic modeling showed reduced tracer volume and blood volume fraction in WMH compared with NAWM, but a significant increase in vascular binding. Vascular [11C]PBR28 binding was also increased compared with normal-appearing white matter of healthy participants free of SVD. Immunohistochemistry showed a diffuse increase in microglial staining (with Iba1) in sampled tissue in SVD compared with control samples, but with only a subset of microglia staining positively for

36 TSPO. Intense TSPO staining (including perivascular macrophages) was observed in the vicinity of
37 damaged small blood vessels. The results suggest an altered phenotype of activated microglia, with
38 reduced TSPO expression, in the areas of greatest white matter ischemia in SVD, with implications
39 for the interpretation of TSPO PET studies in older individuals or those with vascular risk factors.

40 ## 1 Introduction

41 Cerebrovascular small vessel disease (SVD) is increasingly recognised as an important cause of age-
42 related cognitive impairment and dementia. The term defines a heterogeneous group of hereditary
43 and sporadic conditions that impair the brain microcirculation (Iadecola, 2013). Neuropathological
44 findings in SVD affect parenchymal and leptomeningeal small perforating arteries, arterioles,
45 capillaries and, less commonly, small veins and venules (Charidimou et al., 2016). The radiological
46 manifestations include focal lacunar infarcts and ill-defined lesions, hyperintense on T2-weighted
47 MRI. These abnormalities occur in both deep white matter, with relative sparing of subcortical U-
48 fibres, and the deep grey matter of the thalamus and striatum. Diffuse lesions in white matter are
49 often referred to as diffuse white matter hyperintensities (WMH) or leukoaraiosis (Hachinski et al.,
50 1986). Histologically, diffuse lesions correspond to areas of rarefaction of myelin (Fazekas et al.,
51 1993), enlargement of perivascular spaces but general sparing of the neuropil. Hallmark pathology of
52 small vessels in these regions includes endothelial proliferation, thickening and splitting of the walls,
53 small plaque-like accumulations of plasma proteins, perivascular reactive astrocytosis and
54 accumulation of perivascular macrophages (Alafuzoff et al., 2012). Increasingly, interest has shifted
55 towards a critical role for the neurovascular unit in the pathogenesis of SVD (Hassan et al., 2003),
56 with evidence for compromise of the blood-brain barrier (BBB) and cerebral blood flow regulation
57 (Rosenberg, 2009; Holland et al., 2015). This has reignited interest in inflammation, as regulation of
58 immune responses is a cardinal function of the neurovascular unit and blood-brain barrier.

59 Neuropathological evidence supports a role for inflammation in the pathogenesis of SVD. Activated
60 microglia are abundant in areas of damaged white matter, more so than in areas of morphologically
61 unaffected control white matter. Microglia cells show the morphology of activated cells, which
62 suggests immune response and a role in antigen presentation, possibly in response to BBB disruption
63 and extravasation of plasma proteins (Simpson et al., 2007). Epidemiological evidence also suggests
64 an association between systemic inflammation and WMH extent (Sjogren et al., 2001). Microglial
65 activation is associated with increased metabolic activity and mitochondrial biogenesis. The latter
66 leads to enhanced expression of the mitochondrial 18kDa translocator protein (TSPO) (Albrecht et
67 al., 2016). This alteration in protein expression provides a possible molecular marker of microglial
68 activation, which has been exploited by positron emission tomography (PET) studies, using
69 radioligands to investigate microglial activation *in vivo* in the human brain. PET studies in a number
70 of neurodegenerative and neuroinflammatory diseases have shown higher TSPO expression,
71 consistent with *post-mortem* studies showing microglia activation (Jacobs et al., 2012). Moreover,
72 PET imaging studies suggest that focal lacunar infarction, a feature of SVD, initiates microglial
73 activation (Radlinska et al., 2009). However, few data are available on TSPO in the diffuse,
74 progressive form of SVD (Evans et al., 2017). The role of inflammation in diffuse SVD has
75 important therapeutic implications, as WMH progression is associated with cognitive decline and risk
76 of dementia. New treatment avenues to retard progression of diffuse SVD are therefore urgently
77 needed.

78 This study utilized the second generation (Owen et al., 2014) tracer [^{11}C]PBR28 to investigate TSPO
79 binding patterns in individuals with SVD. Based on *post-mortem* observations, we hypothesized that
80 [^{11}C]PBR28 binding would be higher within WMH than normal-appearing white matter (NAWM).

This is a provisional file, not the final typeset article

81 We included a group of participants with symptomatic lacunar stroke within the last 12 months, to
82 investigate whether acute infarction modulated inflammation within SVD. We compared these results
83 with a dataset of [^{11}C]PBR28 brain PET scans from healthy controls. To facilitate interpretation of
84 PET binding results, we also investigated *post-mortem* brains of subjects with neuropathologically-
85 confirmed SVD.

86 2 Methods

87 2.1 SVD participants

88 SVD was defined as the presence of confluent or near confluent (corresponding to Fazekas grade 2 or
89 above) WMH on T2-weighted MRI scans of the brain. Participants were defined as 'asymptomatic' if
90 they had no history of stroke or TIA and were free of cognitive and gait symptoms. Asymptomatic
91 SVD participants were recruited from the local community. Symptomatic SVD patients (individuals
92 with a history of lacunar stroke in the last year) were recruited from a cohort enrolled in the
93 longitudinal STRATEGIC study of cognitive function after stroke (registered with ClinicalTrials.gov
94 as https://www.clinicaltrials.gov/show/NCT03982147). All participants were aged over 50 and fluent
95 in English; we excluded those with large artery infarcts, diagnosis of dementia, active malignancy,
96 major neurological or psychiatric illness (as defined by DSM-IV-TR), previous moderate to severe
97 head injury (Mayo clinic classification of severity) or lack of capacity to consent. Those invited to
98 participate in the PET study all had high or medium TSPO binding status based on Ala147Thr
99 polymorphism genotyping (Fazekas et al., 1993; Owen et al., 2012). In total, six asymptomatic SVD
100 participants and five stroke patients underwent PET (Table 1).

101 The study was approved by the Bromley Research Ethics Committee (ref: 13-LO-1745) and was
102 conducted in accordance with the Declaration of Helsinki. All participants provided written,
103 informed consent.

104

105 [INSERT TABLE 1 HERE]

106

107 2.2 [^{11}C]PBR28 PET imaging

108 Radiopharmaceutical preparation of [^{11}C]PBR28 was performed as previously described (Owen et
109 al., 2014) and the imaging protocol was adopted from previous [^{11}C]PBR28 PET studies (Veronese et
110 al., 2018). Briefly, an initial low-dose computed tomography (CT) scan was acquired for attenuation
111 and scatter correction using a Siemens Biograph™ True Point™ PET/CT scanner (Siemens Medical
112 Systems, Germany). Subjects then received a bolus injection of [^{11}C]PBR28 (injected dose mean±SD
113 349±10 MBq) followed by a 90-minute PET emission scan. PET data were acquired in three-
114 dimensional mode and binned into 26 frames (durations: 8 x 15 s, 3 x 1 min, 5 x 2 min, 5 x 5 min, 5 x
115 10 min). Images were corrected for attenuation and scatter and reconstructed using filtered back
116 projection.

117 In parallel to the PET acquisition, arterial blood was sampled from the radial artery using a combined
118 automatic (from 0 to 15 minutes after tracer injection) and manual approach (samples collected at 5,
119 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 minutes) in agreement with the experimental protocol used
120 in previous publication (Bloomfield et al., 2016). From these blood samples, a time-continuous

121 metabolite-free plasma input function was derived for each individual to describe the tracer delivery
122 to the brain by using Multiblood software (https://github.com/MatteoTonietto/MultiBlood) (Tonietto
123 et al., 2019). PET scans started at a similar time of day to reduce potential effects of circadian rhythm
124 on TSPO density (between 10.00 am and 3.30 pm) (Collste et al., 2016). Cumulative scanner
125 movement, defined as the sum of total frame-to-frame movement during imaging acquisition, was
126 15.2±4.6 (mean±SD) mm and none of the participants showed interframe motion spikes > 5mm. Free
127 plasma fraction (f_p) ranged from 1.4% to 2.7% (mean±SD: 1.8%±0.4%). These numbers are
128 qualitatively comparable with the historical archive of dynamic [11C]PBR28 PET studies
129 (Bloomfield et al., 2016; Nair et al., 2016; Veronese et al., 2018).

130 **2.3 Magnetic Resonance Imaging (MRI)**

131 MRI sequences were acquired using an MR750 3.0 Tesla MR scanner (GE Healthcare, Little
132 Chalfont, Buckinghamshire, United Kingdom). We selected sequences to meet the STandards for
133 ReportIng Vascular changes on nEuroimaging (STRIVE) (Wardlaw et al., 2013). T1-weighted scans
134 were acquired with Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence with
135 repetition time of 7.312ms, echo time of 3.016ms and a flip angle of 11°. Images were acquired in
136 the sagittal plane covering the whole head with field of view (FOV) of 270 x 270 mm and matrix size
137 of 256 x 256 voxels. Slice thickness and slice gap were 1.2 mm. T2-weighted fast recovery fast spin
138 echo (FRFSE) and fluid-attenuated inversion recovery (FLAIR) sequences were acquired to delineate
139 infarcts and other vascular lesions. The FRFSE sequence used a TR of 4380 ms, TE of 54-65 ms and
140 flip angle of 90-111°. The FLAIR sequence used a TR of 8000 msec, TE of 120-130 msec and flip
141 angle of 90-111°. Images were acquired in the axial plane with FOV of 240 x 240 mm and matrix
142 sizes of 320 x 256 and 256 x 128 voxels for FRFSE and FLAIR respectively. Slice positions were
143 aligned for both sequences with 72 slices at 2 mm thickness for FRFSE and 36 slices at 4 mm
144 thickness for FLAIR.. Perfusion measurements were obtained from an arterial spin labelling
145 acquisition. Pseudo-continuous arterial spin labelling (ASL) MRI was obtained (geometry of 1.875 x
146 1.875 x 3 mm and a post-labelling delay of 2525 ms). One patient (P2) was unable to have a research
147 MRI scan and clinical MR images obtained at 1.5T with a comparable FLAIR acquisition were used
148 to define WMH. An additional patient did not have ASL during their research scan, leaving nine out
149 of eleven patients with ASL scans.

150 **2.4 Image processing and analysis**

151 All PET images were corrected for head movement by realigning all the PET frames to a single
152 "reference" space identified by the PET frame with the highest activity as implemented in
153 MIAKAT™ (http://www.miakat.org). Regional time-activity curves were obtained by sampling the
154 dynamic PET image with predefined regions of interest (ROIs). ROIs delineating WMH were drawn
155 manually on FLAIR images by a single rater (PW). White matter was defined using automated
156 segmentation of T1-weighted images in SPM12. NAWM ROIs were defined by subtracting WMH
157 voxels from segmented white matter. For comparison with the post-mortem tissue analysis (see
158 below), a striatum ROI was defined using the Hammersmith atlas, combining the caudate, putamen
159 and nucleus accumbens (Hammers et al., 2003).

160 MR images and their ROIs were co-registered with a reference PET image for each subject to allow
161 analysis of tracer uptake within specified ROIs (Figure 1). Using SPM12 (Functional Imaging
162 Laboratory, University College London, UK), the FLAIR images were co-registered with the high
163 resolution T1-weighted images, which were in turn co-registered with the reference PET image. The
164 ASL images were co-registered with the T1-weighted images in PET space.

This is a provisional file, not the final typeset article

165 ASL images were processed to generate maps of cerebral blood flow (CBF) and a matched proton
166 density weighted image as described previously (Modinos et al., 2018). The proton density images
167 show anatomical features in the same geometry as CBF and were used for co-registration to PET
168 space.

169

170 [INSERT FIGURE 1 HERE]

171

172 Quantification of [^{11}C]PBR28 tissue distribution was performed using the standard 2-tissue
173 compartmental modelling (2TCM) and 2-tissue compartmental model with the inclusion of vascular
174 binding (2TCM-1K) (Rizzo et al., 2014). Both models have been used with [^{11}C]PBR28 PET for
175 TSPO tissue quantification in controls and patients (Bloomfield et al., 2016; Rizzo et al., 2019) and
176 tested in TSPO specific blocking studies (Owen et al., 2014; Veronese et al., 2018). In a principally
177 vascular disease, modelling the vascular compartment could provide useful disease-relevant
178 information, therefore the 2TCM-1K approach had potential advantages for clinical application to
179 SVD. Note that these kinetic models share most of the micro-parameters with the exception of K_b
180 which is explicitly used in 2TCM-1K to account for the vascular TSPO component. Inclusion of an
181 explicit vascular compartment allows the model to account for two possible confounding factors in
182 SVD: reduced tissue blood flow; and binding of TSPO by the endothelium (so that the tracer remains
183 within the vascular compartment). Both blood flow and altered blood-to-tissue extraction are
184 modelled by the K_1 parameter. In addition to K_b, the total volume of distribution of the tracer in
185 tissue (V_T, ml/cm^3), the blood-to-tissue tracer transport constant (K_1, ml/g/cm^3) and the blood-to-
186 tissue volume fraction (V_b, no units) were considered main parameters of interest. A full description
187 of the model kinetic parameters and their mathematical identifiability is reported in original
188 references (Bloomfield et al., 2016).

189 **2.5 Healthy control participants**

190 A dataset of [11C]PBR28 brain PET scans from healthy individuals was obtained from an
191 institutional PET repository NODE (Maudsley Biomedical Research Centre, London, UK). This
192 dataset was used to compare the [^{11}C]PBR28 PET signal in the NAWM and WMH of the SVD
193 participants with healthy WM tissue. Twenty-one healthy controls (age: 38±15 years, gender: 15
194 males/6 females, 3 MABs/18 HABs) were included in this analysis. Radiotracer synthesis,
195 experimental protocol, image acquisition and reconstruction, analysis pipeline and software were
196 consistent across cohorts (Bloomfield et al., 2016). To confirm that the control participants were free
197 of SVD, T1-weighted MR images were inspected, to confirm the absence of diffuse white matter
198 hypointensities or focal lesions > 4 mm in the basal ganglia (Fazekas et al., 1993; Wahlund et al.,
199 2001).

200 **2.6 Post-mortem tissue analysis**

201 Five *post-mortem* brains found to contain neuropathological evidence of sporadic SVD, were
202 selected from the Manchester Brain Bank cohort (ethics: 09/H0906/52+5). The subjects' age ranged
203 between 71 and 97 years; four were female. The individual samples were selected for relatively pure
204 vascular pathology: all had low levels of tau-related pathology consistent with Braak & Braak stage
205 II and low amyloid load (Thal phase III) (Braak and Braak, 1991; Thal et al., 2002; Braak et al.,
206 2006). *APOE* allele was ε3/ε3 in all subjects. None of the brains demonstrated amyloid angiopathy.

207 Cases with watershed infarcts and laminar necrosis were excluded. From the coronal slices stored in
208 10% buffered formalin, we resampled the striatum at the levels of the septum and nucleus
209 accumbens, and at the level of anterior commissure. The demographics and essential clinical
210 information of the five subjects are reported in Table 1.

211 Two control brains from individuals of comparable age were supplied by the UK Parkinson's Society
212 Brain Bank at Imperial College, London, UK (ethics: 18/WA/0238). They were selected from a
213 cohort of over 900 brains using the following stringent criteria: mild SVD, absence of α-synuclein
214 and TDP-43 inclusions, tau Braak stage I-III and low Thal Aβ phase. None of the subjects had
215 previous history of stroke or cerebrovascular disease. Post-mortem delay for all cases was less than
216 48 hours. Similar to SVD cases, samples of the basal ganglia were taken at the levels of septum and
217 anterior commissure. The H&E-stained sections were reviewed to confirm the diagnosis;
218 immunostains for α-synuclein, Tau, Aβ peptide, p62 and TDP-43 were also available for review. The
219 two control donors were males aged 71 and 77 years at the time of death. The cause of death was
220 malignancy in both cases; brain metastases were not present (Table 1).

221 **2.7 Tissue analysis and quantification of microglia/macrophage density and TSPO expression**

222 The tissue samples were routinely processed over three days on a Shandon Citadel 1000 Processor
223 and embedded in molten Histoplast Paraffin wax using a ThermoFisher HistoStar embedding station.
224 Ten consecutive sections number from 1 to 10 were cut at 5 μm from each block on a Shandon
225 Finesse 325 microtome. Section 1 was stained with hematoxylin-eosin (H&E). Sections 2 and 3 were
226 used for immunohistochemistry. Dewaxing, rehydration and antigen retrieval was performed using a
227 Ventana BenchMark Ultra and the reagents supplied by the manufacturer as per the standard
228 preprogramed protocol (Ventana Medical Systems, Roche Group, Tucson, AZ, USA). The anti-
229 ionized calcium binding adaptor molecule 1 (Iba1) polyclonal antibody (Wako, 019-19741) for
230 microglia and macrophages was used at the dilution of 1:5000 and incubated for 32 minutes. The
231 anti-TSPO polyclonal antibody (Abnova, PAB7095) was used at the dilution of 1:250 for 60 minutes.
232 Nuclei were counterstained with hematoxylin and both post counterstained in bluing reagent for 4
233 minutes.

234 **2.8 Quantification of Iba1 and TSPO immunostains**

235 Immunostains were scanned at the Bioimaging Facility at University of Manchester
236 (www.bmh.manchester.ac.uk/research/facilities/bioimaging) using 3D Histech Pannoramic 250 slide
237 scanner (3D Histech ltd, Hungry). Anatomical landmarks were outlined on the digital images and
238 region of interest for quantification were randomly chosen by the 3D Histech program. The regions
239 were selected in the head of caudate, anterior and posterior putamen, globus pallidus and anterior and
240 posterior limbs of the internal capsule. Iba1 and TSPO positive microglia were counted at the
241 magnification of x20 in 60 ROIs overall in each SVD case and control brain. The accuracy of
242 anatomical margins was validated by an experienced neuropathologist (FR). All images from ROIs
243 were then imported to ImageJ using the Kurt De Vos cell counter (https://imagej.NIH.gov/ij, USA)
244 for postproduction editing and evaluation.

245 Two separate automated counting macros coded in JavaScript were run on ImageJ to count the
246 percentage of area occupied by positive staining signal of TSPO or Iba1 in each ROI (See appendix
247 for JavaScript). Each image was separated into the three RGB channels by applying the Color
248 Deconvolution tool using the H DAB vector. The red channel, showing the oxidized DAB brown
249 precipitate indicating positive immunohistochemistry staining for TSPO or Iba1, was selected and
250 converted to a binary image. The threshold was adjusted to minimize background staining artefacts

This is a provisional file, not the final typeset article

251 and applied consistently across each ROI. The percentage area occupied by the positively stained
252 signal indicated by black was calculated with the Analyze Particle tool (Supplementary Figure 1).
253 Each of the two automated counting macros for TSPO and Iba1 were applied uniformly across all
254 ROI following a quality control assessment comparing manual counts with the automated macros for
255 each stain. Double blinded validation was also carried out independently by three co-authors (RW,
256 BO and FR) with multipoint tagging on ImageJ. Only microglial cells with a clearly identifiable
257 nucleus were counted.

258 **2.9 Statistical Analysis**

259 Statistical analyses were performed using SPSS Statistics version 25 (IBM UK Ltd., Portsmouth,
260 UK). For the imaging data analysis, differences in kinetic parameter estimates between the modelled
261 ROIs were tested using paired t-tests. Comparisons between groups were tested using independent
262 sample t-tests. Equality of variances was tested using Levene's test and correction applied to the
263 degrees of freedom for comparisons where this assumption was not met. Data points were excluded if
264 the values were extreme (more than three interquartile ranges from the edge of the interquartile
265 range) or physiologically implausible (K_b values >1 or close to 0 min^{-1}). Exclusions were made on a
266 by-ROI basis, with data points for all parameters for a given ROI being excluded if one parameter
267 was invalid. For the post-mortem study, the effect of SVD on microglia/macrophage density and Iba1
268 expression was tested using two-way analysis of variance (ANOVA). Given the low number of
269 subjects, each region was considered a separate measure, with region and group as fixed effects.

270 **3 Results**

271 **3.1 TSPO PET binding in lesions and NAWM**

272 The main PET findings were consistent between 2TCM-1K and 2TCM models. Therefore, unless
273 otherwise stated, the results below refer to 2TCM-1K (for a full comparison with the 2TCM, please
274 see Supplementary Material).

275 [^{11}C]PBR28 V_T was lower in WMH than in NAWM ($t(10) = 5.76$, $p < .001$). Similarly, the blood
276 volume fraction (V_b) was also reduced in WMH compared with NAWM ($t(10) = 6.39$, $p < .001$). The
277 plasma-to-tissue tracer transport kinetic constant (K_1) was also reduced in WMH ($t(10) = 8.29$, $p <$
278 $.001$). In contrast, vascular TSPO binding (K_b) was higher in WMH than in NAWM ($t(10) = -3.24$, p
279 $< .01$). There was no evidence that TSPO binding differed between symptomatic and asymptomatic
280 individuals. Reduction in V_b and K_1, as well as increase in K_b were confirmed when WHM was
281 compared to WM tissues from healthy controls (V_b t(20) = 5.04, p < .001; K_1 t(20) = 2.29, p = .033;
282 K_b t(15.3) = 2.52, p = .023, equal variances not assumed). Individual data points are shown in Figure
283 2.

284

285 [INSERT FIGURE 2 HERE]

286

287 **3.2 Regional CBF analysis**

288 Regional CBF was reduced in WMH versus NAWM (mean± SD 31.4± 3.8 vs 43.0± 4.5mL/100
289 g/min, $t(8) = 16.07$, $p < .001$, Supplementary Figure 3). There were no significant correlations

between rCBF and any of the kinetic parameters in either WMH or NAWM (Pearson's $|r| <= .55$, $p >= .12$).

3.3 Neuropathological assessment

The five *post-mortem* cases showed features of severe SVD according to the criteria proposed by Skrobot et al (Skrobot et al., 2016). (Figure 3). One of the brains showed a microinfarct in the anterior putamen that was excluded from tissue sampling. The distribution and density of microglial cells in striatum and anterior and posterior limbs of the internal capsule was similar across the five cases. Of microglia positively stained with Iba1, a mean of only 23% also stained positively for TSPO (Table 2, Figure 4). The fraction of TSPO-positive microglia was similar in all regions examined. The breakdown of values in each region is shown in Table 2 and individual values are presented in Figure 5. TSPO was expressed in the endothelium as normally seen in vessels whereas expression was considerably reduced or absent in the tunica media of vessels with fibrosis of their walls. When present, perivascular macrophages were TSPO-positive whereas no detectable TSPO expression was present in perivascular astrocytes.

[INSERT FIGURE 3, FIGURE 4 AND TABLE 2 HERE]

Perforating arteries in the two control brains showed thin walls, only minimal widening of perivascular spaces and mild perivascular astrocytosis. The mean proportion of Iba1 stained microglia also staining positively for TSPO was 63%. The ratio of TSPO to Iba1 density differed significantly between groups ($F(1,15) = 5.79$, $p = .029$) but not regions ($F(2,15) = 2.49$, $p = .116$). This effect appeared to be driven by an increase in Iba1 density in SVD tissue ($F(1,15) = 7.93$, $p = .013$), while TSPO density did not differ between groups ($F(1,15) = 0.25$, $p = .623$).

For comparison with the neuropathological assessment, we modelled PET TSPO tracer binding in the striatum. There were no significant differences between participants with SVD and healthy controls in the striatum with either the 2TCM or the 2TCM-1K model. Of the 11 participants with SVD, only three had one or more lesions >4 mm in the striatum, with one accompanied by widespread smaller lesions. These three participants are distinguished in Figure 2 and do not appear to differ from the rest of the SVD group.

[INSERT FIGURE 5 HERE]

4 Discussion

Expression of TSPO, often increased in the presence of activated microglia (Pannell et al., 2019), was reduced in WMH in comparison with normal-appearing white matter. In contrast, TSPO binding in the vascular compartment was higher in WMH, relative to both normal-appearing tissue in SVD and white matter of healthy individuals free of SVD. Immunohistochemistry in *post-mortem* brain tissue showed a higher number of Iba1-positive microglia in SVD, but a reduction in the proportion of

This is a provisional file, not the final typeset article

328 TSPO-positive microglia. In and around affected small vessels, TSPO expression was found in vessel
329 walls and perivascular macrophages, consistent with the PET binding results. These results suggest
330 an alteration of the phenotype of activated microglia in ischemic WMH, in which microglial
331 activation is uncoupled from TSPO expression. Furthermore, they suggest that TSPO can provide
332 potentially useful information about vascular and perivascular pathology in SVD.

333 A previous *post-mortem* analysis of microglial staining, with careful alignment of histological
334 sampling and imaging abnormalities, was performed in the MRC-CFAS neuropathology cohort
335 (Simpson et al., 2007). Activated microglia were found to be abundant in regions affected by WMH
336 and present at lesser abundance in normal-appearing white matter. However, in the MRC-CFAS
337 study, an activated microglial phenotype was inferred from expression of HLA-DR;
338 immunohistochemistry of TSPO was not performed. In the present study, histological analysis of
339 post-mortem white matter confirmed that microglia are abundant in SVD (Figure 4). However, many
340 of these microglia stained negatively for TSPO (Figure 5). One possible explanation is that the
341 microglial phenotype – and particularly the upregulation of mitochondrial biosynthesis – is altered in
342 the ischemic conditions of visible areas of injury in SVD. Relative ischemia, compared with normal-
343 appearing white matter, was confirmed experimentally in WMH in the patients investigated with
344 PET. Conceivably, microglial activation without enhanced expression of TSPO occurs because
345 regions of WMH are chronically hypoperfused, so that the role of oxidative phosphorylation, and
346 thereby mitochondrial proliferation, in the microglial response is diminished. Alternatively,
347 microglial phenotype may change as tissue injury and repair enters a more chronic phase (i.e. in
348 contrast to acute ischemic injury). Little is known about the role of activated microglia at a late stage
349 remote from injury. Persistent activated morphology could reflect either ongoing tissue remodeling
350 or recurrent immune challenge, for example from cells or molecules that cross a compromised blood-
351 brain barrier. If there is a *chronic* activated microglial phenotype, this might be less tightly coupled to
352 mitochondrial biosynthesis and metabolism. Altered tracer dynamics might have also influenced the
353 pattern of results. The decreases in the modelled tracer transport suggest exactly this. However, even
354 after modelling these effects, there is still an apparent reduction in TSPO binding. Overall, the results
355 point not to a reduction of inflammatory response in WMH, but rather to an altered phenotype of
356 activated microglia, with reduced TSPO expression.

357 We found no evidence that the pattern of TSPO binding was different in individuals with
358 symptomatic stroke in the year leading up to PET. The number of individuals in each subgroup was
359 small so these results must be interpreted with caution. However, the lack of an obvious difference in
360 the scatter plots in Figure 2 argues against the contention that neuroinflammation is limited to those
361 with recent symptoms.

362 Our findings would fit with a view of progression of SVD whereby initial increases in TSPO
363 expression are followed by a chronic phase in which decreased TSPO accompanies worsening
364 hypoperfusion and increased damage to neurons. Imaging studies have shown that leakage of plasma
365 proteins begins early in SVD and creates an inflammatory microenvironment that sustains and
366 maintains tissue damage (Fu and Yan, 2018). Macrophages and activated microglia release proteases,
367 reactive oxygen species (ROS) and reactive nitrogen species that can attack the blood vessel walls,
368 extra cellular matrix and myelin (Rosenberg, 2017; Fu and Yan, 2018). Increased TSPO in microglia
369 initially protects brain tissue from high levels of ROS (Guilarte et al., 2016). During the course of the
370 disease lower TSPO in microglia and macrophages can reflect a progressive reduction of
371 inflammatory response (Mulugeta et al., 2008) but conversely can be instrumental in maintaining
372 tissue damage given its role in the resistance against ROS cytotoxicity. In addition, ROS and nitric
373 oxide intermediates produced by activated microglia are effective in damaging mitochondria and the

374 resulting mitochondrial dysfunction can cause further downregulation of TSPO. Low microglial
375 TSPO in SVD could therefore reflect a chronic 'toxic state' in which microglia-induced ROS exceeds
376 antioxidant defenses with subsequent injury of neurons, ECM and vessel walls (Guilarte et al., 2016).

377 Interestingly, the differences in tracer kinetic modelling parameters between WHM and NAWM were
378 consistent with both standard 2TCM and 2TCM-1K (see Supplementary Material). The latter
379 includes an additional term that separates TSPO tracer binding within parenchymal and vascular
380 compartments. Modelling [^{11}C]PBR28 with 2TCM-1K has shown several advantages compared to
381 standard 2TCM. Firstly, 2TCM-1K leads to a better and more efficient data description (improved fit
382 and lower Akaike coefficient) compared to standard 2TCM in both healthy individuals and patients
383 with CNS diseases (Rizzo et al., 2014; Bloomfield et al., 2016). Secondly, the 2TCM-1K is more
384 sensitive to changes in affinity as demonstrated by its higher sensitivity to changes in rs6971
385 polymorphism (Rizzo et al., 2014). Finally, the 2TMC-1K has a stronger agreement with TSPO
386 mRNA expression than the 2TCM – this was demonstrated both in terms of binding data at baseline
387 (Rizzo et al., 2014) and in terms of displacement after TSPO blocking (Veronese et al., 2018). The
388 work presented here extends existing literature by showing how 2TCM-1K can also be used to
389 investigate TSPO distribution at the intact and disrupted vascular interface. Irrespective of the type of
390 model used for the tracer quantification, both 2TCM and 2TCM-1K showed a reduction of blood
391 volume (V_b) and blood to tissue tracer transport (K_1) in WHM as compared to NAWM and WM
392 tissues in healthy controls, so that the main PET results are consistent across models.

393 Explicit modelling of the vascular compartment led to the striking finding of an increase in the
394 vascular tracer binding constant, K_b, in WMH compared to NAWM and healthy white matter.
395 Elevated K_b most likely reflects a higher density of TSPO in or around vessel walls. TSPO is
396 expressed in endothelium and the extent of WMH in patients with SVD correlates with
397 thrombomodulin, a circulating marker of endothelial cell activation (Hassan et al., 2003). However,
398 endothelial TSPO staining appeared normal in our *post-mortem* SVD specimens. Perivascular
399 macrophages also bind TSPO ligands and were indeed observed in our specimens. Higher
400 [^{11}C]PBR28 signal in the vascular compartment in SVD may be further explained by the formation of
401 pockets delimited by collagen type IV-positive membranes in vessels walls (Forsberg et al., 2018).
402 These pockets contain plasma proteins which can bind and entrap TSPO ligands, delaying their
403 diffusion through vessel walls (Lockhart et al., 2003; Turkheimer et al., 2015). According to this
404 hypothesis, the increase in TSPO PET signal in the vascular compartment in SVD may be driven in
405 party by tracer trapped within the vessel wall rather than a true increase in TSPO expression.

406 The persistence of TSPO tracer in vascular compartment might also reflect the progressive loss of
407 smooth muscle cells in the tunica media and their replacement by fibrous connective tissue, collagen
408 type IV in particular (Veronese et al., 2018), with a possible subsequent increase in resistance to
409 lipophilic tracers.

410 In addition to offering new insight into the pathogenesis of SVD, the current results have important
411 implications for the design and interpretation of PET studies that utilize TSPO as a marker of
412 neuroinflammation. Given the fact that in white matter lesions the TSPO signal was mainly at the
413 interface between brain parenchyma and vascular unit, blood sampling and full compartmental
414 modelling is fundamental to distinguish the contribution of different compartments to the measured
415 PET signal. At the same time, reference region quantification approaches are not likely to be
416 appropriate because of the assumption of a similar blood-to-tissue tracer exchange between target and
417 reference tissue. Therefore, in participants with evidence of cerebrovascular disease, TSPO PET
418 studies should adopt blood sampling and full compartment modelling approaches and avoid analyses

This is a provisional file, not the final typeset article

419 that depend on reference regions. Practical considerations often argue against invasive blood sampling
420 in older groups; the present results show that full modelling strategies are most needed in groups who
421 may find these procedures more difficult to tolerate. The third implication is that TSPO upregulation
422 and microglial proliferation are uncoupled in damaged tissue: TSPO cellular dynamics are more
423 complex than a simple "TSPO upregulation equals microglia activation". The presence of damaged
424 white matter is not confined to those with prior lacunar stroke or a diagnosis of SVD, so these
425 implications extend to other clinical settings. A large proportion of patients with established AD,
426 perhaps as many as 40%, have diffuse WMH. The possibility of activated but TSPO-negative microglia
427 in areas of WMH will require a more careful interpretation of TSPO PET binding results in a wide
428 range of clinical settings.

429 The interpretation of TSPO PET signal as a true marker of microglial activation and therefore
430 neuroinflammation has been a matter of debate in molecular imaging studies (Owen et al., 2017; Notter
431 et al., 2018). Indeed, TSPO signal can be driven by other factors, such as recruitment of peripheral
432 monocytes into the parenchyma, adherence of circulating leucocytes to the vascular endothelium and
433 the expression of TSPO in other CNS cells including astrocytes, vascular endothelial cells and neurons.
434 Potential avenues for future studies might include use of dual tracers with different properties in terms
435 of cell type or compartment specificity. The development of new tracers with specificity for different
436 classes of immune cells or cell surface markers would also be a major advance. The current results add
437 the possibility of TSPO-negative activated microglia to the list of provisos and underscore the value of
438 parallel PET and *post-mortem* analyses.

439 4.1 Strengths and limitations

440 This study has limitations. The sample sizes for both the PET and *post-mortem* studies are small.
441 This study used a full PET design with dynamic acquisition and arterial blood sampling using high-
442 specific TSPO tracer as [^{11}C]PBR28. Arterial blood sampling is invasive, which limits its use with
443 participants who are elderly or ill. The intention was to provide a preliminary study adopting a
444 comprehensive PET methodology, which could be used to plan larger studies and provide a starting
445 point to explore less invasive methods, which have been developed (Garcia-Lorenzo et al., 2018;
446 Schain et al., 2018; Zanotti-Fregonara et al., 2019) but require more validation work before extending
447 them to SVD disease. The healthy control participants were younger than the group with SVD, on
448 average. Several TSPO studies show a positive association between age and TSPO brain expression
449 (Kumar et al., 2012; Schuitemaker et al., 2012; Paul et al., 2019), raising a confounding effect of age
450 on the differences we report between the SVD and healthy controls (see Supplementary Figure 4).
451 However, a recent study on a large sample (N=140) of [^{11}C]PBR28 PET scans shows that this effect
452 might be limited to cortical regions only (Tuisku et al., 2019). Measurement of cerebral blood flow
453 with arterial spin labelling made it possible to explore the relationships between tissue blood flow
454 and TSPO binding. However, for both methods, signal-to-noise ratio is relatively low in white matter,
455 limiting the precision with which these associations can be explored. The PET regions of interest
456 were matched as closely as possible to the anatomical landmarks used for post mortem tissue
457 sampling, but a fixed frame of reference based on post-mortem MRI was not available for this study.
458 A future study would also be strengthened by obtaining systemic markers of inflammation from
459 individuals undergoing PET.

460 4.2 Conclusion

461 ElevatedTSPO binding provides evidence of inflammatory activation localized to vessel walls and
462 perivascular spaces in SVD. Reduced TSPO expression despite microglial proliferation and tissue
463 pathology consistent with inflammation may reflect mitochondrial deficiency in microglia as a result

464 of chronic hypoxia or chronic oxidative stress. Our findings add further evidence for a pivotal role of
465 the neurovascular unit in the pathogenesis of SVD and should prompt extra caution when interpreting
466 TSPO PET in older individuals or those with vascular risk.

467 5 Acknowledgements

468 The authors wish to thank the participants in the PET study and the donors to the Manchester Brain
469 Bank and the UK Parkinson's Society Brain Bank for their essential contributions to this work. We
470 gratefully acknowledge the assistance of staff at Invicro London, UK and the NIHR Wellcome Trust
471 King's Clinical Research Facility in scanning participants.

472 6 Author Contributions

473 PW recruited participants, acquired and processed MR data, analyzed MR and PET statistics and
474 contributed to the paper. MV analyzed PET data and contributed to the paper. NM recruited
475 participants and acquired PET data. FT assisted in the design of the experiments, interpretation of the
476 data and preparation of the manuscript. ER assisted in the design of the experiments, interpretation of
477 the data and preparation of the manuscript. CB assisted in the design of the experiments,
478 interpretation of the data and preparation of the manuscript. SW assisted in the design of the
479 experiments, interpretation of the data and preparation of the manuscript. AH selected,
480 immunostained, analyzed and quantified normal control brains. BO, RW performed tissue
481 processing, immunostaining and quantification of Iba1 and TSPO in SVD brains. TM, OH
482 contributed PET data from healthy controls. FR designed the methodology of tissue analysis, selected
483 the cases and contributed to manuscript writing. MO designed and oversaw the PET and MRI studies
484 and contributed to the paper.

485 7 Funding

486 This study was supported by the National Institute for Health Research (NIHR) Biomedical Research
487 Centre at South London and Maudsley NHS Foundation Trust and King's College London; Medical
488 Research Council, UK, grant reference MR/K022113/1; the European Commission Horizon 2020
489 Programme (grant agreement no. 667375).

490 8 Conflict of Interest

491 The authors declare that the research was conducted in the absence of any commercial or financial
492 relationships that could be construed as a potential conflict of interest.

493 9 Supplementary Material

494 Supplementary information is available online.

495 10 References

496 Alafuzoff, I., Gelpi, E., Al-Sarraj, S., Arzberger, T., Attems, J., Bodi, I., et al. (2012). The need to
497 unify neuropathological assessments of vascular alterations in the ageing brain: multicentre survey by
498 the BrainNet Europe consortium. *Exp Gerontol* 47(11)**,** 825-833. doi: 10.1016/j.exger.2012.06.001.

499 Albrecht, D.S., Granziera, C., Hooker, J.M., and Loggia, M.L. (2016). In Vivo Imaging of Human
500 Neuroinflammation. *ACS Chem Neurosci* 7(4)**,** 470-483. doi: 10.1021/acschemneuro.6b00056.

This is a provisional file, not the final typeset article

501 Bloomfield, P.S., Selvaraj, S., Veronese, M., Rizzo, G., Bertoldo, A., Owen, D.R., et al. (2016).
502 Microglial Activity in People at Ultra High Risk of Psychosis and in Schizophrenia: An
503 [(11)C]PBR28 PET Brain Imaging Study. *Am J Psychiatry* 173(1)**,** 44-52. doi:
504 10.1176/appi.ajp.2015.14101358.

505 Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H., and Del Tredici, K. (2006). Staging of
506 Alzheimer disease-associated neurofibrillary pathology using paraffin sections and
507 immunocytochemistry. *Acta Neuropathol* 112(4)**,** 389-404. doi: 10.1007/s00401-006-0127-z.

508 Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta*
509 *Neuropathol* 82(4)**,** 239-259.

510 Charidimou, A., Pantoni, L., and Love, S. (2016). The concept of sporadic cerebral small vessel
511 disease: A road map on key definitions and current concepts. *Int J Stroke* 11(1)**,** 6-18. doi:
512 10.1177/1747493015607485.

513 Collste, K., Forsberg, A., Varrone, A., Amini, N., Aeinehband, S., Yakushev, I., et al. (2016). Test-
514 retest reproducibility of [(11)C]PBR28 binding to TSPO in healthy control subjects. *Eur J Nucl Med*
515 *Mol Imaging* 43(1)**,** 173-183. doi: 10.1007/s00259-015-3149-8.

516 Evans, N.R., Tarkin, J.M., Buscombe, J.R., Markus, H.S., Rudd, J.H.F., and Warburton, E.A. (2017).
517 PET imaging of the neurovascular interface in cerebrovascular disease. *Nat Rev Neurol* 13(11)**,** 676-
518 688. doi: 10.1038/nrneurol.2017.129.

519 Fazekas, F., Kleinert, R., Offenbacher, H., Schmidt, R., Kleinert, G., Payer, F., et al. (1993).
520 Pathologic correlates of incidental MRI white matter signal hyperintensities. *Neurology* 43(9)**,** 1683-
521 1689. doi: 10.1212/wnl.43.9.1683.

522 Forsberg, K.M.E., Zhang, Y., Reiners, J., Ander, M., Niedermayer, A., Fang, L., et al. (2018).
523 Endothelial damage, vascular bagging and remodeling of the microvascular bed in human
524 microangiopathy with deep white matter lesions. *Acta Neuropathol Commun* 6(1)**,** 128. doi:
525 10.1186/s40478-018-0632-z.

526 Fu, Y., and Yan, Y. (2018). Emerging Role of Immunity in Cerebral Small Vessel Disease. *Front*
527 *Immunol* 9**,** 67. doi: 10.3389/fimmu.2018.00067.

528 Garcia-Lorenzo, D., Lavisse, S., Leroy, C., Wimberley, C., Bodini, B., Remy, P., et al. (2018).
529 Validation of an automatic reference region extraction for the quantification of [(18)F]DPA-714 in
530 dynamic brain PET studies. *J Cereb Blood Flow Metab* 38(2)**,** 333-346. doi:
531 10.1177/0271678X17692599.

532 Guilarte, T.R., Loth, M.K., and Guariglia, S.R. (2016). TSPO Finds NOX2 in Microglia for Redox
533 Homeostasis. *Trends Pharmacol Sci* 37(5)**,** 334-343. doi: 10.1016/j.tips.2016.02.008.

534 Hachinski, V.C., Potter, P., and Merskey, H. (1986). Leuko-araiosis: an ancient term for a new
535 problem. *Can J Neurol Sci* 13(4 Suppl)**,** 533-534.

536 Hammers, A., Allom, R., Koepp, M.J., Free, S.L., Myers, R., Lemieux, L., et al. (2003). Three-
537 dimensional maximum probability atlas of the human brain, with particular reference to the temporal
538 lobe. *Hum Brain Mapp* 19(4)**,** 224-247. doi: 10.1002/hbm.10123.

539 Hassan, A., Hunt, B.J., O'Sullivan, M., Parmar, K., Bamford, J.M., Briley, D., et al. (2003). Markers
540 of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis. *Brain* 126(Pt 2)**,** 424-
541 432. doi: 10.1093/brain/awg040.

542 Holland, P.R., Searcy, J.L., Salvadores, N., Scullion, G., Chen, G., Lawson, G., et al. (2015).
543 Gliovascular disruption and cognitive deficits in a mouse model with features of small vessel disease.
544 *J Cereb Blood Flow Metab* 35(6)**,** 1005-1014. doi: 10.1038/jcbfm.2015.12.

545 Iadecola, C. (2013). The pathobiology of vascular dementia. *Neuron* 80(4)**,** 844-866. doi:
546 10.1016/j.neuron.2013.10.008.

547 Jacobs, A.H., Tavitian, B., and consortium, I.N. (2012). Noninvasive molecular imaging of
548 neuroinflammation. *J Cereb Blood Flow Metab* 32(7)**,** 1393-1415. doi: 10.1038/jcbfm.2012.53.

549 Kumar, A., Muzik, O., Shandal, V., Chugani, D., Chakraborty, P., and Chugani, H.T. (2012).
550 Evaluation of age-related changes in translocator protein (TSPO) in human brain using (11)C-[R]-
551 PK11195 PET. *J Neuroinflammation* 9**,** 232. doi: 10.1186/1742-2094-9-232.

552 Lockhart, A., Davis, B., Matthews, J.C., Rahmoune, H., Hong, G., Gee, A., et al. (2003). The
553 peripheral benzodiazepine receptor ligand PK11195 binds with high affinity to the acute phase
554 reactant alpha1-acid glycoprotein: implications for the use of the ligand as a CNS inflammatory
555 marker. *Nucl Med Biol* 30(2)**,** 199-206.

556 Modinos, G., Egerton, A., McMullen, K., McLaughlin, A., Kumari, V., Barker, G.J., et al. (2018).
557 Increased resting perfusion of the hippocampus in high positive schizotypy: A pseudocontinuous
558 arterial spin labeling study. *Hum Brain Mapp* 39(10)**,** 4055-4064. doi: 10.1002/hbm.24231.

559 Mulugeta, E., Molina-Holgado, F., Elliott, M.S., Hortobagyi, T., Perry, R., Kalaria, R.N., et al.
560 (2008). Inflammatory mediators in the frontal lobe of patients with mixed and vascular dementia.
561 *Dementia and Geriatric Cognitive Disorders* 25(3)**,** 278-286. doi: 10.1159/000118633.

562 Nair, A., Veronese, M., Xu, X., Curtis, C., Turkheimer, F., Howard, R., et al. (2016). Test-retest
563 analysis of a non-invasive method of quantifying [(11)C]-PBR28 binding in Alzheimer's disease.
564 *EJNMMI Res* 6(1)**,** 72. doi: 10.1186/s13550-016-0226-3.

565 Notter, T., Coughlin, J.M., Sawa, A., and Meyer, U. (2018). Reconceptualization of translocator
566 protein as a biomarker of neuroinflammation in psychiatry. *Mol Psychiatry* 23(1)**,** 36-47. doi:
567 10.1038/mp.2017.232.

568 Owen, D.R., Guo, Q., Kalk, N.J., Colasanti, A., Kalogiannopoulou, D., Dimber, R., et al. (2014).
569 Determination of [(11)C]PBR28 binding potential in vivo: a first human TSPO blocking study. *J*
570 *Cereb Blood Flow Metab* 34(6)**,** 989-994. doi: 10.1038/jcbfm.2014.46.

571 Owen, D.R., Narayan, N., Wells, L., Healy, L., Smyth, E., Rabiner, E.A., et al. (2017). Pro-
572 inflammatory activation of primary microglia and macrophages increases 18 kDa translocator protein
573 expression in rodents but not humans. *J Cereb Blood Flow Metab* 37(8)**,** 2679-2690. doi:
574 10.1177/0271678X17710182.

This is a provisional file, not the final typeset article

575 Owen, D.R., Yeo, A.J., Gunn, R.N., Song, K., Wadsworth, G., Lewis, A., et al. (2012). An 18-kDa
576 translocator protein (TSPO) polymorphism explains differences in binding affinity of the PET
577 radioligand PBR28. *J Cereb Blood Flow Metab* 32(1)**,** 1-5. doi: 10.1038/jcbfm.2011.147.

578 Pannell, M., Economopoulos, V., Wilson, T.C., Kersemans, V., Isenegger, P.G., Larkin, J.R., et al.
579 (2019). Imaging of translocator protein upregulation is selective for pro-inflammatory polarized
580 astrocytes and microglia. *Glia*. doi: 10.1002/glia.23716.

581 Paul, S., Gallagher, E., Liow, J.S., Mabins, S., Henry, K., Zoghbi, S.S., et al. (2019). Building a
582 database for brain 18 kDa translocator protein imaged using [(11)C]PBR28 in healthy subjects. *J*
583 *Cereb Blood Flow Metab* 39(6)**,** 1138-1147. doi: 10.1177/0271678X18771250.

584 Radlinska, B.A., Ghinani, S.A., Lyon, P., Jolly, D., Soucy, J.P., Minuk, J., et al. (2009). Multimodal
585 microglia imaging of fiber tracts in acute subcortical stroke. *Ann Neurol* 66(6)**,** 825-832. doi:
586 10.1002/ana.21796.

587 Rizzo, G., Veronese, M., Tonietto, M., Bodini, B., Stankoff, B., Wimberley, C., et al. (2019).
588 Generalization of endothelial modelling of TSPO PET imaging: Considerations on tracer affinities. *J*
589 *Cereb Blood Flow Metab* 39(5)**,** 874-885. doi: 10.1177/0271678X17742004.

590 Rizzo, G., Veronese, M., Tonietto, M., Zanotti-Fregonara, P., Turkheimer, F.E., and Bertoldo, A.
591 (2014). Kinetic modeling without accounting for the vascular component impairs the quantification
592 of [(11)C]PBR28 brain PET data. *J Cereb Blood Flow Metab* 34(6)**,** 1060-1069. doi:
593 10.1038/jcbfm.2014.55.

594 Rosenberg, G.A. (2009). Inflammation and white matter damage in vascular cognitive impairment.
595 *Stroke* 40(3 Suppl)**,** S20-23. doi: 10.1161/STROKEAHA.108.533133.

596 Rosenberg, G.A. (2017). Extracellular matrix inflammation in vascular cognitive impairment and
597 dementia. *Clin Sci (Lond)* 131(6)**,** 425-437. doi: 10.1042/CS20160604.

598 Schain, M., Zanderigo, F., Ogden, R.T., and Kreisl, W.C. (2018). Non-invasive estimation of
599 [(11)C]PBR28 binding potential. *Neuroimage* 169**,** 278-285. doi: 10.1016/j.neuroimage.2017.12.002.

600 Schuitemaker, A., van der Doef, T.F., Boellaard, R., van der Flier, W.M., Yaqub, M., Windhorst,
601 A.D., et al. (2012). Microglial activation in healthy aging. *Neurobiol Aging* 33(6)**,** 1067-1072. doi:
602 10.1016/j.neurobiolaging.2010.09.016.

603 Simpson, J.E., Ince, P.G., Higham, C.E., Gelsthorpe, C.H., Fernando, M.S., Matthews, F., et al.
604 (2007). Microglial activation in white matter lesions and nonlesional white matter of ageing brains.
605 *Neuropathol Appl Neurobiol* 33(6)**,** 670-683. doi: 10.1111/j.1365-2990.2007.00890.x.

606 Sjogren, M., Blomberg, M., Jonsson, M., Wahlund, L.O., Edman, A., Lind, K., et al. (2001).
607 Neurofilament protein in cerebrospinal fluid: a marker of white matter changes. *J Neurosci Res*
608 66(3)**,** 510-516. doi: 10.1002/jnr.1242.

609 Skrobot, O.A., Attems, J., Esiri, M., Hortobagyi, T., Ironside, J.W., Kalaria, R.N., et al. (2016).
610 Vascular cognitive impairment neuropathology guidelines (VCING): the contribution of
611 cerebrovascular pathology to cognitive impairment. *Brain* 139(11)**,** 2957-2969. doi:
612 10.1093/brain/aww214.

613 Thal, D.R., Rub, U., Orantes, M., and Braak, H. (2002). Phases of A beta-deposition in the human
614 brain and its relevance for the development of AD. *Neurology* 58(12)**,** 1791-1800. doi:
615 10.1212/wnl.58.12.1791.

616 Tonietto, M., Rizzo, G., Veronese, M., Borgan, F., Bloomfield, P.S., Howes, O., et al. (2019). A
617 Unified Framework for Plasma Data Modeling in Dynamic Positron Emission Tomography Studies.
618 *IEEE Trans Biomed Eng* 66(5)**,** 1447-1455. doi: 10.1109/TBME.2018.2874308.

619 Tuisku, J., Plaven-Sigray, P., Gaiser, E.C., Airas, L., Al-Abdulrasul, H., Bruck, A., et al. (2019).
620 Effects of age, BMI and sex on the glial cell marker TSPO - a multicentre [(11)C]PBR28 HRRT PET
621 study. *Eur J Nucl Med Mol Imaging* 46(11)**,** 2329-2338. doi: 10.1007/s00259-019-04403-7.

622 Turkheimer, F.E., Rizzo, G., Bloomfield, P.S., Howes, O., Zanotti-Fregonara, P., Bertoldo, A., et al.
623 (2015). The methodology of TSPO imaging with positron emission tomography. *Biochem Soc Trans*
624 43(4)**,** 586-592. doi: 10.1042/BST20150058.

625 Veronese, M., Reis Marques, T., Bloomfield, P.S., Rizzo, G., Singh, N., Jones, D., et al. (2018).
626 Kinetic modelling of [(11)C]PBR28 for 18 kDa translocator protein PET data: A validation study of
627 vascular modelling in the brain using XBD173 and tissue analysis. *J Cereb Blood Flow Metab* 38(7)**,**
628 1227-1242. doi: 10.1177/0271678X17712388.

629 Wahlund, L.O., Barkhof, F., Fazekas, F., Bronge, L., Augustin, M., Sjogren, M., et al. (2001). A new
630 rating scale for age-related white matter changes applicable to MRI and CT. *Stroke* 32(6)**,** 1318-1322.
631 doi: 10.1161/01.str.32.6.1318.

632 Wardlaw, J.M., Smith, E.E., Biessels, G.J., Cordonnier, C., Fazekas, F., Frayne, R., et al. (2013).
633 Neuroimaging standards for research into small vessel disease and its contribution to ageing and
634 neurodegeneration. *Lancet Neurology* 12**,** 822-838. doi: 10.1016/S1474-4422(13)70124-8.

635 Zanotti-Fregonara, P., Kreisl, W.C., Innis, R.B., and Lyoo, C.H. (2019). Automatic Extraction of a
636 Reference Region for the Noninvasive Quantification of Translocator Protein in Brain Using (11)C-
637 PBR28. *J Nucl Med* 60(7)**,** 978-984. doi: 10.2967/jnumed.118.222927.

638 11 Figure legends

639 **Figure 1: Image processing pipeline.** Top: WMH and infarcts were drawn in T2-weighted FLAIR
640 images, which were co-registered with T1-weighted images along with the ROIs. Green = deep
641 WMH. Blue = periventricular WMH. Red = infarct lesion. Second row: grey and white matter were
642 segmented using T1-weighted images, which were co-registered to PET space (third row) along with
643 the tissue ROIs and the ROIs from the FLAIR images. Bottom row: ASL proton density images (left
644 image) were co-registered to PET space along with CBF maps (right three images). Green = white
645 matter. Blue = grey matter. Bottom: PET images.

646 **Figure 2: TSPO binding differs between normal appearing white matter (NAWM) and white**
647 **matter hyperintensities (WMH).** Top row: group frequency maps of WMH (red) and NAWM
648 (green)with voxel intensity indicating number of participants with corresponding tissue type, and the
649 atlas region defining the striatum (blue). Plots show volume of tracer (V_T), tissue-to-blood ratio (V_b),
650 plasma to tissue tracer transport (K_1) and vascular-bound tracer (K_b) for individual participants.
651 Crosses represent healthy controls (HC). Filled and hollow circles represent individuals in the SVD
652 group with (WMH+) or without (WMH-) a history of lacunar stroke, either in the whole brain for

This is a provisional file, not the final typeset article

653 WMH and NAWM ROIs or in the striatum for the striatum ROI. Horizontal line: mean. * $p < .05$.
654 *** $p < .001$.

655 **Figure 3: Histological confirmation of small vessel disease.** The globus pallidus shows widening
656 of perivascular spaces, loose-texture neuropil and white matter due to florid reactive astrocytosis (A,
657 hematoxylin-eosin – x4); perforating arteries demonstrate thickened walls; the tunica media is
658 replaced by fibrous connective tissue (B, hematoxylin-eosin – x20)

659 **Figure 4: Staining for Iba1 and TSPO.** Panels A-F show whole mount sections from the anterior
660 (A-C) and posterior basal ganglia (D-E) from a brain with severe SVD. The sections are stained with
661 hematoxylin-eosin (A, D), and with immunochemistry for Iba1 (B, E) and TSPO (C, F). The bar
662 indicates 1cm. The framed areas show the inner segment of the globus pallidus. Pictures G-I show a
663 x20 magnification of the framed area. Perforating arteries have thickened walls (G, HE – x20); there
664 is florid microglial and macrophagic response (red cells) (H, Iba1 immunostain – x20); TSPO
665 expression is low and limited to a minority of microglia cells. In contrast, endothelial cells are
666 intensely positive (arrow) (I, TSPO immunostain – x20).

667 **Figure 5: Fewer microglia express TSPO in SVD than control tissue.** A: individual measurements
668 of the pan-microglial marker Iba1 (hollow markers) and TSPO (filled markers) in SVD and healthy
669 controls. In SVD, there are greater numbers of microglia but a smaller proportion express TSPO; B:
670 ratios of TSPO:Iba1 density in each group. The ratio is significantly lower in SVD over all regions.
671 Horizontal line = mean.

672

673

674 **12 Tables**

675 **12.1 Table 1. Participant demographics.**

Group	ID	Age at scan / death	Sex	Extent of white matter lesions	Clinical diagnosis	TSPO tracer affinity	Tau Braak stage	APOE
PET Asymptomatic SVD	P1	73	F	Fazekas DWM grade 2	Healthy	High	n/a	n/a
	P2	78	F	Fazekas DWM grade 2	Healthy	High	n/a	n/a
	P3	85	F	Fazekas DWM grade 2	Healthy	Mixed	n/a	n/a
	P4	69	F	Fazekas DWM grade 2	Healthy	High	n/a	n/a
	P5	90	M	Fazekas DWM grade 2	Healthy	High	n/a	n/a
	P6	72	F	Fazekas DWM grade 3	Healthy	High	n/a	n/a
PET Symptomatic SVD	P7	58	M	Fazekas DWM grade 2	Lacunar stroke	High	n/a	n/a
	P8	86	M	Fazekas DWM grade 2	Lacunar stroke	High	n/a	n/a
	P9	51	M	Fazekas DWM grade 3	Lacunar stroke	High	n/a	n/a
	P10	79	M	Fazekas DWM grade 2	Lacunar stroke	Mixed	n/a	n/a
	P11	55	M	Fazekas DWM grade 2	Lacunar stroke	High	n/a	n/a
PET controls	N = 21	M 37.8 SD 15.7	6 female 14 male	Fazekas DWM grade 0 No striatal lacunes	Healthy	3 mixed 18 high	n/a	n/a
Post-mortem controls	PDC013	77	M	Mild ageing changes	Ageing	n/a	II	n/a
	C073	77	M	Mild ageing changes	Hepato-carcinoma	n/a	II	n/a
Post-mortem patients	DPM10/24	92	F	Severe SVD	Vascular dementia	n/a	III	ε3ε3
	DPM16/07	71	F	Severe SVD	Ageing	n/a	I	ε3ε3
	DPM16/19	97	F	Severe SVD with microinfarction	Ageing	n/a	II	ε3ε3
	DPM16/15	92	F	Severe SVD with ischaemic lesions	Vascular dementia	n/a	II	ε3ε4
	DPM16/02	90	M	Severe SVD with microinfarctions	Ageing	n/a	II	ε3ε3

676

677

This is a provisional file, not the final typeset article

678

679 **12.2 Table 2. Immunohistochemical measures**

Group	ROI	Iba1 density	TSPO density	Density ratio
SVD	Caudate	4.60	0.73	17%
	Internal Capsule	10.21	0.72	8%
	Pallidus	7.62	2.43	56%
	Putamen	4.15	0.38	11%
	Mean	**6.65**	**1.07**	**23%**
HC	Internal Capsule	3.66	1.24	34%
	Pallidus	2.41	1.70	72%
	Putamen	1.68	1.35	83%
	Mean	**2.58**	**1.43**	**63%**

680