

**Positron Emission Tomography Imaging of
Neuroinflammation in Multiple Sclerosis with a
Second Generation Translocator Protein PET Radioligand**

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ABSTRACT

This thesis describes a new approach for molecular imaging of neuroinflammation in Multiple Sclerosis (MS). My aim was to use the 2nd generation TSPO radioligand ¹⁸F-PBR111 to explore the potential of Positron Emission Tomography (PET) targeting the 18-kDa Translocator Protein (TSPO), as an *in vivo* biomarker of activated microglia in MS patients.

This thesis addresses three research objectives. First, I characterised ¹⁸F-PBR111 PET signal in healthy controls' brains and tested how it is affected by the *TSPO* gene polymorphism at rs6971. Second, I measured ¹⁸F-PBR111 uptake across white matter volumes segmented using structural MRI measures related to MS neuropathology. Third, I explored how ¹⁸F-PBR111 uptake in the hippocampus correlated with depressive symptoms and to the brain functional connectivity of the hippocampus.

Eleven patients with relapsing-remitting MS and 22 age-matched healthy controls underwent ¹⁸F-PBR111 PET and MRI scans. Structural and functional MRI sequences were used to define conventional MS neuropathological markers and for the assessment of functional connectivity, respectively.

I discovered that white matter ¹⁸F-PBR111 PET signal in healthy volunteers varied with TSPO genotype and correlated positively with age. In patients with MS, signal intensity in MRI-defined lesions was higher than that in normal-appearing white matter and correlated with the historical rate of progression of their disability. Hippocampal ¹⁸F-PBR111 uptake was higher in the MS patient group than in healthy volunteers and correlated with both depressive symptoms and functional connectivity of the hippocampus with frontal, temporal and parietal cortex.

I thus discovered that this 2nd generation TSPO PET radiotracer, used in humans for the first time in our study, is sensitive to MS neuropathology consistent with recognized patterns of microglial activation and that differences between subjects can be related to disability progression. I also have discovered a novel relationship between this measure of hippocampal microglial activation and affective symptoms of MS.

to my family

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Alessandro Colasanti

November 2014

Declaration of originality

I declare that the work described in this thesis is that of the author and that all work by others is appropriately referenced.

None of this work has been submitted for any other qualification at this or any other university.

Alessandro Colasanti

November 2014

Statement of contribution

I was the principal contributor to the research project illustrated in this thesis from the earliest phases of the design and preparation of the studies. I prepared the initial draft of the project and outlined the plan for the studies in the “research proposal” of my Wellcome Trust fellowship application. I designed and wrote the first drafts of the study protocol, and coordinated multiple reviews of the study plans with all the co-investigators, and eventually I submitted the final study protocol to the Research Ethics Committee (REC). I also designed and completed all the additional documentation for the ethics application, including the IRAS form, the Site-Specific application forms, the R&D application form, the Participant Information Sheet, the letter to General Practitioners and the advertisement material. I wrote and processed the application for both the Administration of Radioactive Substances Advisory Committee (ARSAC) and the Research & Development offices. I processed the application to the REC and presented the application at the REC meeting. I replied to both REC and ARSAC committees’ comments and finally obtained definitive approvals. I contributed to the preparation of the numerous legal contracts necessary for the setup of the study, including those between GlaxoSmithKline, Imanova Ltd, Imperial College London, Nottingham University, UCL, the NIHR/Wellcome Trust Imperial Clinical Research Facility, Bayer and ANSTO.

I have been the Chief Investigator (as well as local Principal Investigator) of the study reported here (study protocol reference: EMI115241). I have continuously ensured compliance with Good Clinical Practice and I held the ultimate responsibility for the study conduct and management of all research sites involved in the research, for the entire duration of the study.

I have set up, coordinated, and actively managed the identification and recruitment of study subjects. I conducted initial phone-based pre-screening interviews with all subjects interested in taking part in the study. I met all subjects for explanation of the study procedures, and obtained the informed consent and approved their inclusion in the study, based on the review of the inclusion criteria. I have been responsible of the medical governance of the study. I have led the screening process, interviewed and conducted a complete medical examination of all subjects. I reviewed blood results and any clinical incidental findings, and liaised with General Practitioners and consultant neurologists to share any useful clinical information based on incidental findings. I have administered questionnaires, clinical rating scales and psychiatric diagnostic interviews.

I was responsible of all phases of PET radioligand administration and PET and MRI scanning. Specifically, in all cases I injected the PET radiotracer and the Gadolinium contrast. I was present and supervised the scanning procedures for all MRI and PET scans.

I run a significant part of the PET imaging analysis. Specifically, my involvement included:

- the inspection of all individual PET imaging study data
- the inspection of the regional and blood Time Activity Curves for quality control and identification of outliers
- the comparison of various kinetic modeling methods for selection of the quantification method to be used in the final analysis.
- the exploration of various methodologies for identification of a suitable pseudo-reference region

- the setup and optimization of the process of lesion masking of patients with multiple sclerosis
- the characterisation of PET signal distribution in voxel-wise parametric maps
- the use of voxel-wise group statistics (using FSL randomise) to study differences in signal distribution across and within groups.

I collaborated to the process of quantification of PET signal and related pre-processing analysis and I understood the rationale and practicalities of all aspects related to these. I ideated, supervised and coordinated the process of quantification of PET signal across various MRI-defined volumes of interest (atlas-based brain regions, T2-FLAIR lesions, DIR lesions, MTR-based segmentations).

I run all fMRI analysis using the FSL software. The analysis included pre-processing (registration, motion correction, filtering), extraction of hippocampal time-series, and use of voxel-wise multiple regression models (see Chapter 6 for further details). I have examined and summarized the outcomes from the statistical maps generated by the imaging software.

I conceived and run all statistical analyses, including statistical tests on group comparisons and on correlations between clinical and demographic data, PET data, and structural and functional MRI parameters, as well as clinical parameters.

I provided the initial interpretation of all findings, and coordinated the discussion and review of the findings with all collaborators to the project. I have been the principle author and drafted the first version of the articles and book chapters, on which Chapters 1, 2, 4, 5 and 6 are based. I have significantly contributed to write the research article on which Chapter 3 is based.

Publications derived from this work

Original research articles

Guo Q, **Colasanti A**, Owen DR, Onega M, Kamalakaran A, Bennacef I, Matthews PM, Rabiner EA, Turkheimer FE, Gunn RN. Quantification of the specific translocator protein signal of ^{18}F -PBR111 in healthy humans: a genetic polymorphism effect on in vivo binding. J Nucl Med 2013; 54(11): 1915-23

Colasanti A, Guo Q, Muhlert N, Giannetti P, Onega M, Newbould RD, Ciccarelli O, Rison S, Thomas C, Nicholas R, Muraro PA, Malik O, Owen DR, Piccini P, Gunn RN, Rabiner EA, Matthews PM. In Vivo Assessment of Brain White Matter Inflammation in Multiple Sclerosis with ^{18}F -PBR111 PET. J Nucl Med 2014; 55(7): 1112-1118.

Book Chapter

Colasanti A, Piccini P. PET imaging in Multiple Sclerosis: focus on the Translocator Protein. In: Dierckx R, Otte A, de Vries E, van Waarde A (eds), PET and SPECT in Neurology 2014; Springer-Verlag Berlin Heidelberg.

In preparation

Colasanti A, Guo Q, Wall M, Giannetti P, Onega M, Newbould RD, Bishop C, Ciccarelli O, Nicholas R, Muraro PA, Malik O, Owen DR, Young AH, Gunn RN, Piccini P, Rabiner EA, Matthews PM. Hippocampal neuroinflammation in Multiple Sclerosis is associated with depressive symptoms and altered functional connectivity: a combined TSPO PET-fMRI study.

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List of abbreviations

2TC: Two Tissue Compartment

ANCOVA: Analysis of Covariance

ARSAC: Administration of Radioactive Substances Advisory Committee

BBB: Blood-Brain Barrier

BDI: Beck Depressive Inventory

BOLD: Blood oxygen level dependent

CB₂: cannabinoid-2

CNS: Central Nervous System

C_V: Coefficient of variation

DMT: Disease modifying treatment

DSM: Diagnostic and Statistics Manual of Mental Disorders

DVR: Distribution Volume Ratio

EAE: Experimental autoimmune encephalitis

EDSS: Expanded Disability Status Scale

FLAIR: Fluid Attenuated Inversion Recovery

fMRI: Functional Magnetic Resonance Imaging

f_p: Plasma free fraction

GABA_A: Gamma-aminobutyric acid-A

HAB: High Affinity Binder

HPLC: High-performance liquid chromatography

LAB: Low Affinity Binder

LPS: Lipopolysaccharide

MAB: Mixed Affinity Binder

MBq: Megabecquerel

MDE: Major Depressive Episode

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

MSSS: Multiple Sclerosis Severity Score

MTR: Magnetization Transfer Ratio

NLHM: Non lesional high MTR

NLLM: Non lesional low MTR

N.s.: Non significant

PET: Positron Emission Tomography

PBR: Peripheral Benzodiazepine Receptor

POB: Plasma-over-blood

PVC: Partial Volume Correction

SNP: Single Nucleotide Polymorphism

SPM: Statistical Parametric Mapping

SNR: Signal-to-Noise Ratio

SUV: Standardized Uptake Value

TAC: Time Activity Curve

TSPO: 18-kDa Translocator Protein

VOI: Volume of Interest

V_{ND} : Non-displaceable volume of distribution

V_T : Total Volume of Distribution

Chapter 1. Background

1.1 Multiple Sclerosis

1.1.1 Brief history

Individual case reports of Multiple Sclerosis (MS) were described already in the 19th century. The first clinical description that is now recognized with confidence as a case of MS is that of Augustus d'Este (1794-1848), an illegitimate grandson of King George III of England, who kept a diary for 26 years in which he recorded the progressive course of symptoms, with transient visual impairment, and subsequent episodes of motor symptoms and weakness of the lower extremities compromising his ability to walk (Landtblom et al., 2010). For decades, similar conditions characterised by episodic and progressive neurological deterioration, were labelled as 'paraplegia'. Jean-Martin Charcot provided a definitive description of MS in the mid 19th century, by detailing macroscopic and microscopic illustrations of his histopathological observations that included lesions, axonal transections and remyelination, and named the disease 'Sclerose en plaques'. It was through a series of Charcot's public lectures in 1868, in which he illustrated the key features of MS, that the concept of MS as a discrete disease became rapidly established (Murray, 2009). By the beginning of the 20th century MS had become one of the most common reasons for admission to a neurological ward.

1.1.2 Epidemiological and clinical considerations

MS is a chronic, debilitating disease and represents the major, non-traumatic, cause of neurological disability.

By 2010 the prevalence of MS was approximately 3 per 1000 in women and 1 per 1000 in men, while the incidence was approximately 10 per 100.000/year in women and 5 per 100.000 in men. It is estimated that MS affects approximately 2.5 million individuals, of which 125.000 are in the UK (Mackenzie et al., 2014). The annual costs per person with MS range between \$6.000 and \$80.000 (Sharac et al., 2010), thus accounting for an estimated 1.2 billion expenditure per annum in the UK.

For approximately 85% of MS patients the disease starts with a course of recurrent and reversible neurological deficits. Relapses usually last no more than a few months and the patient regains neurological function. Relapses are defined by the occurrence of a new neurological symptom or worsening of an old one, lasting at least 24 hours, without fever, and which follows a period of clinical stability or of improvement of at least 30 days. The signs and symptoms are variable, including sensory, motor, visual, cognitive, and emotional alterations. This phase of the disease is termed relapsing-remitting MS. After a period that usually ranges between 8 and 20 years, the majority of relapsing-remitting MS patients enters a second phase of the disease, named secondary progressive MS, characterised by continuous, irreversible neurological decline without intermittent relapses. In about a quarter of patients MS never affects daily routine activities while up to 15% of patients become severely disabled within a short time. A proportion of individuals, accounting for 10–20 % of patients with MS, exhibit a progressive disease from onset without episodes of relapse and remission. This is classified as primary progressive MS.

Current diagnostic criteria incorporate evidence from Magnetic Resonance Imaging (MRI) and specify that individuals must have a minimum of two attacks affecting more than one anatomical site, but, assuming that the initial presentation is suggestive of MS, the second lesion needs not necessarily to be clinically expressed (McDonald

et al., 2001). Individuals are classified in the categories of MS, not MS, or possible MS.

The onset of the disease is most common in the third or fourth decade, but in 2% of patients MS presents before 10 years of age, and in 5% before 16 years of age. Overall, the life expectancy is at least 25 years from disease onset, and death usually results from unrelated causes. When sensory or visual symptoms dominate the course of disease, particularly in young women, the prognosis is relatively good, and there is complete recovery from individual episodes. However, in cases where the disturbance of coordination and balance are the most prominent features, or in cases of men with late onset of disease, the prognosis is less positive. The occurrence of frequent and prolonged relapses with incomplete recovery at onset, and a short interval between the initial episode and the first relapse, represent adverse prognostic features (Compston and Coles, 2002).

MS has a profound negative impact on the quality of life of patients, caregivers, and families, and can restrict professional and personal activities even in early stages. Relapses may result in hospitalization and be associated with a level of disability that disrupts work, social, and family life. Fixed disability in MS is acquired through two distinct mechanisms: incomplete recovery from relapse and disease progression, however the main determinant of disability is the onset of the progressive phase. Current treatments do not effectively combat the inexorable physical, cognitive, and quality-of-life deterioration that many secondary progressive MS patients inevitably face. Disability in MS is commonly quantified in half-point increments using a clinician-measured scale, the Kurtzke Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). The EDSS quantifies disability in a number of functional systems and allows raters to assign a score for each functional system using a 0–10 scale. The

functional systems are: pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, and cerebral. EDSS scores of ≤ 6.5 refer to people with MS who are ambulatory whereas EDSS scores of > 6.5 are defined by the impairment to ambulation. Therefore, this scale measures disease progression predominantly by focusing on deficits in ambulation.

1.1.3 Non-motor symptoms of Multiple Sclerosis

Because of the widespread neuropathological involvement, the range of symptoms observed in MS patients is broad, and leads to impairments also in systems other than those regulating sensory and motor functions. The disease also causes cognitive deterioration and neuropsychiatric symptoms. Classes of symptoms are expressed independently, and for example, the manifestation of cognitive and neuropsychiatric symptom is not necessarily related to motor disability (Zarei, 2006).

The prevalence of cognitive impairments in MS is high, with rates ranging from 43% to 70% (Rao et al., 1991). Deficits can occur at any time throughout the disease course. The most prominent cognitive symptoms include deficits in complex attention, efficiency of information processing, executive functioning, and long-term memory. Processing speed, and visual learning and memory are most commonly affected in MS. Verbal skills such as word naming and comprehension are not typically affected. General intelligence seems to remain intact in most patients (Chiaravalloti and DeLuca, 2008; Rao, 1995).

Affective symptoms are the most prevalent neuropsychiatric manifestation of MS. Depression is the most common psychiatric syndrome, and is a cause of serious morbidity and increased mortality in patients with MS (D'Alisa et al., 2006; Feinstein, 1997). Several studies have concluded that the prevalence of depression in MS

exceeds that associated with having one or more other long-term disabling conditions. For example, a survey of 115,071 adults living in Canada confirmed an elevated prevalence of Major Depression in patients with MS (annual prevalence, 16%), relative to both healthy individuals and those with other long-term medical illnesses (annual prevalence, 4% and 9% respectively). Patients with MS aged 18–45 years had a one in four chance of developing depression over the course of 1 year, while for individuals without MS, the rate of depression was one in eleven. The relative risk ratio for Major Depression among patients with MS, after adjustment for age and sex, was 2.3 (Patten et al., 2003). In another community sample of 1,374 patients with MS, depression was present in 41.8% of cases (Chwastiak et al., 2002). Studies in hospital-based samples found a prevalence of depression two-to-five times higher than that found in the general population (Feinstein et al., 2014). A possibly unusual feature is that, despite the greater prevalence of depression amongst females, an association between depression and female gender has not been observed amongst people with MS.

Depression appears to be less common in patients with clinically isolated syndrome, the first neurological episode potentially indicative of MS, but there is not a clear relationship between disease progression, neurological disability and depression: higher rates of depression have been reported in relapsing–remitting MS relative to progressive MS (Feinstein et al., 2014) and depression severity is higher in MS patients without physical disability relative to controls (Haase et al., 2003).

Structural brain changes on MRI account for approximately 40% of the variance in reports of MS-related depression (Feinstein et al., 2004). Genetic, biochemical, and immunological markers of disease activity also have been associated with depression in MS (Feinstein et al., 2014). Together, these data suggest that depression is a direct

consequence of the brain pathology underlying MS.

1.1.4 Neuropathology of Multiple Sclerosis

MS is characterised by the presence of inflammation, demyelination and neurodegeneration at multiple sites throughout the central nervous system (CNS). The term ‘neuroinflammation’ is widely used in my thesis, although is not easy to define. While it is often used to specifically indicate a CNS-specific glial response of the innate immunity, namely the activation of microglia and astroglia such as that observed in chronic neurodegenerative diseases, it is worth recognizing that the inflammatory pathology in MS is not only restricted to glial activation, but also encompasses various acute “classic” inflammatory phenomena such as the presence of perivascular infiltrates of cells, and a prominent involvement of the adaptive immune system. Accordingly, the term ‘neuroinflammation’ is used in this thesis to indicate diverse inflammatory responses taking place in the brain, including both those of glial as well as peripheral origin.

In MS, focal areas of inflammatory demyelination in which lymphocytes infiltrate perivascular spaces, and myelin, oligodendrocytes and axons are destroyed, cause the reversible disability in relapsing-remitting MS. The blood-brain barrier (BBB) is compromised and lesion areas become edematous. Astrogliosis eventually occurs in the demyelinating plaques and results in a gliotic scar.

Ex vivo pathology studies show that the acute, active lesions are characterised by a hypercellular inflammatory core, marked by prominent lymphocyte infiltration with a high density of activated microglia or macrophages distributed evenly throughout the lesion (Trapp et al., 1999). In chronic active lesions, microglia are increased relative to distant normal white matter tissue and are more concentrated at the lesion edge

than within the lesion. A hypercellular margin, characterised by a high density of activated microglia surrounding demyelinating plaques, is a consistent finding across neuropathological studies (Boyle and McGeer, 1990; Lucchinetti et al., 2000; Marik et al., 2007).

Historically, MS has been considered primarily as a white matter disorder, however renewed examination of cortical grey matter revealed that the prevalence of cortical lesions is high and that demyelination takes place also in the cerebral cortex and deep grey matter of MS patients, and is associated with neuronal loss with scarce inflammatory infiltration (Geurts and Barkhof, 2008; Kidd et al., 1999; Magliozzi et al., 2010). On the other hand, lymphocytic infiltrates in the leptomeninges have been commonly observed in postmortem MS brain (Magliozzi et al., 2007).

Diffuse changes, away from macroscopic lesions, are visible in the normal appearing white and grey matter. These observations included: the presence of microglia activation and axonal pathology associated with changes at the Node of Ranvier, i.e. the disruption of paranodal axoglial junctions (Howell et al., 2010); and the so-called “pre-active lesions” (De Groot et al., 2001), focal areas of activated microglia without apparent loss of myelin that may be at risk for the development of acute inflammatory lesions (Henderson et al., 2009; Marik et al., 2007).

Acute perivascular inflammatory infiltrates associated with changes in BBB permeability are responsible for acute relapses, while the resolution of inflammation and remyelination is thought to contribute to the clinical recovery and remission during the relapsing-remitting phase (Kutzelnigg and Lassmann, 2014).

The secondary progressive phase appears to be characterised by a relatively intact BBB, and inflammation becomes increasingly compartmentalized in the perivascular

and subarachnoid spaces. The clinical progression is thought to be a consequence of an accumulating burden of transected axons and damaged neurons associated with more advanced progression of brain atrophy (Trapp and Nave, 2008). However, diffuse tissue damage in the non-lesional brain tissue, unrelated to axonal damage, also contributes to disease progression. There is evidence that grey matter pathological changes importantly contribute to clinical disease severity (Reynolds et al., 2011). These changes are already present in early stages of the disease, and become more prominent when the disease progresses (Chen et al., 2004a; De Stefano et al., 2003).

1.1.5 Role of microglia

In MS, the areas of tissue damage and demyelination are rich in activated microglia (Bauer et al., 1994). Microglia have a mesodermal origin and represent the resident immunocompetent cells in the CNS. They have been described as sensors for pathological events in the CNS (Kreutzberg, 1996). Microglia in the healthy, mature, CNS have a ramified morphology consisting of a small soma with fine cellular processes. This has been classically associated with microglial “resting” state. Pathological signals such as infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, as well as any other disturbance of brain homeostasis indicating real or potential danger to the CNS, all can evoke rapid and profound changes in the microglial cell shape, gene expression and the functional behavior which summarily is defined as “microglial activation”.

Microglia activation is characterised by a set of stereotypical changes, such as change in morphology, migration of microglia towards the site of damage following chemotactic gradients, proliferation, rearrangement of cell surface molecules for cell-

cell and cell-matrix interactions, and release of pro-inflammatory molecules. Release of chemoattractive factors recruits and guides immune cell populations to the CNS, and presentation of antigens to T-lymphocytes can subsequently trigger an adaptive immune response. Further, microglia's phagocytic activities clear tissue debris, damaged cells, or antibody-coated and opsonised antigens. In addition to their phagocytic role, microglia synthesize and secrete potential neurotoxins that may cause neuronal damage. Also, the range of microglial activities also covers production of neurotrophic factors and the physical association with endangered neurons (Kettenmann et al., 2011; Kreutzberg, 1996; Venneti et al., 2006).

Microglia contribute to MS pathology through several mechanisms including the presentation of antigens and secretion of proinflammatory cytokines, initiating an acute inflammatory response; the clearance of damaged tissue, a property potentially linked both to recovery of function and to recurrence of relapses; and the production of toxic mediators leading to irreversible tissue injury associated with the progressive disease phase (Jack et al., 2005). Microglia may also exert a protective function in MS as they can promote tissue regeneration and repair (Muzio et al., 2007).

Activated microglia may represent an early stage of tissue injury, which precedes the formation of demyelinated plaques, but at the same time it can contribute to progressive neurodegeneration. For these reasons, microglial activation has the potential to become a useful tool to monitor early neuroinflammatory processes, disease progression and response to treatment in MS.

Histopathological data have also evidenced the presence of microglial activation in cortical lesions, which included significant microglial distribution and association of microglia with neuronal elements (Peterson et al., 2001).

1.2 Neuroimaging hallmarks of inflammation in Multiple Sclerosis

The most frequently used imaging tool to identify *in vivo* biomarkers in MS is MRI. MRI has an established role in the diagnosis of MS, and has been used to investigate the natural course of the disease and monitor treatment effects in clinical trials. In relapsing-remitting MS patients, the effect of a treatment on MRI-based measures and its effect on the frequency of relapses and short term progression of disability are strongly correlated, supporting the use of MRI parameters as surrogates for clinical end points (Sormani et al., 2014; Sormani et al., 2009).

However, changes in MRI measures of inflammatory activity correlate weakly with disability progression in secondary-progressive MS (Goodin, 2006). At high levels of disability, a plateauing relationship is commonly observed between T2-weighted hyperintensities and disability, indicating that other factors should be considered when assessing the more advanced phase of the disease (Li et al., 2006).

Although data derived from post-mortem pathology suggest widespread cortical involvement in MS, it is currently difficult to accurately assess the contribution of cortical pathology with routinely available MRI imaging techniques. T1-weighted and T2-weighted MRI detects only a minority of cortical lesions. This may be due to the different pathophysiology of cortical grey matter lesions, compared to those in the white matter, with less inflammatory cell infiltration and absent BBB damage (Peterson et al., 2001). A detailed study comparing the number of cortical lesions detected by histopathology and post-mortem MRI, applying dual-echo T2-weighted spin-echo images and Fluid Attenuated Inversion Recovery (FLAIR), yielded detection rates of FLAIR images of only 5% for intracortical, 41% for leukocortical,

and 38% for deep grey matter lesions (Geurts et al., 2005a). The detection of periventricular and juxtacortical lesions has been greatly improved with double inversion recovery and phase sensitive inversion recovery (Calabrese et al., 2010; Geurts et al., 2005b; Nelson et al., 2007), however intracortical and subpial lesions remain difficult to detect with current MR imaging resolution. The use of high-field 7 Tesla MRI also holds promises to improve the detection of cortical lesions (Tallantyre et al., 2008; Tallantyre et al., 2010).

Despite the importance of MRI in transforming the clinical diagnosis of MS and the development strategies for new drugs, MRI measures are also limited by their lack of neuropathological specificity. For example, hyperintensities on T2-weighted images non-specifically reflects edema, demyelination or astrogliosis (Arnold and Matthews, 2002).

Microglia constitute an attractive cellular target with an improved pathological specificity for imaging of neuroinflammation. Also, the possibility of visualization of activated microglia would have the potential of enabling the characterisation of neuroinflammatory involvement in normal appearing white matter and in cortical grey matter as well, which can ultimately contribute to predict disease progression and study the relationship between cortical pathology and related clinical symptoms, such as emotional and cognitive alterations.

1.3 Targets for molecular imaging in Multiple Sclerosis

Molecular imaging, based on the *in vivo* administration of radiolabelled ligands that bind selectively to a target of interest, offers the potential of high specificity for molecular markers of cellular and metabolic processes. The high selectivity of Positron Emission Tomography (PET) allows microdosing of the radiotracer, ensuring high safety and tolerability for the subjects who undergo the procedure. Currently, there is a great potential for the application of molecular imaging in MS in the assessment of inflammatory responses, myelin imaging and quantitative measurement of neuronal function. Molecular imaging could be also used to test novel therapeutics, in order to ascertain whether they reach the target and have the desired pharmacodynamic action (Matthews et al., 2011).

Although for more than a decade there has been a great interest and intense research in the identification of suitable PET targets for imaging the brain in MS, only a limited number of targets have been investigated in living patients to date. The most extensively characterised PET target is the 18-kDa Translocator Protein (TSPO). Preliminary evidence also indicates the potential use of the adenosine 2A receptors (A2ARs) and some myelin components as additional PET targets for imaging in MS. Other targets hold promise for future application, for example receptors such as the gamma-aminobutyric acid-A (GABA_A) receptor, cannabinoid-2 (CB₂) receptor, acetylcholine and some purinergic receptors, and cellular components such as phosphatidylserine. So far, however, these have been only tested *in vitro*, in preclinical experimental models of autoimmune encephalomyelitis, or in neuroinflammatory and neurodegenerative conditions other than MS.

1.4 The Translocator Protein

1.4.1 *Physiology and expression in the brain*

The TSPO was formerly known as the peripheral benzodiazepine receptor (PBR). It was identified as a benzodiazepine receptor that was pharmacologically, anatomically, structurally, and physiologically distinct from the classic benzodiazepine receptor found in the CNS. The TSPO is a protein localized on the outer mitochondrial membrane, and although is primarily located in the mitochondria, it has been also found in red blood cells (which are devoid of mitochondria) or in the cell membrane and in the nucleus and perinuclear area in different cell lines (Chen and Guilarte, 2008).

The TSPO has several biological functions, the most characterised being the control of cholesterol transport associated with steroid synthesis. The TSPO is abundantly expressed in steroidogenetic tissues where it mediates transport of cholesterol from cytosol into mitochondria (Papadopoulos et al., 2006), where the synthesis of steroids from cholesterol takes place. The other physiological functions attributed to the TSPO are heme biosynthesis, control of autophagy and apoptosis, synthesis of bile acids, calcium flow, production of reactive oxygen species, chemotaxis and cellular immunity, and mitochondrial respiration [for review see (Chen and Guilarte, 2008)]. The TSPO appears to be involved in basic housekeeping functions and was hypothesized to be essential for embryonic development, although a recent report suggested that TSPO global knock-out mice are viable and are capable of steroidogenesis (Tu et al., 2014).

A number of endogenous ligands that bind to the TSPO have been proposed. One putative endogenous ligand is the diazepam binding inhibitor or endozepine (Guidotti

et al., 1983). Cholesterol is also considered an endogenous ligand for the TSPO with nanomolar affinity (Lacapere et al., 2001). Other potential endogenous ligands are the porphyrins (protoporphyrin IX, mesoporphyrin IX, deuteroporphyrin IX, hemin), which exhibit a very high affinity for the TSPO (Snyder et al., 1987).

In normal conditions the TSPO is highly expressed in peripheral tissues, particularly where steroids are synthesized. In the normal human brain, the expression of the TSPO is low. A study mapped the cellular localization and the degree of expression of the TSPO by immunohistochemistry in post-mortem brain tissue of subjects without neurological conditions. The TSPO was mainly expressed in macrophages and microglia, astrocytes, oligodendrocytes, endothelial cells and smooth muscle cells. TSPO was present in subpial and subependymal glia, meninges (vessels, macrophages and sometimes arachnoid cells), ependymal cells, and choroid plexus. Some punctate staining was found in neuronal cell bodies too (Cosenza-Nashat et al., 2009).

Autoradiography studies using radiolabelled TSPO ligands demonstrated that the expression of the TSPO is enhanced on activated microglia and, in the case of a disrupted BBB, on invading cells of mononuclear-phagocyte lineage, including macrophages and monocytes (Chen and Guilarte, 2008; Venneti et al., 2006). Some studies have suggested the presence of increased TSPO expression on astrocytes, however the relative contributions of astrocytes versus microglia to the binding of TSPO radioligands in neuroinflammation has been a subject of continuing debate (Venneti et al., 2006).

The lack of cellular specificity of TSPO binding implicates that various cell types are likely to contribute significantly to the signal obtained by PET with TSPO-specific radioligands. The global detectable signal is most likely resulting from the

combination of TSPO radioligand binding to microglia, macrophages, monocytes, astrocytes and endothelial cells. A recent study (Rizzo et al., 2014) suggested that the presence of TSPO binding sites on the endothelial layer of blood vessels is of particular interest for imaging purposes, as it might potentially affect the accuracy of quantification of PET data using a second-generation high affinity TSPO PET radioligand.

The mechanisms responsible for increased TSPO expression in microglia in the CNS are not known. In the periphery, such as pancreatic islet cells, Leydig cells, and cultured astrocytes, proinflammatory cytokines cause an increase in TSPO mRNA and TSPO ligand binding. It has been proposed that the cellular upregulation of TSPO may serve as a protective strategy against cytokine toxicity. Since activated microglia themselves are sources of cytokines, the increase in TSPO expression has been proposed to represent an autocrine-paracrine phenomenon (Venneti et al., 2006). A recent study demonstrated that specific TSPO ligands increased the proliferation and phagocytosis of resting microglial cells and led to increased production of reactive oxygen species, while, conversely TSPO ligands reduced the expression of proinflammatory genes and cytokine release of activated microglia (Choi et al., 2011).

1.4.2 Autoradiography studies in Multiple Sclerosis

To ascertain whether increases in TSPO radiotracers binding are specific to activated microglia in the brain of MS patients, a number of autoradiography studies have been conducted in post-mortem brain tissues obtained from MS patients. Focal increases in binding of the TSPO radioligands ^3H -PK11195, ^3H -DAA1106 and ^3H -PBR28 have been found in the brain of MS patients (Banati et al., 2000; Benavides et al., 1988; Owen et al., 2010; Venneti et al., 2008). Banati and coworkers demonstrated the

highest ^3H -PK11195 binding in MS plaques, but areas of increased binding were also found on activated microglia outside the histologically-defined borders of MS plaques, as well as in areas of normal appearing grey matter. These experiments have been also able to elucidate the relative contribution of reactive astrocytes and activated microglia to the binding of TSPO ligands, as most of the studies showed that the increased binding of ^3H -PK11195 and ^3H -DAA1106 was mostly co-localized with immunohistochemistry markers of activated microglia, rather than astrocytes (Banati et al., 2000; Owen et al., 2010; Venneti et al., 2008; Vowinckel et al., 1997). Further, these studies demonstrated that ^3H -DAA1106 and ^3H -PBR28 bind to TSPO with higher affinity for TSPO, relative to ^3H -PK11195.

Based on the evidence from autoradiography studies that TSPO radiotracer binding reflects presence of activated microglia, PET with TSPO radiotracers has been used to visualize neuroinflammatory processes *in vivo*. Figure 1.1 illustrates the use of TSPO PET imaging to visualize microglia, reflecting neuroinflammatory pathology, in MS patients.

Post-mortem studies	Radioligand	Findings
Benavides et al., 1987	³ H-PK11195	Areas of high specific binding colocalized with demyelinating plaques within the white matter
Vowinckel et al., 1997	³ H-PK11195	Binding correlated with CD68-labeled activated microglia
Banati et al., 2000	³ H-PK11195	Areas of high specific binding colocalized with demyelinating plaques within the white matter and with oil red O-positive macrophages and EBM11-positive microglia
Venneti et al., 2008	³ H-PK11195 ³ H-DAA1106	Binding correlated with CD68-labeled activated microglia
Owen et al., 2010	³ H-PK11195 ³ H-PBR28	Areas of high specific binding colocalized with demyelinating plaques within the white matter

Table 1.1 TSPO autoradiography studies

Correlation between TSPO radioligand binding and correlation with immunohistochemistry markers of activated microglia and demyelination in post-mortem MS brain.

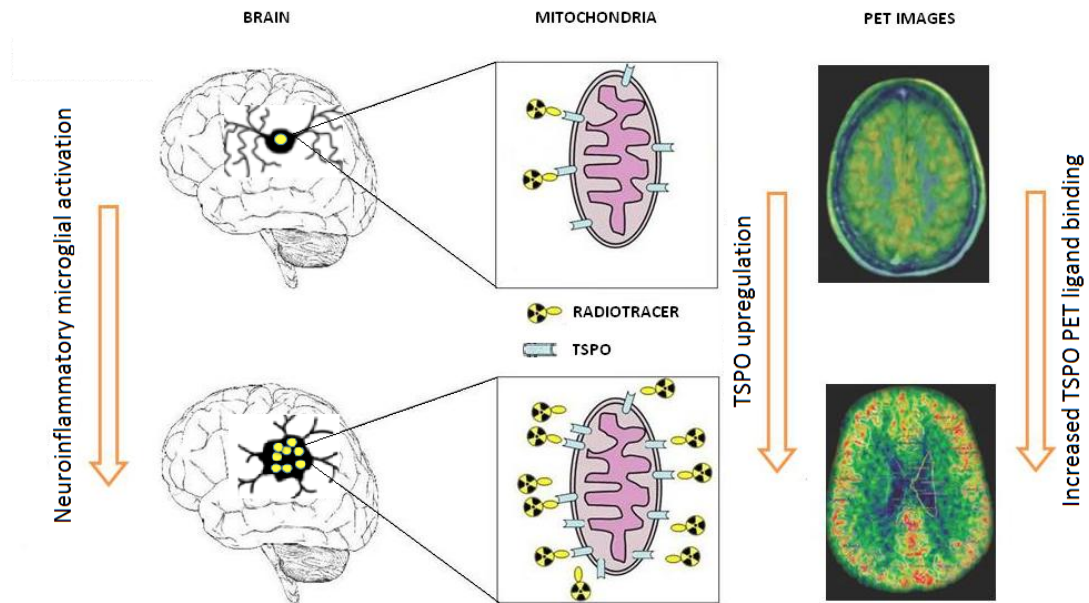


Figure 1.1 Schematic representation of the rationale underlying the use of TSPO PET imaging to visualize neuroinflammation in MS

The activation of microglia due to neuroinflammatory processes is associated with the upregulation of TSPO, leading to the increase of available TSPO for PET radioligand binding. The brain images on the right have been obtained with PET and ^{11}C -(R)-PK11195 in a healthy volunteer (top) and a patient with MS (bottom) (Politis et al., 2012).

1.4.3 First generation TSPO PET studies in Multiple Sclerosis

The most studied TSPO radiotracer is ^{11}C -(R)-PK11195, which has been used for more than a decade. Newer radiotracers have been recently tested, and preliminary data on their application as tools for imaging in MS is now available.

Studies have showed an increased uptake of ^{11}C -(R)-PK11195 in various neurological disorders other than MS, namely Human Immunodeficiency Virus encephalitis, Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple system atrophy, stroke, amyotrophic lateral sclerosis, CNS neoplasms, and traumatic brain injuries (reviewed in Cagnin et al., 2007 and Venneti et al., 2006).

Nine ^{11}C -(R)-PK11195 PET studies in MS patients have been reported. The studies are summarized in Table 1.2. In general, an increased ^{11}C -(R)-PK11195 uptake was found in MS brain compared to matched healthy controls. While in healthy volunteers, the brain uptake of ^{11}C -(R)-PK11195 was found to be extremely low, in MS patients areas with increased uptake were consistently observed to correspond to white matter Gadolinium-enhancing lesions (Banati et al., 2000; Debruyne et al., 2002; Debruyne et al., 2003; Vowinckel et al., 1997). An increased ^{11}C -(R)-PK11195 binding has been observed in the majority of hypointense T1-weighted MRI lesions (Giannetti et al., 2014). Moreover, studies indicated that there were focal areas of increased ^{11}C -(R)-PK11195 uptake also in normal appearing grey and white matter areas, thalamus, and periventricular white matter indicating that TSPO PET was able to visualize also diffuse inflammatory processes in areas which appeared normal on MRI (Banati et al., 2000; Rissanen et al., 2014). The overlap between MRI lesions and increased radioligand uptake was higher during relapses (Banati et al., 2000), consistent with the role of activated microglia in active acute MS lesions (Peterson et

al., 2001). A positive correlation has been suggested between ligand uptake and disease duration, disability, and brain atrophy (Banati et al., 2000; Debruyne et al., 2003; Versijpt et al., 2005), however the correlations were not consistently replicated across different studies. Also, the observed differences between MS patients versus controls in ^{11}C -(R)-PK11195 normal-appearing grey and white matter parenchymal binding have not been consistent across studies. Although the studies by Banati found an increased radioligand uptake in the thalamus and brain stem of MS patients compared to healthy controls (Banati et al., 2000; Rissanen et al., 2014), in Debruyne's study the authors didn't find any between-groups differences in the areas outside lesions (Debruyne et al., 2003). Also, the latter study found a decreased uptake in T2-lesions compared to normal appearing tissue, while an increased ^{11}C -(R)-PK11195 uptake was associated with relapses. The authors suggested that the upregulation of TSPO may be transient, however issues related to different quantification methods may account for the differences found in this study compared to others.

Interestingly a recent study, which was specifically focused on cortical pathology, found an increase in cortical grey matter ^{11}C -(R)-PK11195 uptake in MS patients relative to healthy controls, which was more pronounced in secondary progressive than relapsing-remitting patients (Figure 1.2). There was a significant correlation between high cortical ^{11}C -(R)-PK11195 uptake and disability as measured with EDSS, and this relationship was stronger for the subgroup of secondary progressive MS patients (Politis et al., 2012). The distribution of increased signal was not uniform across the cortical grey matter, and the cortical regions with highest increase included pre-central and post-central gyri. The uptake in the white matter was also increased in MS patients relative to controls, however there was no relationship between disability

and white matter uptake. This *in vivo* evidence of cortical involvement in MS pathology, predictive of MS disability, is consistent with recent neuropathological studies that described the presence of subpial grey matter demyelination secondary to the presence of substantial meningeal inflammation in some cortical sulci (Magliozzi et al., 2010). Importantly, meningeal inflammation was associated with an increased density of activated microglia and substantial neuronal loss in grey matter lesions of the precentral gyrus of secondary progressive MS patients (Howell et al., 2011; Magliozzi et al., 2007; Magliozzi et al., 2010), which is consistent with the findings of the PET imaging evidence (Politis et al., 2012).

Only one longitudinal study has been conducted so far. The authors investigated the effects of 1 year-treatment with glatiramer acetate, an immunosuppressive agent commonly used as a treatment of relapsing-remitting MS, on the uptake of ^{11}C -(R)-PK11195 in nine previously untreated relapsing-remitting MS patients. A significant decrease was noted in cortical gray matter and cerebral white matter, and a trend towards decreased uptake was seen in the putamen and thalamus (Ratchford et al., 2011). However, these finding should be considered preliminary as the study didn't include a control group and the sample size was small.

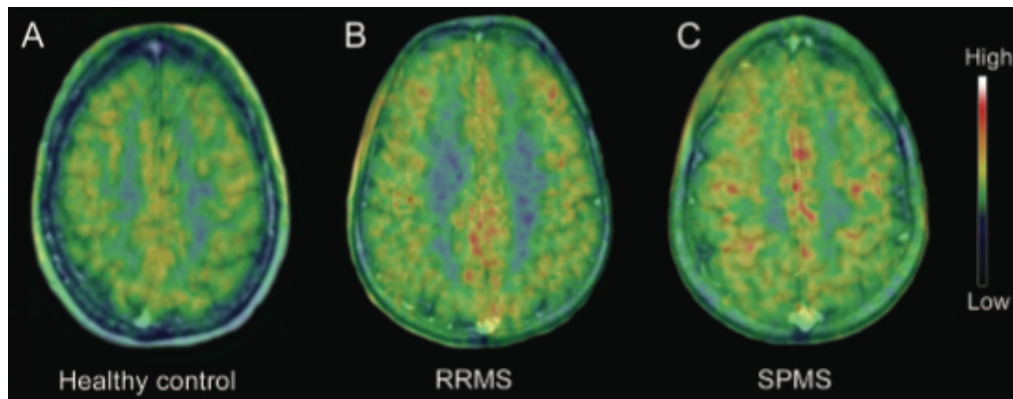


Figure 1.2 Examples of ^{11}C -(R)-PK11195 PET images

These images display the TSPO density in the brain of (A) a healthy 40-year-old woman, (B) a 46-year-old man with a 12-year history of relapsing-remitting MS and (C) a 46-year-old woman with secondary progressive MS and a 26-year disease duration (from Politis et al., 2012).

The first and best characterised TSPO tracer ^{11}C -(R)-PK11195 is hampered by a poor signal-to-noise (SNR) ratio, which complicates its quantification (Banati et al., 2000; Shah et al., 1994). This is a result of a combination of factors, the most significant of which is that ^{11}C -(R)-PK11195 binds non-specifically to other sites in addition to TSPO (Owen et al., 2010). Moreover, ^{11}C -(R)-PK11195 also suffers from low brain penetration (Debruyne et al., 2003; Vas et al., 2008). As a consequence of these factors, only very low signal from ^{11}C -(R)-PK11195 is found in the normal brain, resulting in a poorly defined pattern of binding.

The quantification of ^{11}C -(R)-PK11195 is further complicated by the ubiquitous distribution of TSPO, with a lack of a region that is devoid of TSPO binding. This complicates the definition of a normal reference tissue and becomes particularly problematic in diseases that are disseminated throughout the brain, such as MS.

The most common method used for quantification of ^{11}C -(R)-PK11195 brain uptake is cluster analysis, which was developed originally by the Hammersmith Hospital group in London. To derive a pseudo-reference Time Activity Curve (TAC), ^{11}C -(R)-PK11195 binding in control subjects has been used to represent a minimum or basal level of TSPO binding. It has been assumed that ^{11}C -(R)-PK11195 uptake in control subjects would have a similar shape to a reference TAC, representative of normal tissue, in patients. By applying cluster analysis to patient studies, several TACs could be extracted and their shapes were compared against the control curve. The cluster TAC that appears most consistent with the control TAC is used as a reference curve for the patient study. A refined method, named Supervised clustering analysis (SuperPK) (Turkheimer et al., 2007) has been based on the characterisation of kinetic classes of ^{11}C -(R)-PK11195 uptake. Other modelling techniques include the normalisation to either the white or grey matter. The former is based on the notion

that ^{11}C -(R)-PK11195 uptake in the white matter provides a stable level activity that can be used as a reference for evaluating the binding level in other regions. The latter consists of sampling different grey matter regions, with the region with lowest determined uptake taken as the reference region (Debruyne et al. 2003).

Blood input compartmental modelling has been also used with a blood input function, which is always considered the gold standard method of quantification. However, the accurate quantification of a blood input function was found to be complicated due to the highly variable kinetic behavior of ^{11}C -(R)-PK11195 in plasma. This unstable kinetic behavior has been attributed to variable plasma protein binding, in particular high binding to alpha1-acid glycoprotein (Lockhart et al., 2003). This can potentially contribute to the access of ^{11}C -(R)-PK11195 to the brain parenchyma in diseases with BBB breakdown, such as MS, therefore providing a source of brain uptake of ^{11}C -(R)-PK11195 which is independent of TSPO.

Due to the limitations reported above, there has been great interest in the development of newer TSPO radioligands with improved imaging characteristics.

1.4.4 Second generation TSPO PET radioligands

Only three TSPO PET radiotracers, other than ^{11}C -(R)-PK11195, have been used in MS patients to my knowledge. A study compared the uptake of ^{11}C -(R)-PK11195 and that of the tracer ^{11}C -vinpocetine in 4 MS patients. The global uptake of vinpocetine was approximately 50% higher compared to ^{11}C -(R)-PK11195. The regional and the perilesional uptakes were also investigated. The binding was higher for ^{11}C -vinpocetine in the frontal lobe, thalamus, and perilesional regions. Interestingly, the correlation between the binding values of the two tracers within the same regions was weak. Also, the kinetic behaviours of the ligands were also different in the

perilesional region, perhaps indicating that the two ligands bind differentially to the TSPO (Vas et al., 2008). However, the limitations inherent to small sample size and the choice of the reference region may have accounted for the discrepancies observed.

The recently developed TSPO radioligand ^{11}C -PBR28, with high affinity for TSPO in vitro and high brain penetration, was applied to MS patients in a study by Oh and coworkers (2010). Eleven MS patients and 7 healthy controls were studied, and a follow-up scan in 6 MS patients was also obtained. The results indicated that parenchymal ^{11}C -PBR28 binding did not differ between MS patients and healthy controls, however an altered distribution of TSPO expression in the MS brain was suggested, based on a higher white matter-to-grey matter ratio of ^{11}C -PBR28 uptake in MS patients relative to controls. No relationship was observed between ^{11}C -PBR28 binding and MRI-based lesion load. Focal increased binding was observed to correspond to Gadolinium-enhancing lesions, and, importantly, the comparison between baseline and follow-up scans indicated that increased focal ^{11}C -PBR28 binding preceded the development of new Gadolinium-enhancing lesions.

A recent investigation using the novel tracer ^{18}F -FEDAA1106 in 9 MS patients in acute relapse and 5 healthy controls, also did not report any significant differences between patients and controls in any examined region of interest, and there was no evidence of any higher uptake in MRI-identified MS lesions relative to surrounding areas (Takano et al., 2013).

Other potential novel TSPO radiotracers that are promising for application in MS patients include: ^{18}F -PBR111, ^{11}C -DPA713 and ^{18}F -PBR06, which are promising ligands tested in autoradiography assays on post-mortem MS brain tissues (Owen et al., 2011) and evaluated through ^{11}C -DAC and the SPECT tracer ^{123}I -CLINDE have

been successfully tested in the rodent model of MS, the experimental autoimmune encephalomyelitis. There are a variety of other TSPO ligands which are currently tested in healthy subjects and for other neurological conditions, for a comprehensive review see (Chauveau et al., 2008).

Some of the 2nd generation TSPO radioligands that have been introduced offer higher specific to non-specific signal. A study of our group, using a biomathematical model based on *in silico/in vitro* data (Guo et al., 2009; Guo et al., 2012) predicted that the 2nd generation TSPO radioligands ¹⁸F-PBR111, ¹¹C-PBR28, and ¹¹C-DPA713 would perform better *in vivo* than ¹¹C-(R)-PK11195 and that these radioligands would give greater power to detect group differences than ¹¹C-(R)-PK11195, and as a result, fewer subjects may be required to detect mild-to-moderate TSPO increase in neurological populations (Guo et al., 2012).

1.4.5 Variation in the affinity of novel radioligands for TSPO

The use of 2nd generation TSPO radioligands, in particular ¹¹C-PBR28, became complicated by the discovery of the existence of apparent “non-binders”, subjects that appear not to bind ¹¹C-PBR28. In vitro radioligand binding studies using post-mortem human brain tissue revealed substantial population variation in the affinity of ¹¹C-PBR28 for the TSPO. “Low Affinity Binders” (LABs) have a marked reduction in affinity for ¹¹C-PBR28 ($K_i \sim 188$ nM) in comparison to “High Affinity Binders” (HABs, $K_i \sim 3.4$ nM). Hence LABs do not produce a measurable specific signal in PET studies with ¹¹C-PBR28 and appear as “non-binders”(Owen et al., 2010). A third group was also identified [“Mixed Affinity Binders” (MABs)], who express the HAB and LAB binding sites in approximately equal number (Owen et al., 2010). This variation in binding affinity is not specific to ¹¹C-PBR28; all the tested 2nd generation

TSPO radioligands behave in a similar manner (Owen et al., 2011). In contrast, ^{11}C -(R)-PK11195 binding in the brain doesn't vary between HABs, MABs, and LABs. The reason for this is not clear, but one hypothesis is that different binding sites within TSPO molecule, for different radiotracers, account for this difference between ^{11}C -(R)-PK11195 and 2nd generation tracers.

Interestingly, the variation in binding affinity of 2nd generation tracers was found to be due to a single nucleotide polymorphism (SNP), rs6971, in the gene encoding the TSPO (Owen et al., 2012), which causes a substitution of threonine and alanine at position 147 (Ala147Thr). This substitution is common in Caucasians: 49% of subjects are Ala-Ala (HABs), 42% are Ala-Thr (MABs), and 9% are Thr-Thr (LABs), but its prevalence varies across ethnic groups. Since the binding class is consistent within each subject for all the 2nd generation ligands tested to date, genotyping the rs6971 polymorphism can help the quantitative assessment of TSPO ligand binding, by allowing binding affinity to be predicted at screening. The functional role of the rs6971 SNP is unclear, however in a case-control study based on two large datasets, we previously found an association between this polymorphism and manifestation of Bipolar Disorder suggesting that rs6971 is a functional SNP (Colasanti et al., 2013).

Despite the possibility of predicting the classes of binding affinity at screening is very important for the application of 2nd generation ligands in basic and pharmacological research, the presence of a variation in binding affinity across the population may still be impractical for the clinical application of these tracers. For example, genotyping would be necessary in all the patients undergoing PET imaging with ^{11}C -PBR28, in order to distinguish those who are HABs from those who are MABs, and in approximately 10% of the population (LABs) the specific signal of ^{11}C -PBR28 would not be measurable in any case.

Author	Subjects	PET ligand	Results
Vowinckel et al. (1997)	2 MS	^{11}C -PK11195	High focal ^{11}C -PK11195 binding in MRI-Gadolinium active lesion
Banati et al. (2000)	12 MS; 8 HV	^{11}C -PK11195	Higher global and Gadolinium-active lesions binding in MS relative to HVs
Debruyne et al. (2003)	22 MS; 7 HV	^{11}C -PK11195	No group difference in global ^{11}C -PK11195 binding; higher binding in Gadolinium-active lesions relative to normal appearing brain. Higher binding in T2 lesions of MS patients during relapses compared to HVs. Positive correlation between ^{11}C -PK11195 binding and disease duration
Versijpt et al. (2005)	24 MS; 8 HV	^{11}C -PK11195	Positive correlation between ^{11}C -PK11195 binding in normal appearing white matter and brain atrophy. Negative correlation between binding in T2 lesions and atrophy
Vas et al. (2008)	4 MS	^{11}C -PK11195 ^{11}C -vinpocetine	Higher global uptake for ^{11}C -vinpocetine relative to that of ^{11}C -PK11195. Differences between ligands in binding in peri-plaque region
Oh et al. (2010)	11 MS; 7 HV	^{11}C -PBR28	No group difference in global ^{11}C -PBR28 binding; higher binding in Gadolinium-active lesions relative to normal appearing brain. Increased ^{11}C -PBR28 binding preceded appearance of Gadolinium-enhancement
Ratchford et al. (2012)	9 MS	^{11}C -PK11195	Decrease in global and regional ^{11}C -PK11195 binding after 1 year of glatiramer acetate treatment
Politis et al. (2012)	16 MS; 8 HV	^{11}C -PK11195	Higher cortical grey matter ^{11}C -PK11195 binding in secondary-progressive MS. In secondary-progressive MS, positive correlation between cortical grey matter binding and MS disability
Takano et al. (2013)	9 MS; 5 HV	^{18}F -FEEDA1106	No differences between MS patients and healthy controls
Giannetti et al. (2014)	19 MS	^{11}C -PK11195	Higher ^{11}C -PK11195 binding in 76% of black-holes. In secondary-progressive MS, positive correlation between black-holes binding and disability
Rissanen et al. (2014)	10 MS, 8HV	^{11}C -PK11195	Higher ^{11}C -PK11195 binding in periventricular & normal-appearing WM and thalamus in secondary progressive MS

Table 1.2 PET studies using TSPO radioligands in MS patients

1.5 Rationale and Aims of the thesis

1.5.1 Rationale for the choice of ^{18}F -PBR111

More than 50 candidate 2nd generation TSPO PET radioligands have been introduced in the last five years (reviewed in Chauveau et al., 2008). However, when the current project was designed, data on *in vivo* application were available only for a limited number of radioligands. Of these, a number had been tested in humans (^{18}F -FEDAA 1106; ^{11}C -PBR28; ^{11}C -AC5216; ^{18}F -PBR06; ^{11}C -DPA713; ^{11}C -Vinpocetine), while for others only data on application in rodents and non-human primates was available (^{18}F -PBR111, ^{18}F -FEPPA, ^{18}F -DPA714; ^{11}C -DAA1106).

The analysis of existing evidence from human and preclinical applications, as well as *in vitro* observations of our group, led us to select ^{18}F -PBR111 for initial evaluation in studies of MS patients and healthy controls. Fluorine 18-radiolabelled tracers offer the advantage over similar carbon-11 molecules of a longer radioisotope half-life that could allow the use of the radiotracer also in PET centers where *de novo* radiosynthesis is not possible.

Initial characterisation in biodistribution studies in rodents suggested ^{18}F -PBR111, amongst various fluorine-18 candidates in the public domain, had optimal characteristics in terms of selectivity and specificity of its signal for TSPO. In a study using an acute lesional model of neuroinflammation in rats, ^{18}F -PBR111 was found to be metabolically stable and display high *in vivo* binding to TSPO (Fookes et al., 2008). Furthermore, a pilot study in baboons demonstrated that ^{18}F -PBR111 produced a robust TSPO-specific, displaceable signal in the brain (Carson et al., 2010).

Autoradiography work by our group showed that differences in binding affinity between HABs, MABs, LABs were substantial across all the 2nd generation TSPO ligands tested (DAA1106, DPA713, PBR06, PBR111, PBR28), however PBR111, together with DAA1106 and DPA713 displayed the smallest difference in affinity between HABs and LABs. The *in vitro* affinity of PBR111 in LABs was 4 fold smaller than that in HABs, however our prediction was that the affinity in LABs would be still sufficiently high ($K_i=62$ nM) to yield a measurable signal *in vivo*.

The possibility of obtaining measurable signal in LABs differentiates ¹⁸F-PBR111 from another excellent radiotracer, ¹¹C-PBR28, that displays an extremely high *in vitro* affinity to TSPO in HABs ($K_i=3$ nM), but very low in LABs ($K_i=237$ nM), which is unlikely to be sufficient for *in vivo* measurements. Taken together, the observations from *in vitro* work led to the prediction that the use *in vivo* of ¹⁸F-PBR111 would require correction for variation in binding affinity, similarly to that of all other 2nd generation radiotracers, and would allow the inclusion of LABs, as well as HABs and MABs subjects, in my PET study.

Our prediction was that ¹⁸F-PBR111 would be a more sensitive radiotracer compared to the 1st generation TSPO radioligand ¹¹C-(R)-PK11195. An analysis by our group, using a biomathematical approach based on *in vitro* data, predicted that the within-subject variability for ¹⁸F-PBR111 would be lower than that of ¹¹C-(R)-PK11195 for HABs and MABs in both normal and diseased states (Guo et al., 2012). This implied that for between-subject studies may demand smaller sample sizes with ¹⁸F-PBR111 to detect significant differences in TSPO density when binding class information is known *a priori*. At the beginning of the recruitment of participants in my study, it was discovered that binding class could be easily identified using a single genetic test

from peripheral blood assays, making the stratification of subjects across binding affinity classes much simpler.

A recent study by Mattner and coauthors examined the binding of ^{18}F -PBR111 in experimental autoimmune encephalitis (EAE), a rodent model of relapsing-remitting MS (Mattner et al., 2013). Combined postmortem histologic analysis and in vivo PET imaging with ^{18}F -PBR111 revealed an increased radioligand uptake in EAE that co-localized with markers of activated microglia and macrophages, and paralleled the temporal dynamics of their up regulation during experimentally-induced relapses. In summary, preclinical evidence strongly indicates that ^{18}F -PBR111 is a suitable PET radiotracer for imaging the innate immune response in relapsing-remitting MS patients.

The study presented in this thesis is the first to use ^{18}F -PBR111 in human subjects to my knowledge. The safety of ^{18}F -PBR111 administration in human subjects was assessed through the evaluation of preclinical dosimetry and toxicology data. Dosimetry data were acquired from a study in adult male baboons and extrapolated to human application (Verschuer et al., 2012). Based on these data the effective dose of ^{18}F -PBR111 was estimated at 0.02 mSv/MBq, leading to an exposure of a maximum effective dose of approx. 4 mSv yielded by administration of 200 MBq of ^{18}F -PBR111 for each PET scan per participant. With regard to toxicology data, the Australian Nuclear Science and Technology Organization (ANSTO) has investigated the safety of PBR111 in an extended single-dose toxicology study in rats and reported no toxic or mutagenic effects (ANSTO, Personal Communication). Based on these data, and taking into consideration factors such as feasibility of manufacture, safety margins and levels which do not produce a pharmacological effect, it was established

that the maximum clinical mass dose to be administered in human subjects was 10 μ g, which was deemed largely sufficient for my PET imaging trials.

1.5.2 Rationale for the integration of different imaging modalities

My study combined PET imaging with structural and functional MRI. Subjects underwent the MRI scan on the same day as the PET scan. PET imaging suffers from limited spatial resolution and lack of the spatial information necessary for precise localization of anatomical regions of interest. In order to allow the quantification of PET signal in specific anatomical regions, MRI images are usually acquired in conjunction with PET to allow co-registration (see details in Chapter 2) of the MRI as an anatomical reference.

Additionally, the use of structural MRI in patients with MS allows the anatomical localization of demyelinating lesions. The typical MRI hallmarks of pathology in MS are hyperintense areas observed with T2-weighted FLAIR sequences. Abnormal T2-signal detected on MRI correspond to MS demyelinating plaques at postmortem examination (Stewart et al., 1984).

Imaging hallmarks of acute relapsing MS are the Gadolinium-enhancing lesions. T1-weighted scans using Gadolinium enhancement reflect the presence of local disruption of the BBB with concomitant inflammatory mononuclear-cell infiltrates. The rationale of Gadolinium-enhancement is that Gadolinium-chelates do not cross an intact BBB while in the presence of BBB leakage the Gadolinium-chelate enters the interstitial CNS compartment. This will result in an increase of the T1-weighted MRI signal relative to that observed with an intact BBB. Based on structural T2-FLAIR and post-Gadolinium T1-MRI images, I have assessed the TSPO PET signal both

within and outside demyelinating and acutely inflammatory lesions in the white matter of MS patients (presented in Chapter 4).

We have also acquired Magnetization Transfer parameters, in order to better characterise inflammatory changes occurring outside lesions. Magnetization Transfer contrast represents proton interactions between free fluid and macromolecules, such as myelin, and could be used as a marker of ultrastructural brain tissue integrity. The most widely used Magnetization Transfer contrast parameter is the magnetization transfer ratio (MTR), representing the percentage reduction of MRI signal when applying off-resonance radiofrequency irradiation. The combined use of MTR and T2-FLAIR, presented in Chapter 4 of this thesis, aimed to allow the segmentation of the white matter in lesional and non-lesional areas (based on T2-FLAIR parameters), and the ulterior classification of non lesional-areas into normal and abnormal areas (see Chapter 4 for further details). The intensity of ^{18}F -PBR111 PET signal was measured and compared across all these MRI-defined regions of interests.

My study also included the use of functional MRI (fMRI), based on the blood oxygen level dependent (BOLD) signal. fMRI gives an indirect measure of neuronal function, specifically consisting in aggregate neuronal excitation and inhibition in grey matter circuits (Logothetis, 2008). MS lesions may affect the function of neuronal networks in several ways: early acute inflammatory responses lead to permanent axonal changes (Trapp et al., 1998); furthermore, fMRI studies highlighted an adaptive and compensatory role of brain circuit plasticity in response to brain lesions that can limit the clinical expression of the disease (Cifelli and Matthews, 2002; Reddy et al., 2000). By measuring patterns of spontaneous fluctuations of BOLD signal during resting state in the absence of a task, fMRI can be used to inform on the architecture and interconnectivity of distributed brain networks, specifically the degree of functional

correlation between distant areas. Resting-state functional imaging has the advantage of a short acquisition time (i.e. less than 10minutes) therefore the addition of resting-state fMRI sequences causes only minimal additional discomfort to patients already undergoing imaging procedures. Alterations in functional connectivity have been identified in MS, as well as in many other diseases, even in the absence of evident structural modifications, indicating high sensitivity of the method (Barkhof et al., 2014). I studied the functional connectivity of the hippocampus using resting-state fMRI (see chapter 6), to provide an indirect measure of neuronal function, and explore the neuronal mechanisms underlying the association between hippocampal neuroinflammation and manifestation of affective symptoms in MS patients.

1.5.3 Aims of the thesis

Neuroimaging techniques allows the study of the correlation between brain pathology and the manifestation of clinical symptoms, in vivo, in patients with MS. MRI has been extensively used and represents the gold standard method for diagnosis, assessment of natural course of disease and monitoring of treatment efficacy in MS patients. However the abnormalities identified with MRI reflect a wide range of underlying neuropathological processes and MRI-based measures explain only a modest part of the variability associated with clinical expression of MS. Examples of this are the poor correlation between extent of radiological involvement and rate of development of disabilities (the clinico-pathological paradox), and the weak association between imaging findings and manifestation of non-motor implications of MS, such as the development of affective symptomatology.

Molecular imaging, using PET, is an alternative neuroimaging approach that allows a higher pathological specificity, and possibly precision, of the measurement. The first

examples of application of molecular imaging, and specifically the use of PET imaging to visualize microglia through TSPO targeting radiotracers, yielded promising results, although it was recognized that more research was necessary for identification of optimal PET radioligands with improved SNR compared to the first generation of radiotracers.

The aim of my PhD research project is to explore the potential of TSPO-targeted PET as a tool to visualize in vivo neuroinflammatory processes in MS patients. I selected a novel TSPO PET radioligand (^{18}F -PBR111), based on optimal in-vitro characteristics and promising preclinical applications, and tested it in a sample of healthy controls and patients with relapsing-remitting MS.

The study presented in the thesis is a pilot investigation and follows three principal study objectives, as detailed below. The findings of these analyses are presented separately in chapters 3 to 6.

Objective 1: Characterisation of the uptake of ^{18}F -PBR111 in the brain of healthy subjects (Chapter 3):

We examined the kinetic behavior of the radioligand in the brain and plasma, and tested whether it yielded a measurable specific signal in the healthy brain; we investigated the effects on ^{18}F -PBR111 signal of age and the genetic polymorphism of *TSPO* gene, rs6971, known to determine the affinity of most TSPO radioligands.

Objective 2: Characterisation of ^{18}F -PBR111 signal in lesional and non-lesional white matter in MS patients (Chapter 4):

^{18}F -PBR111 uptake was quantified across lesional and non-lesional areas of the brain white matter using a segmentation based on structural MRI measures, and was compared between MS patients and healthy controls, and across lesional and non-lesional white matter areas within MS patients; finally, I examined the relationship between rate of development of disability and TSPO signal in MRI-defined lesions.

Objective 3: Characterisation of ^{18}F -PBR111 signal in the hippocampus of MS patients and its relationship to depressive symptoms and neuronal function (Chapters 5 and 6):

I compared ^{18}F -PBR111 uptake in the hippocampus of MS patients to that of healthy volunteers, and explored the correlation between hippocampal ^{18}F -PBR111 signal and depressive symptoms (Chapter 5); in Chapter 6, I analysed the relationship between the strength of functional connectivity of the hippocampus to the rest of the brain measured with resting-state fMRI, and both hippocampal ^{18}F -PBR111 signal and depressive symptoms.

Chapter 2. Participants and general methodology

2.1 Design of the study

This was an open-label study in patients with relapsing-remitting MS and healthy volunteers. All subjects were genotyped for the rs6971 SNP, that determines variations in affinity between subjects (HAB, MAB, LAB) for second-generation TSPO radioligands (Owen et al., 2012).

The study took place at Imanova Centre for Imaging Sciences, Hammersmith Hospital, London. All participants underwent an MRI scan and an ^{18}F -PBR111 PET scan on the same day. Four healthy volunteers and one MS patient (all HABs) underwent a second ^{18}F -PBR111 PET scan on the following day in order to evaluate the reproducibility of the PET signal (presented in Chapter 4).

Disability was assessed by an experienced neurologist using the EDSS. The Multiple Sclerosis Severity Score (MSSS) was calculated based on EDSS scores and disease duration according to Roxburgh *et al.* (2005).

The study was approved by the Essex 1 Research Ethics Committee (Ref no. 11/EE/0026) and the Administration of Radioactive Substances Advisory Committee (ARSAC). All subjects gave written informed consent and were given financial compensation for the time spent in their participation in the study.

2.2 Study subjects

2.2.1 Healthy volunteers

Healthy controls were recruited from a database of volunteers. For healthy volunteers, exclusion criteria included any medical, neurological, or psychiatric illness at

screening or any concomitant pharmacological treatments. All structural MRI images were inspected for unexpected findings of potential clinical significance or features that might confound PET co-registration or quantitative analysis by an experienced clinical neuroradiologist.

Twenty-five healthy volunteers in total were studied. Three healthy subjects did not have a PET scan, due to the failure of PET radioligand synthesis (n=2) or failure of arterial catheterization (n=1). Out of the 22 subjects who completed the PET scan, 21 were included in the analysis presented in Chapter 3 for the characterisation of ^{18}F -PBR111 binding (the analysis of one subject's dataset was completed only after the completion of that study). Only 11 healthy volunteers, chosen to match MS patients for age and rs6971 genotype, were included in the case-control analysis presented in Chapter 4. The whole sample of 22 subjects with available PET data was used for the analysis presented in Chapter 5. Only fifteen healthy controls underwent fMRI scan and were included in the analysis presented in Chapter 6.

2.2.2 Multiple Sclerosis patients

Patients with MS were referred by consultant neurologists. All patients had relapsing-remitting MS based on neurological history and previous evaluations. No subject received steroid treatment in the month before the PET scans and patients receiving disease-modifying treatments did not change in treatment for at least 3 months prior to scanning.

Thirteen MS patients were included in total. For two patients, PET data could not be analysed due to failure of metabolite analysis. The remaining sample of 11 subjects was used for the analysis presented in Chapter 4 and 5. All 13 MS patients' data were used for the fMRI analysis presented in Chapter 6.

2.3 Magnetic Resonance Imaging

Subjects underwent MRI scanning on the same day as PET scanning, approximately 2 hours before the beginning of the PET scan. MRI data was acquired on a 3T Siemens Verio (Siemens Healthcare, Erlangen Germany) clinical MRI scanner equipped with a 32-channel phased-array head coil, over a period of 60 minutes. Data acquired included T1-weighted scans (with and without Gadolinium), T2-weighted FLAIR, MTR, and resting state fMRI data.

T1-weighted structural images were applied for PET co-registration and used the Alzheimer's Disease Neuroimaging Initiative recommended parameters with a parallel imaging factor of 2 in 5m:09s (Jack et al., 2008). T1-weighted images were acquired pre- and post- iv Gadolinium-chelate administration (0.1 mmol/kg Gadoteric Acid, Dotarem®). Resting-state fMRI data was acquired with a double-echo gradient echo EPI sequence, capturing 150 volumes in 5m:06s.

Details on specific protocols used for T2-weighted FLAIR images and MTR maps are presented in Chapter 4, while the protocol for the resting-state fMRI and functional connectivity analysis are presented in detail in Chapter 6.

2.4 Positron Emission Tomography

2.4.1 Radioligand synthesis

^{18}F -PBR111 was labelled with fluorine-18 by a simple one-step tosyloxy-for-fluorine nucleophilic aliphatic substitution, followed by purification by semi-preparative high-performance liquid chromatography (HPLC) and reformulation. The synthetic procedure of ^{18}F -PBR111 was adapted from a previously described method (Fookes et al., 2008). A fully automated procedure was developed in-house using a Siemens Explora GN module coupled with a semi-preparative HPLC system. Figure 2.1 illustrates the radiosynthesis of ^{18}F -PBR111.

^{18}F -Fluoride, produced using a Siemens RDS-111 Eclipse cyclotron equipped with a fluoride target loaded with oxygen-18 enriched water, was obtained by means of the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction. The ^{18}F -fluoride in solution in oxygen-18 enriched water was then trapped onto a DW-TRC-L trap and release cartridge (O.R.T.G.). Following that first step the fluoride was released into the reactor using 1.0 mL of a solution consisting of 8.3 mL of acetonitrile, 0.8 mL of water, 250 mg of K_{222} and 50 mg of K_2CO_3 . The content of the reactor was evaporated a first time and then the evaporation process was repeated 2 times following the addition of 1 mL of acetonitrile each time. The tosyloxy precursor (6.0-10.0 mg in solution in 1.0 mL of anhydrous acetonitrile) was then added to the reactor containing the “dry” ^{18}F -fluoride. The reaction mixture was heated for 5 min at 100 °C and then cooled to 50 °C before diluting with water. The crude reaction mixture was then diluted with water (3 ml) and loaded onto the HPLC injection loop for purification on an Agilent Eclipse XDB C18 column (5 μm , 250 $\times$ 9.4 mm) with ammonium formate buffer (pH 4)/ CH_3CN (55:45, v/v) at 9.5 mL/min. The fraction containing ^{18}F -PBR111 was

collected in water (20 ml) and loaded onto an activated Waters Sep-Pak[®] Classic C18 cartridge (Waters Corp.) for reformulation. Following an initial SepPak[®] column wash with water, ¹⁸F-PBR111 was eluted off the column with ethanol and 0.9% saline solution for injection to lead to ¹⁸F-PBR111 formulated in 11 mL of maximum 20% (v/v) ethanol in 0.9% saline solution for injection. In the final step a sterile filtration through a 0.2 µm sterile filter (Millipore Millex GV, 0.22 µm, 33 mm) was performed to deliver the final dose as a sterile and pyrogen-free solution.

Typically, the total ¹⁸F-PBR111 synthesis procedure, including HPLC purification and Sep-Pak[®]-based formulation, was accomplished in less than 60 min. Up to 4.2 GBq of ¹⁸F-PBR111 (>97% radiochemically pure, n>20) were obtained starting from 10 GBq of ¹⁸F-fluoride with a specific radioactivity of up to 480 GBq/µmol.

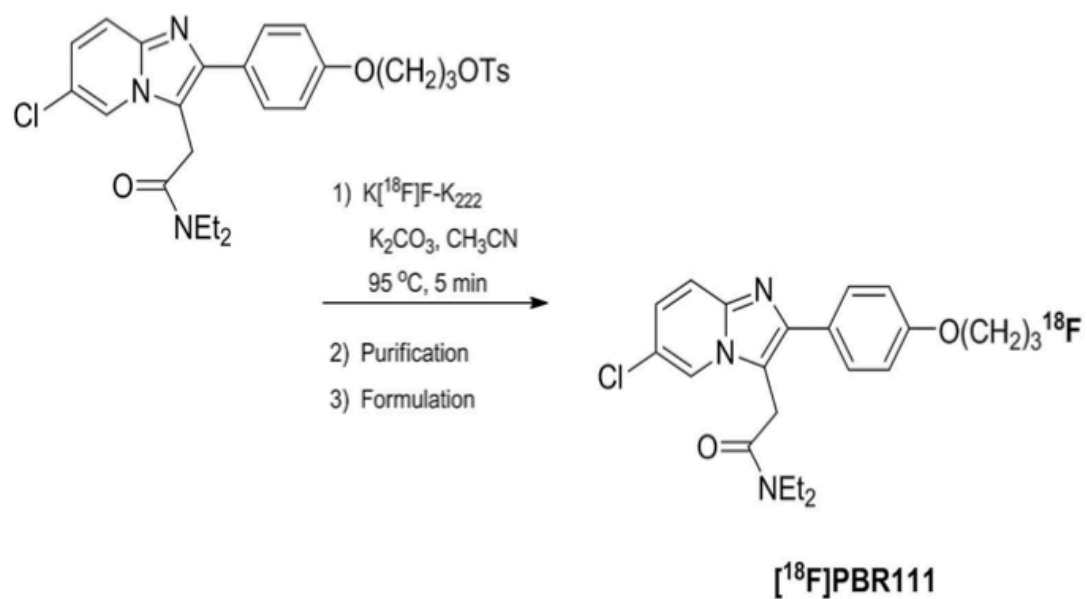


Figure 2.1 Radiosynthesis of ^{18}F -PBR111

2.4.2 PET protocol

Median injected mass of ^{18}F -PBR111 was 0.33 μg (range 0.16 – 8.66) in healthy volunteers and 0.50 μg (range 0.2 – 1.9) in MS patients.

PET data were reconstructed using filtered back projection including corrections for attenuation and scatter (based on a low-dose Computerised Tomography acquisition). Dynamic data were binned into 29 frames (durations: 8 x 15 s, 3 x 1 min, 5 x 2 min, 5 x 5 min, 8 x 10 min).

Arterial blood data were sampled via the radial artery to enable generation of an arterial plasma input function. A continuous sampling system (ABSS Allogg, Mariefred, Sweden) was used to measure whole blood activity each second for the first 15 minutes of each scan. Discrete blood samples were manually withdrawn at 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 minutes after scan start to facilitate measurement of whole blood and plasma activity. Samples taken at 5, 10, 15, 20, 30, 50, 70, 90 and 120 minute time points were also analysed using HPLC to determine the fraction of radioactivity corresponding to intact parent compound in arterial plasma. The first three discrete blood samples were used to calibrate the continuous blood data, and then the continuous and discrete data sets were used to form a whole blood activity curve covering the duration of the scan. Discrete plasma samples were divided by the corresponding whole blood samples to form plasma-over-blood (POB) data. A constant POB model was fitted (i.e. average of POB values). This POB value was then multiplied by the whole blood curve to generate a total plasma curve. Parent fraction data were fitted to a sigmoid model $f = ((1 - (t^3 / (t^3 + 10^a)))^b + c) / (1 + c)$ where t is time and a , b and c are fitted parameters. The resulting fitted parent fraction profile was multiplied by the total plasma curve and

then smoothed post-peak using a tri-exponential fit to derive the required parent plasma input function. For each scan, a time delay was fitted and applied to the input function to account for any temporal delay between blood sample measurement and the tomographic measurements of the tissue data.

2.4.3 *Image analysis*

Dynamic PET data were corrected for motion via frame-to-frame image registration and aligned with the individual's structural T1 MRI image using Statistical Parametric Mapping-5 (SPM5, Wellcome Trust Center for Neuroimaging, <http://www.fil.ion.ucl.ac.uk/spm>) with a mutual information cost function.

The Clinical Imaging Center neuroanatomical atlas (Tziortzi et al., 2011) was nonlinearly deformed into the individual's space, via T1-weighted MRI data mapping, to obtain a personalized anatomical parcellation of regions of interests. Details on Volumes of Interest (VOIs) used for each of the analyses are presented separately in each chapter. Each VOI was then applied to the dynamic PET data to derive regional TACs.

2.4.4 *Kinetic analysis*

A two-tissue compartment (2TC) kinetic model with a metabolite-corrected plasma input function was applied to the dynamic PET data using a fixed blood volume correction of 5%. For each VOI examined, the total Volume of Distribution (V_T) was estimated from the rate constants as described previously (Gunn et al., 2001). The Logan graphical method (Logan et al., 1990) employing a plasma input, using a fixed blood volume correction of 5% and a linear start time at 35 minutes was also used to estimate the V_T of each VOI and further applied at the voxel level to produce

parametric V_T maps. Model fitting and parameter estimation were performed using software implemented in Matlab R2008b (The MathWorks, Inc.).

The choice and definition of VOIs is explained separately for each analysis in Chapters 3 to 6.

A recent study using ^{11}C -PBR28 in healthy subjects (Rizzo et al., 2014) explored the use of an improved quantification model, based on the hypothesis of the existence of an additional irreversible component from the blood to the endothelium. The study findings suggested that the addition of that irreversible component in the model provides some potential advantages in terms of accuracy of measurements. Our study, however, is testing the use of ^{18}F -PBR111 for the first time in humans, and since there are no available data on the quantification of ^{18}F -PBR111, we prefer to choose the application of a simpler 2TC kinetic model that, in our view, relies on fewer assumptions and therefore is preferable for a pilot investigation. Since the use of the additional irreversible component for kinetic modeling, as suggested by Rizzo et al, has not been tested in clinical populations, we feel it is important that its use is first applied to patients using a better characterised tracer such as ^{11}C -PBR28, before its application to novel tracers.

2.5 Statistical analysis

The statistical analyses used in this thesis include both parametric and non-parametric statistical tests. The use of non-parametric statistics, including use of medians and ranges for descriptive statistics, non-parametric comparisons for paired and independent group comparisons, and Spearman's tests for correlations, was generally preferred considering the small sample size of our study population, and the non-normal distribution of the variables analysed in our studies. In fact, for smaller studies (e.g. with <30 cases), Altman (1999) has recommended use of a non-parametric tests to best guard against false positives (Type I error) unless there is confidence that the data are normally distributed, which we cannot have in this case. In fact, in our analysis of [^{18}F]-PBR111 PET scans in healthy controls we found that ^{18}F -PBR111 V_T was not normally distributed as demonstrated by visual inspection of data and Shapiro-Wilk normality test (White matter V_T : $p=0.032$; $n=21$), hence we preferred to describe the distribution of the V_T data using non-parametric statistics, and to use non-parametric statistics for comparison of V_T data between genetic groups (Chapters 3 and 4), between cases and controls (Chapter 4), and between VOIs within the same subjects. Similarly, we used non-parametric statistical tests for study of correlation between V_T data and clinical variables (Chapter 4) and for correlations of clinical variables to each other (Chapter 5). However, a possible concern can be that with non-parametric statistics Type II error rates (false negatives) might arise.

Parametric statistics was used in cases where a general linear model approach was needed (for example Analysis of Covariance (ANCOVA) in chapters 3 and 5), and for Linear Regression and partial correlation analyses, where the independent effects of multiple regressors on the variables of interest were studied (Chapters 3 and 5).

Chapter 3. Influence on genotype and age on ^{18}F -PBR111 *in vivo* binding signal in healthy volunteers

3.1 Introduction

Microglia activation may be used as an index of neuroinflammatory processes in imaging studies, hence a method to quantify microglia activation *in vivo* would prove of potential utility as a diagnostic tool or a pharmacodynamic marker in drug development.

PET has the unique ability to quantify microglial density in the human brain non-invasively, by targeting the TSPO. PK11195, a selective antagonist for the TSPO, has been labeled with ^{11}C , and used as PET radioligand for more than 15 years to image neuroinflammation (Cagnin et al., 2007). The ^{11}C -(R)-PK11195 signal was found to be elevated in a variety of neuroinflammatory brain diseases (Debruyne et al., 2003; Edison et al., 2008; Gerhard et al., 2005; Pavese et al., 2006). However, the low brain extraction and the poor SNR of ^{11}C -(R)-PK11195 images have led to the search for improved TSPO PET imaging agents (see Chapter 1 for further details).

In the past 5 years numerous 2nd generation TSPO ligands have been proposed (Chauveau et al., 2008; Endres et al., 2009; Kreisl et al., 2010; Mizrahi et al., 2012). Although these ligands were claimed to have increased delivery across the BBB or higher SNR over ^{11}C -(R)-PK11195, initial human studies have demonstrated considerable variability in their binding across subjects, including the presence of the so called “non-binders”, healthy subjects who demonstrated very little if any binding of ^{11}C -PBR28 (Kreisl et al., 2010). All 2nd generation ligands tested in vitro in human

tissue samples bind TSPO with either high affinity or low affinity or a mix of high and low affinity binding sites (HABs, MABs, LABs) (Owen et al., 2011; Owen et al., 2010). This finding has retrospectively explained the ‘non-binders’ phenomenon, as the affinity of ^{11}C -PBR28 to LABs was only 188 nM (Owen et al., 2010), and thus no specific binding in these subjects would be expected. The presence of MABs in the population also explained the variability in the PET signal across healthy volunteers, as these subjects may be expected to have significantly lower binding of the 2nd generation ligands, from HABs. Our group has demonstrated that these different binding patterns in humans are fully explained by the rs6971 polymorphism in the TSPO gene (Owen et al., 2012). The frequencies of this polymorphism vary across ethnic groups with the Caucasians having the highest frequency for LABs (HABs:MABs:LABs=49:42:9) compared to African Americans (56:38:6) and Han Chinese and Japanese (94:6:0.001). Our hypothesis that the rs6971 polymorphism explained the large between subject variability seen with 2nd generation TSPO PET ligands, was consistent with *in vivo* data showing differences between HAB and MAB binding for TSPO ligands, ^{18}F -FEPPA and ^{11}C -PBR28 (Kreisl et al., 2013a; Mizrahi et al., 2012).

^{18}F -PBR111 was previously evaluated *in vivo* in the non-human primate brain, which demonstrated high delivery, fast kinetics and high SNR ($\text{BP}_{\text{ND}} \sim 4$), based on the blockade of the ^{18}F -PBR111 binding with unlabeled PK11195 (7 mg/kg i.v.) (Carson et al., 2010). An *in silico* biomathematical model predicting the *in vivo* performance of radioligands in humans (Guo et al., 2009), enabled us to account for the influence of genetic polymorphisms in the target protein. The model predicted that ^{18}F -PBR111 would show moderate to high specific binding in the healthy human brain across genetic groups (Guo et al., 2012). Evidence of the ubiquitous

expression of TSPO throughout the brain (Chen et al., 2004b) and the global reduction in ^{18}F -PBR111 binding following the administration of unlabeled PK11195 in the non-human primate (Carson et al., 2010), indicated that any reference region approach will be confounded by a contribution from specific binding.

Here the characterisation of ^{18}F -PBR111 binding in healthy human volunteers is presented. I test the hypothesis that *in vivo*, the ^{18}F -PBR111 signal is dependent on the rs6971 polymorphism in the TSPO gene, and evaluate the effects of age on binding in the human brain.

3.2 Methods

3.2.1 Subjects

In total, 21 healthy volunteers, aged between 28 and 62 years (51 ± 10 yrs, 14 females, 7 males) with an average body weight of 77.2 ± 22.4 kg, were included in the current analysis.

3.2.2 Definition of Volumes of Interest

Our attention focused on regions of moderate and high binding based on previous literature (Fujita et al., 2008): brain stem, thalamus, hippocampus, global cortical regions, cerebellum, and caudate. The latter was chosen as representative of low binding regions. Due to defluorination of the metabolite from ^{18}F -PBR111 (Fookes et al., 2008), VOIs for frontal, parietal, occipital, temporal cortex and cerebellum were eroded of 5 mm away from the skull to avoid spill in from uptake outside the brain (Carson et al., 2003). Each VOI was then applied to the dynamic PET data to derive regional TACs.

3.2.3 Kinetic analysis

The total V_T was estimated using a 2TC kinetic model with a metabolite-corrected plasma input function (Gunn et al., 2001) and with the Logan graphical method (Logan et al., 1990) (details presented in Chapter 2).

In order to account for free fraction in plasma (f_p), the V_T/f_p was obtained in addition to V_T , to consider correction for plasma protein binding effects across subjects. V_T and V_T/f_p were compared with the averaged standardized uptake value from 90 to 120 min ($\text{SUV}_{90_120 \text{ min}}$).

To investigate the scanning duration required for reproducible estimates of V_T , time stability analysis was performed by analysing scan durations ranging from 30 to 120 min., in 10 min. increments.

3.2.4 Statistical analysis

The Mann-Whitney U and Kruskal-Wallis Tests for independent samples were used to evaluate the difference in V_T between the different genetic groups. Linear regression and Analysis of Covariance (ANCOVA) were used to characterize respectively the effect of age on V_T within each genetic group, and the effect of age on the difference in V_T between the different genetic groups.

3.3 Results

3.3.1 Plasma and kinetic analysis

In plasma, the concentration of ^{18}F -PBR111 peaked at about 1 min. followed by a rapid decrease, which was well described by a tri-exponential model (Figure 3.1A). One metabolite appeared quickly following parent administration. The parent compound accounted for about 40% of the total concentration in plasma at 30 min., and the parent fraction further reduced and reached between 9.3% to 36.4% ($20.1\% \pm 8.7\%$) at 120 min (Figure 3.1B). The metabolite has been previously identified as a 3- ^{18}F -fluoropropionic acid, which is more polar than the parent compound (Fookes et al., 2008). A bone uptake activity was observed and was consistent with defluorination reported for this metabolite in the literature (Fookes et al., 2008). The metabolite profile did not vary across genetic groups. The f_p was measured for 8 HABs (0.05 ± 0.02), 6 MABs (0.07 ± 0.02) and 4 LABs (0.06 ± 0.01). No significant difference was found in f_p across the different genetic groups (Figure 3.2).

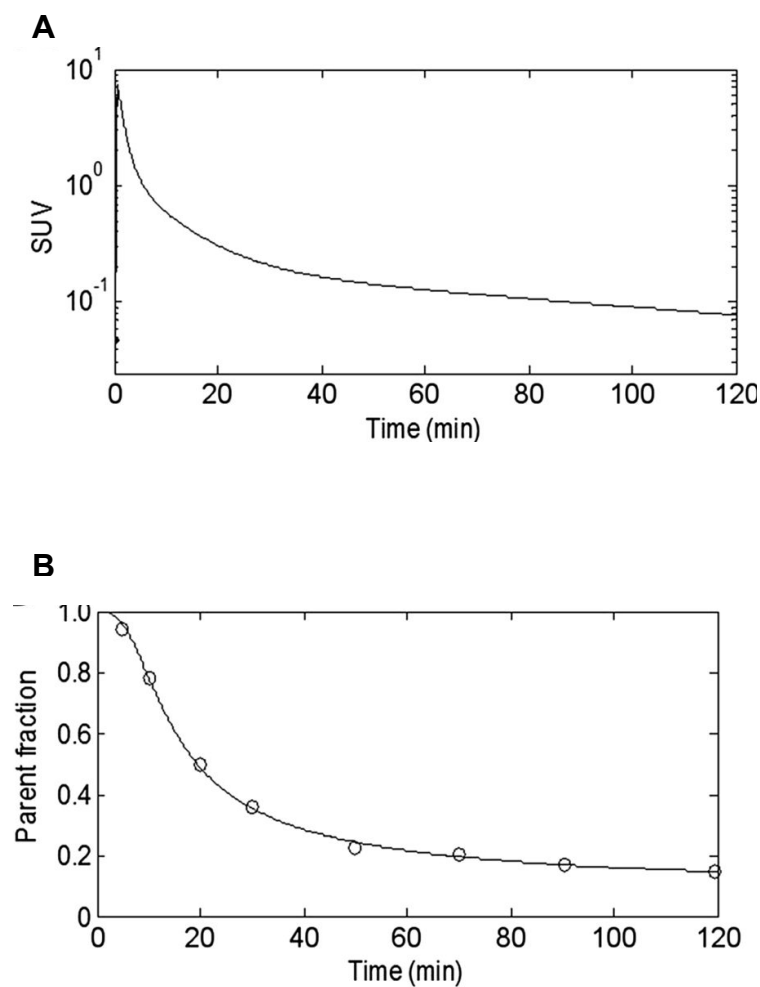


Figure 3.1 Measurements of radiotracer concentrations and parent fraction in the plasma

(A) ^{18}F -PBR111 plasma concentration fitted with a triexponential model after peak, in a representative subject with High Affinity Binding (HAB) status; (B) Concentration of the plasma parent fraction of ^{18}F -PBR111 fitted with a sigmoid parent fraction model, in a representative subject with HAB status.

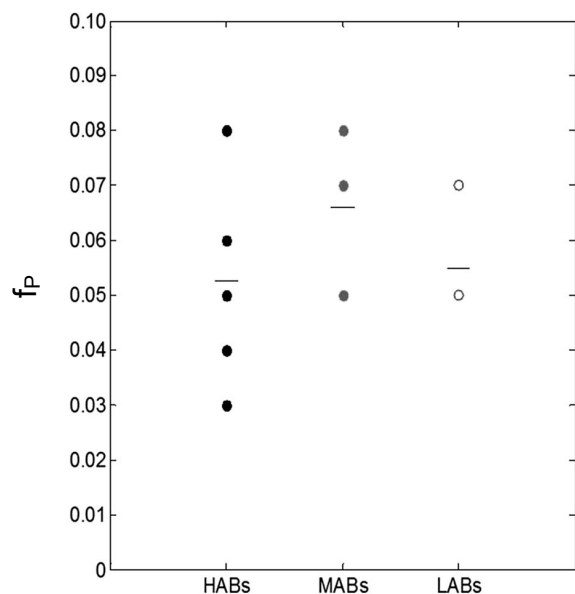


Figure 3.2 Plasma free fraction (f_p) for different genetic groups: HABs, MABs, and LABs

The figure illustrates the mean concentration estimates of plasma free fraction (f_p) of ^{18}F -PBR111 across each binding status group. No significant difference was found between groups. HAB: High Affinity Binder; MAB: Mixed Affinity Binder; LAB: Low Affinity Binder.

After injection, ^{18}F -PBR111 readily entered the brain. The distribution of the radioactivity within the brain was widespread, with slightly higher signal in thalamus, brain stem, hippocampus, followed by other cortical regions, cerebellum and lowest in striatum, consistent with previous findings with ^{11}C -PBR28 (Figure 3.3) (Fujita et al., 2008).

The concentration of the ligand in tissue peaked at around 2 min. after injection for all subjects and washed out with different elimination rates corresponding to the different genetic groups (Figure 3.4). In HABs, the concentration in a high binding region such as brain stem, decreased to 50% of the peak at 60 min. whilst in MABs and LABs, the washout was faster and the concentration decreased to 50% of the peak at 40 min. and 20 min. respectively.

2TC fits to the TACs across VOIs in a representative HAB subject are shown in Figure 3.5A. The time stability analysis of the 2TC model demonstrated an increase in V_T extending beyond the 120 min. scan duration used in our studies (Figure 3.5B). This effect was present in all brain regions and for all genetic groups. A duration of scan of 60 min. would lead to 10% to 20% underestimation of V_T in comparison to a value obtained from the full 120 min. acquisition.

3.3.2 Genetic influence on the *in vivo* binding signal

The comparison of the $\text{SUV}_{60-90\text{min}}$, V_T and V_T/f_p values in grey matter masked regions of interest across different genetic groups is presented in Figure 3.6. Although we observed a between-groups difference for all three parameters, $\text{SUV}_{90-120\text{min}}$ didn't reach significance whilst we observed significant differences for V_T between all groups (HABs vs MABs: $p=0.025$; HABs vs LABs: $p=0.005$ and MABs vs LABs: $p=0.014$). The correction for plasma free fraction (V_T/f_p) did not reduce the between

subjects variability. Significant differences were found for V_T/f_p between HABs and both MABs and LABs across all regions, but between MABs and LABs were only seen in the hippocampus and brain stem. For these reasons, only the V_T will be reported in the following analyses of this chapter. The average absolute difference in V_T between HABs and MABs (1.54 ± 0.23) was similar to the one observed between MABs and LABs (1.14 ± 0.17), in line with the hypothesis that MABs express half of the high affinity sites and half of the low affinity sites.

A spatially normalised averaged V_T parametric map for each genetic group is presented in Figure 3.3, illustrating the global difference in V_T across the different genetic groups consistent with the results from the VOI analysis.

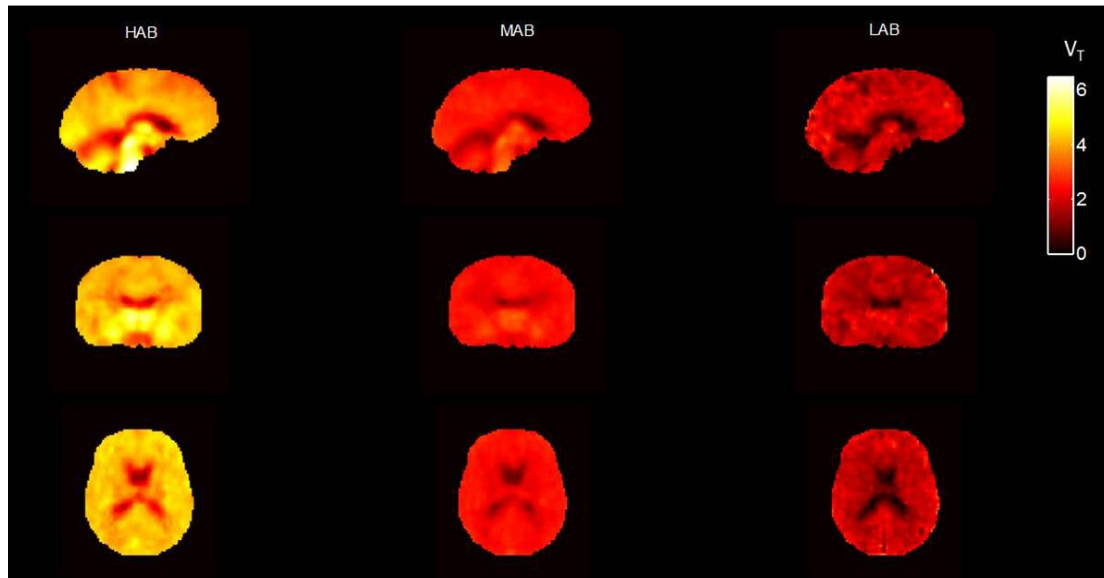


Figure 3.3 Spatially normalised average ^{18}F -PBR111 V_T parametric maps for each genetic group.

The figure displays the spatially normalised averaged voxel-wise ^{18}F -PBR111 V_T parametric maps for each binding group, illustrating the global difference in ^{18}F -PBR111 V_T between the different groups. HAB: High Affinity Binder; MAB: Mixed Affinity Binder; LAB: Low Affinity Binder.

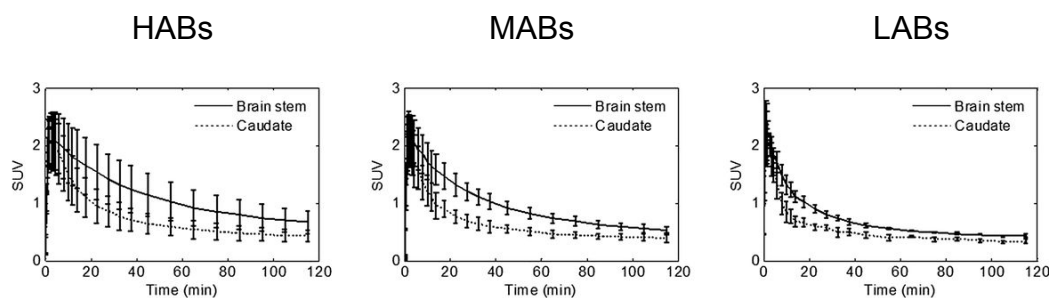


Figure 3.4 Averaged time-activity curves in brain stem and caudate across genetic groups

The figure illustrates the averaged time-activity curves (TACs) for ^{18}F -PBR111 across TSPO binding groups. Brainstem and Caudate are displayed as representative VOIs with relatively high and low TSPO binding, respectively. HAB: High Affinity Binder; MAB: Mixed Affinity Binder; LAB: Low Affinity Binder. The Error bar represents the Standard Deviation (SD) at each frame.

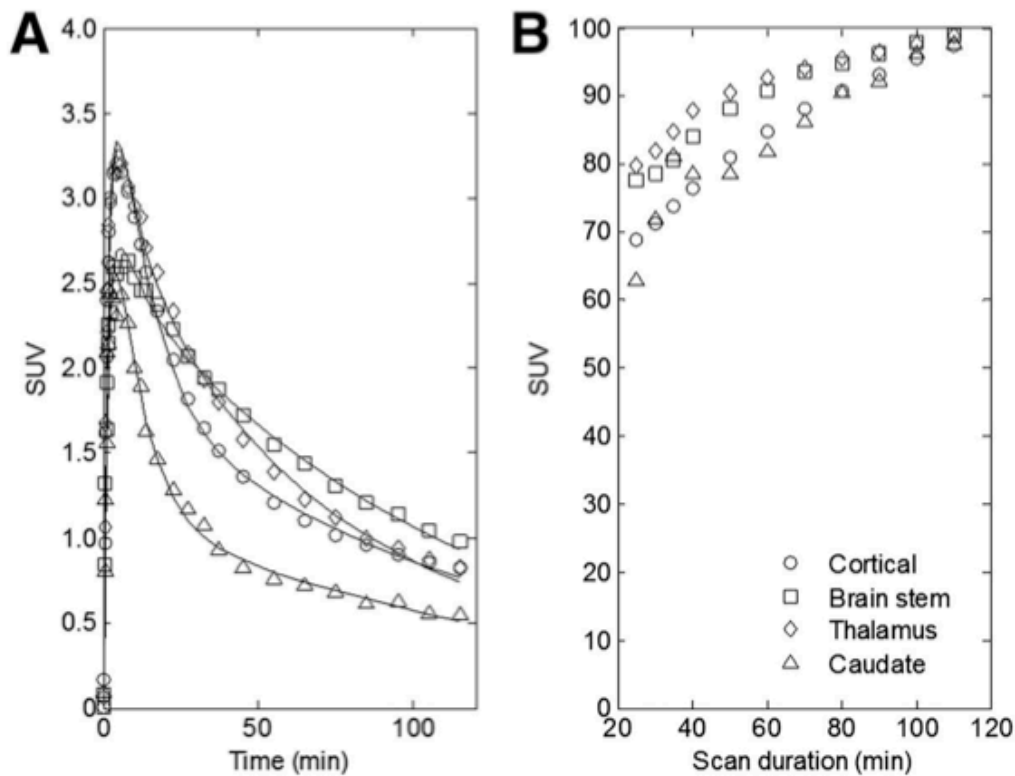


Figure 3.5 Kinetic model fits to HAB time-activity curves and time-stability analysis

The figure illustrates the fits of the 2 Tissue Compartment (2TC) model to the ^{18}F -PBR111 Time Activity Curves (TACs) across various Volumes of Interests (VOIs) in a representative High Affinity Binder subject. (A) Unconstrained 2 Tissue Compartment model fit to time-activity curves. (B) Assessment of time-stability of ^{18}F -PBR111 V_T for unconstrained 2TC model. The time stability analysis of the 2TC model demonstrated an increase in V_T extending beyond the 120 min. scan duration used in our studies. This effect was present in all brain regions and for all genetic groups.

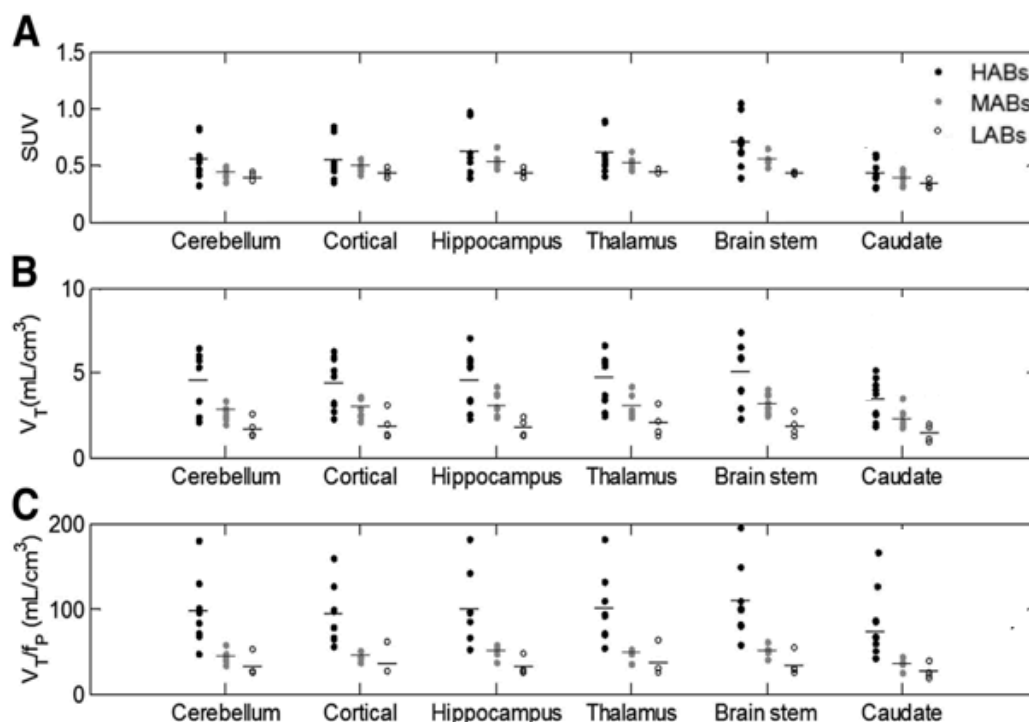


Figure 3.6 Comparison of $\text{SUV}_{90-120 \text{ min}}$, V_T , and V_T/f_p values between genetic groups across VOIs

The figure illustrates ^{18}F -PBR111 Standard Uptake Values (SUV), Volume of Distribution (V_T), and Volume of Distribution corrected for plasma free fraction (V_T/f_p) across TSPO binding groups, and across Volumes of Interest (VOIs). HAB: High Affinity Binder; MAB: Mixed Affinity Binder; LAB: Low Affinity Binder. Horizontal lines indicate mean values for each genetic group.

3.3.3 *Relationship with age*

A substantial variability in V_T was still found within each genetic group, especially in HABs. We found a significant correlation between age and V_T in all VOIs except caudate for HABs, and in the brainstem, hippocampus, and cerebellum for MABs. The linear regression slope in HABs was 0.09, and was about 2-fold higher than the slope estimated for MABs (0.04), suggesting that the effect of age is more evident in subjects with higher specific binding (Figure 3.7). The relationship between V_T and age was not studied in LABs because of the small sample size ($n=4$) and limited age range of participants (44-56 years old). When ANCOVA was used to correct for the age effect within each binding class, the standard error of the mean in HABs was reduced by 40%.

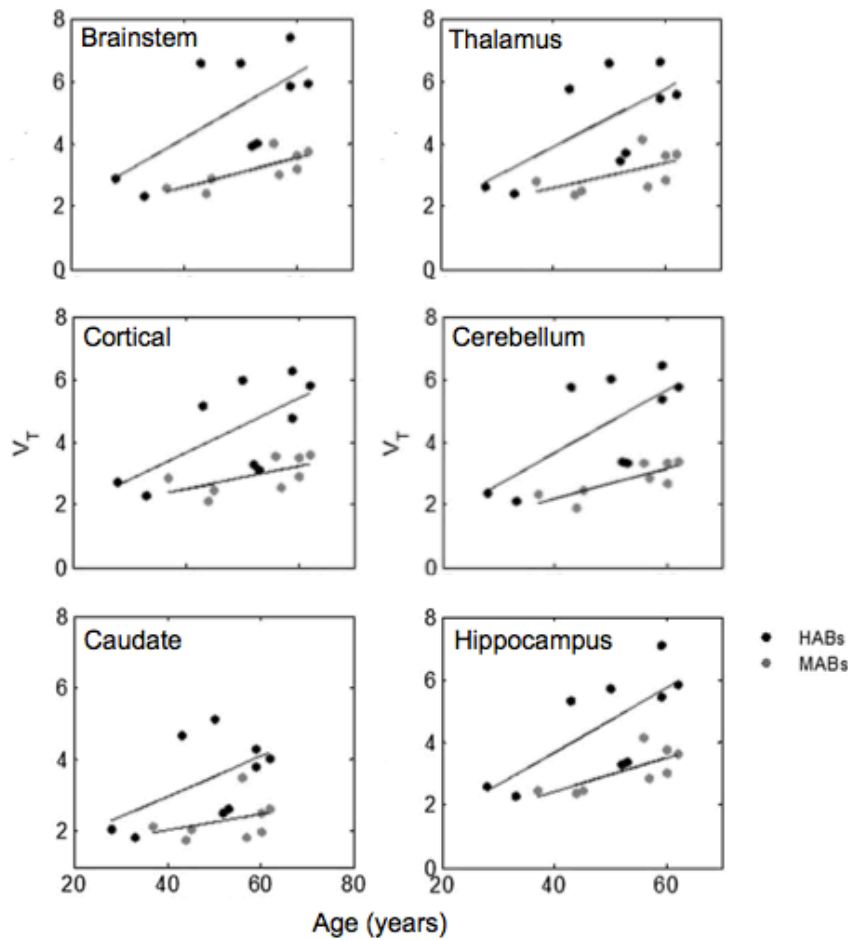


Figure 3.7 Correlations between V_T and age in HABs and MABs across the different VOIs

Pearson's coefficients and significance across the different VOIs: Brain Stem, HAB: $r=0.68$, $p<0.05$, MAB: $r=0.78$, $p<0.05$. Thalamus, HAB: $r=0.73$, $p<0.05$; MAB: $r=0.57$, Non Significant (N.S.). Cortical regions, HAB: $r=0.67$, $p<0.05$; MAB: $r=0.61$, N.S. Cerebellum, HAB: $r=0.70$, $p<0.05$; MAB: $r=0.81$, $p<0.05$. Caudate, HAB: $r=0.55$, N.S., MAB: $r=0.37$, N.S. Hippocampus, HAB: $r=0.73$, $p<0.05$; MAB: $r=0.74$, $p<0.05$.

3.4 Discussion

The binding of ^{18}F -PBR111 in the healthy human brain was characterised. Our team previously identified a strong effect of the rs6971 polymorphism in the TSPO gene on the binding affinity of PBR111 to TSPO in human tissue *in vitro* (Owen et al., 2011). Here, we observed the same effects of the rs6971 polymorphism on the *in vivo* binding of ^{18}F -PBR111, in line with the prediction based on the genetic status of the subjects.

The distribution of the signal yielded by ^{18}F -PBR111 was similar to that of other TSPO tracers previously evaluated in humans, such as ^{11}C -R-PK11195 and ^{11}C -PBR28.

The 2TC kinetic model fitted the time activity curves well except for a slight underestimation of the tail. This observation agrees with the interpretation of the time stability analysis which shows that V_T increases with scan duration. This could be due to some small irreversible component, with radiometabolite getting into the brain being a possibility. A similar observation has been made with other 2nd generation TSPO tracers, such as ^{11}C -PBR28, ^{11}C -DPA713 and ^{18}F -PBR06 in humans (Endres et al., 2009; Fujimura et al., 2009; Fujita et al., 2008).

Another potential explanation for this observation is the presence of binding of TSPO to endothelial cells. Previous studies, using both ^{11}C -R-PK11195 and ^{11}C -PBR28, demonstrated that the additional inclusion of a vascular component in the kinetic model might provide more precision in the quantification of TSPO specific signal (Rizzo et al., 2014; Turkheimer et al., 2007). Furthermore, due to the particularly high TSPO density in the vascular endothelium, a concentration gradient is likely to occur through the brain parenchyma, where the ligand binds non-specifically to lipids on its

way to the binding sites. It is possible that with high lipophilicity tracers (e.g. with ^{11}C -PBR28), the concentration of the free tracer at the binding site may be lower than its average tissue concentration, leading to decreased in-vivo affinity of the tracer to TSPO. This provides some support to the hypothesis that similarly to ^{11}C -PBR28, the high affinity binding of ^{18}F -PBR111 to the endothelial TSPO might be responsible of the observed underestimation of the data with the 2TC model we used.

As the increase in V_T of ^{18}F -PBR111 was global, as with other tracers, the scan can likely be shortened to 60 min allowing for a consistent underestimation of less than 20%. However, when comparing different groups, analysis should always be performed with the data from the same scan durations and the degree of underestimation in different clinical populations needs to be evaluated.

We compared ^{18}F -PBR111 binding across genetic groups using three outcome measures, namely $\text{SUV}_{60-90\text{min}}$, V_T and V_T/f_p . Significant differences, consistent with our predictions based on *in vitro* data were observed between HABs, MABs and LABs using both V_T and V_T/f_p , but not using SUVs. In our data, f_p did not differ across genetic groups in the healthy subjects and the variability between subjects in V_T was not reduced after correction for the free fraction in plasma. Therefore, V_T estimated from a 2TC model appeared to be the most appropriate measure to quantify the total binding of TSPO.

Within each genetic group, there was still variability in V_T across subjects, especially in HABs. We found that this variability could be partly explained by a correlation between age and binding.

Our observation of an association between age and TSPO density is consistent with the results of some previous human imaging studies (Gulyas et al., 2009), although

not consistently observed using other radiotracers (Suridjan et al., 2014), and is further supported by preclinical data indicating a progressively increased TSPO density in aging rats using serial longitudinal microPET assessments (Walker et al., 2015). These observations are line with the notion of increased microglia activation associated with aging (Ogura et al., 1994), an association that appear to vary across brain regions (Hart et al., 2012).

By using ANCOVA to correct for the effect of age, the standard error of the mean V_T for HABs was reduced by 40%. Thus age effect should be considered carefully in TSPO study design by either carefully matching subjects for age or by applying an appropriate correction for age related changes. Although the relationship between V_T and age across the full age range is unlikely to be linear, a linear regression was used as a first order approximation. We found the slope of HABs was about 2-fold higher than that seen for MABs, which is consistent with the previously measured affinity ratio between high and low binding sites *in vitro*.

We additionally observed regional differences in ^{18}F -PBR111 binding suggesting that microglia density is not homogenous across the healthy human brain. Our findings are in line with earlier post-mortem observations (Mittelbronn et al., 2001).

Finally, the fluorine-18 radionuclide provides advantage for use of the radioligand in multicenter clinical trials, however the approach we used for quantification requires arterial input methods, which would limit its use to centers with adequate infrastructure and expertise. In addition, there is an element of defluorination present, and care should be taken when evaluating most superficial cortical layers close to bone.

In conclusion, ^{18}F -PBR111 PET showed a measurable and quantifiable specific signal in the healthy human brain for TSPO. A proportion of the inter-subject variability in the PET signal for healthy volunteers can be attributed to the genetic variation at the rs6971 locus and age of the subjects. Having accounted for these effects, the ligand can be used for the quantitative assessment of TSPO expression related to neuroinflammatory process in the brain.

Chapter 4. Assessment of white matter inflammation in Multiple Sclerosis patients with ^{18}F -PBR111

4.1 Introduction

A prominent neuropathological feature of MS is the activation of microglia, resident brain innate immune response cells, in the white matter particularly within demyelinating lesions, but also extending into adjacent white matter tissue (Henderson et al., 2009; Kutzelnigg et al., 2005; Marik et al., 2007; van Horssen et al., 2012).

MRI markers specifically associated with microglial and macrophage activation are limited. Correlational post-mortem neuropathology and MRI in MS have confirmed that T1-weighted Gadolinium contrast enhancement and associated T2-weighted hyperintensity changes in white matter reflect the adaptive immune response, but do not specifically inform on relative macrophage or microglial involvement (De Groot et al., 2001). T2-weighted white matter hyperintensities also are non-specifically associated with other pathological features including demyelination, axonal loss and astrogliosis (Gouw et al., 2011). A recent study found that subtle reductions in the MRI MTR in peri-lesional white matter volumes are associated with increased microglia density (Moll et al., 2011). However, decreases in MTR reflect other changes in tissue microstructure, such as demyelination, as well and do not provide a specific biomarker for innate immune activation.

PET radioligand binding to TSPO in brains of patients with MS primarily reflects activated microglia and macrophages (Banati et al., 2000; Cosenza-Nashat et al., 2009). However, the interpretation of the findings of early studies, using the first

generation PET tracer ^{11}C -(R)-PK11195, is limited by the rather poor SNR for this tracer and non-specific binding, which challenge accurate quantitation of the signal (Banati et al., 2000; Debruyne et al., 2003; Vas et al., 2008; Vowinckel et al., 1997).

A number of second generation TSPO PET radioligands with improved SNR relative to ^{11}C -(R)-PK11195, have been developed (Chauveau et al., 2008). Unexpectedly, the first two studies using newer TSPO radioligands did not report increases in white matter binding of MS patients relative to healthy controls (Oh et al., 2011; Takano et al., 2013). However, the analyses did not take into consideration the population variation in binding of radioligands associated with the rs6971 *TSPO* gene SNP (Owen et al., 2012). Heterogeneity of binding across carriers of different alleles may have contributed to the failure to differentiate brain TSPO radioligand binding between MS patients and healthy controls successfully.

^{18}F -PBR111 is a 2nd generation TSPO ligand with promising imaging characteristics (Fookes et al., 2008). A recent study showed increased ^{18}F -PBR111 uptake corresponding specifically to activated microglia in EAE (Mattner et al., 2013). We have developed a robust approach for quantitative *in vivo* assessment of specific ^{18}F -PBR111 binding to TSPO (see previous chapter) (Guo et al., 2013).

Here, I have used ^{18}F -PBR111 PET co-registered with MRI to evaluate regional binding to white matter in healthy volunteers and in MS patients who were stratified genetically according to the rs6971 SNP. Based on post-mortem descriptions of activated microglia and macrophage distributions (Filippi et al., 2012; Henderson et al., 2009; Moll et al., 2011), I predicted increased ^{18}F -PBR111 binding in regions of T2-hyperintense lesions, in the immediate peri-lesional volumes, and in non-lesional volumes with reduced MTR, compared to that in the white matter of healthy

volunteers and in the white matter with a normal MTR (“normal appearing”) in MS patients. The reproducibility of ^{18}F -PBR111 signal also was assessed in five subjects (four healthy volunteers and one MS patient).

4.2 Methods

4.2.1 Subjects

The sample included patients with relapsing-remitting MS and age-matched healthy volunteers (n=11 per group). All subjects were genotyped for the rs6971 SNP, that determines variations in affinity between subjects (who express either a high affinity (HAB) or low affinity (LAB) or co-expression of both binding states (MAB)) for second-generation TSPO radioligands (Owen et al., 2012). Patients with MS and healthy volunteers were matched for the rs6971 SNP. The demographic variables, TSPO binding affinity class, and clinical characteristics of study participants are presented in Table 4.1.

Focal, non-specific alterations in the white matter signal intensity were found in one healthy volunteer and the subject was referred for further evaluation. This volunteer had no past or present neurological symptoms and the physical and neurological examination was unremarkable. No other abnormalities were found. The neuroradiologist did not judge the findings to be clinically significant or to suggest pathology. This subject has been included in the analysis. Another healthy volunteer, who underwent two scans in consecutive days to assess ^{18}F -PBR111 PET reproducibility, had diffuse white matter hyperintensities at MRI that were deemed compatible with the presence of cerebral small vessel disease. The subject was excluded from the between groups (MS vs. healthy controls) analyses, but was included in the analysis of the test-retest reproducibility of the signal.

In one MS patient (4.1, case 7) I found 2 Gadolinium-enhancing lesions on T1-weighted MRI. No contrast enhancement was found in any of the other patients.

4.2.2 Study design

Each participant underwent an MRI scan and a ^{18}F -PBR111 PET scan on the same day. Four healthy volunteers and one MS patient (all HABs) underwent a second ^{18}F -PBR111 PET scan on the following day in order to evaluate the reproducibility of the PET signal.

Disability was assessed by an experienced neurologist using the EDSS. The MSSS was calculated based on EDSS scores and disease duration according to Roxburgh *et al.* (2005). The EDSS and MSSS scores of one patient were not considered in the analysis as most disability for the patient had been attributed previously to an incomplete traumatic spinal cord injury causing weakness in lower limbs, which was antecedent to the onset of MS.

Healthy Volunteers ^a				Relapsing-Remitting MS patients						
Case	binding affinity class	Age	Gender	binding affinity class	Age	Gender	EDSS	Disease Duration	MSSS	DMT
1	HAB	52	F	HAB	48	F	-	8	-	natalizumab
2	LAB	51	M	LAB	39	F	4	20	3.0	interferon β
3	HAB	42	F	HAB	40	F	4	11	4.9	natalizumab
4	HAB	59	M	HAB	55	F	2	20	0.9	interferon β
5	LAB	65	M	LAB	53	F	7	20	7.4	-
6	MAB	57	M	MAB	59	F	3	16	2.6	interferon β
7	HAB ^b	28	M	HAB	41	F	2.5	14	2.3	-
8	HAB ^b	28	M	HAB ^b	28	M	2	7	3.2	-
9	MAB	36	F	MAB	41	F	6	4	9.1	interferon β
10	HAB	33	F	HAB	42	F	6	1.5	9.6	interferon β
11	HAB ^b	52	F	HAB	50	F	4	2	8.6	interferon β
Mean age		45.7 years		45.1 years						

Table 4.1 Demographic variables, TSPO binding affinity class, and clinical characteristics of study participants

EDSS: Expanded Disability Status Scale; MSSS: Multiple Sclerosis Severity Score; DMT: Disease Modifying Treatment; HAB: High Affinity Binder; MAB: Mixed Affinity Binder; LAB: Low Affinity Binder.

^a a twelfth healthy volunteer, (genotype, HAB; age: 50 y.; gender: female) was included in the test-retest analysis but was excluded from the comparison between-groups due to white matter abnormalities consistent with presence of cerebral small vessel disease (see Subjects section). ^b These subjects underwent two scans in consecutive days to assess test-retest reproducibility.

4.2.3 MRI

The general characteristics of the MRI scanner and the T1-weighted protocol (with and without contrast) have been presented in Chapter 2.

Volumetric T2-weighted FLAIR images were acquired using a 1mm isotropic resolution 3D SPACE sequence (Mugler and Brookeman, 2003), with a 250x250x160mm FOV, TE=395ms, TR=5s, TI=1800ms, turbo factor of 141, 256x256x160 matrix, and parallel imaging factor of 2 in 5m:52s. MTR maps were acquired using a 3D spoiled gradient echo sequence [fast low angle shot (FLASH)]. Two volumes with 1mm isotropic resolution were acquired using 256x240x192mm FOV, TR of 27ms and a flip angle of 5° to give pseudo proton density weighting (PDw), a parallel imaging factor of 2, 6 echoes acquired using 630Hz/pixel bandwidth with TEs every 1.95ms from 1.95 to 11.7ms in 7m:20s. Each high-bandwidth echo was summed to increase SNR without introducing off-resonance effects of low readout bandwidth (Helms and Dechent). One volume used a 12.24ms duration Gaussian RF pulse at 2.2 kHz off resonance with a nominal flip angle of 540° to add magnetization transfer weighting (MTw). MTR maps were calculated using the signals from the MTw and PDw acquisitions by the equation:

$$MTR = 100 \cdot (S_{PDw} - S_{MTw}) / S_{PDw} \quad [1]$$

4.2.4 PET

Details on radioligand synthesis and PET protocol have been provided in Chapter 2. The injected activity was similar in healthy volunteers and MS patients (164.6 MBq ± 9 and 169.7 MBq ± 12.4 [mean ± SD], respectively).

In three cases (Table 4.1, cases n. 2, 3, 11), patients were not able to tolerate the whole duration of the scan due to increased discomfort related to neurological symptoms (i.e. muscular spasms and pain). In these cases I considered the V_T obtained from the analysis of the first 70 minutes of dynamic acquisition post injection. However, this is likely to lead to underestimation of V_T for these cases, as demonstrated by our previous analysis of time stability of V_T (Guo et al., 2013). Within subject comparisons of V_T obtained with acquisitions of 70 minutes vs 120 minutes in my sample indicated that scan duration of 70 min would lead to 7 to 35 % (median 16%) underestimation of V_T in comparison to values obtained from the full 120min acquisition. On the basis of these data, I applied a correction to the V_T of the 3 patients who could not complete the scan, increasing their V_T values of 16%.

4.2.5 *Definition of Volumes of Interest*

In healthy volunteers and MS patients, the whole white matter was segmented automatically from each individual's structural T1 MRI image using SPM5 (Wellcome Trust Center for Neuroimaging, <http://www.fil.ion.ucl.ac.uk/spm>).

To define demyelinating lesions in MS patients' brains, T2-FLAIR hyperintense white matter volumes were segmented using JIM version 6.0 (Xinapse Systems, Northants) by an experienced MRI analyst (Figure 4.1 a, b). The white matter of patients, segmented using SPM5, was filled with T2-FLAIR lesions to complete the whole white matter mask.

The remaining white matter in the MS patients (remaining after exclusion of the T2-FLAIR hyperintense lesions, Figure 4.1b) was further segmented to three VOIs as follows:

- perilesional volumes, including voxels within a 6 mm-thick volume disposed around T2-hyperintense lesions (Figure 4.1c);
- non-lesional low MTR (NLLM) voxels, with MTR ranging between 90-98% of the non-lesional mean white matter MTR (Moll et al., 2011) (Figure 4.1d);
- non-lesional high MTR (NLHM) voxels, showing $\text{MTR} \geq 98\%$ of the non-lesional mean white matter MTR (Figure 4.1e); the NLHM VOI was assumed to be representative of the normal white matter tissue (Moll et al., 2011).

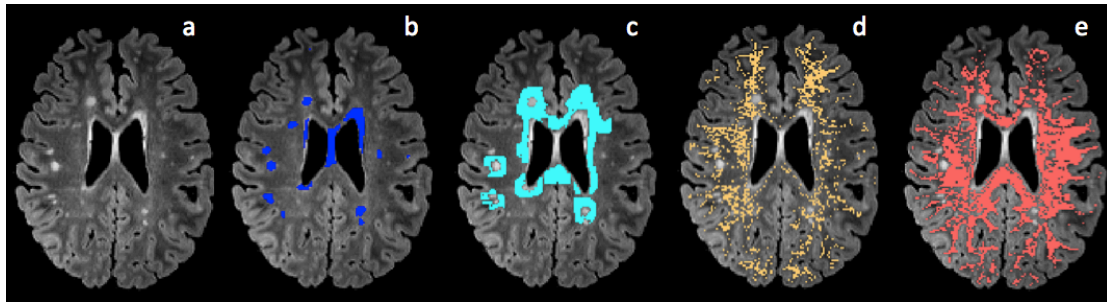


Figure 4.1 **Definition of Volumes of Interest**

a) T2-FLAIR image of a representative MS patient (case 1, Table 4.1); hyperintense areas correspond to demyelinating lesions; **b)** Lesions (dark blue); **c)** perilesional areas (light blue) corresponding to the intersection of 6 mm diameter sphere traced around lesions within the image plane; **d)** Non-lesional voxels with MTR values ranging between 90-98% of the mean MTR of non lesional white matter tissue (NLLM) (copper); **e)** Non-lesional voxels with MTR values ranging above 98% of the mean MTR of non lesional white matter tissue (NLHM), corresponding to normal appearing white matter (red).

4.2.6 Image analysis

Details on image analysis and kinetic analysis have been provided in Chapter 2. As previously described, the V_T was quantified using 2TC kinetic model with a metabolite-corrected plasma input function. The Logan graphical method employing a plasma input (Logan et al., 1990) was applied at the voxel level to produce parametric V_T maps (see Chapter 2 for further details).

4.2.7 Statistical analysis

All analyses were conducted using SPSS 20. I expressed ^{18}F -PBR111 V_T for the whole white matter as V_T^{WM} . For each subject the mean ^{18}F -PBR111 V_T uptake of all lesions larger than 100 voxels (V_T^{L}) and perilesional volumes (V_T^{PL}), respectively, was computed. Uptakes of ^{18}F -PBR111 in NLLM and NLHM were expressed as V_T^{NLLM} and V_T^{NLHM} , respectively.

Between-subjects comparisons. ^{18}F -PBR111 V_T^{WM} was compared between binding affinity classes (HABs, MABs, LABs) by using non-parametric comparison for independent samples (Kruskal-Wallis Test). I compared ^{18}F -PBR111 V_T^{WM} of MS patients to that of healthy controls by using a two-tailed Wilcoxon Signed-Rank Test. Healthy volunteers' ^{18}F -PBR111 V_T^{WM} was compared to each of MS patients' VOI (V_T^{L} , V_T^{PL} , V_T^{NLLM} , V_T^{NLHM}) separately using two-tailed Wilcoxon Signed-Rank Tests. For those subjects (four healthy volunteers, and one MS patient) who underwent two scans in consecutive days, the mean ^{18}F -PBR111 V_T of the two visits for all VOIs have been used in all between- and within- subject analyses.

Within-subjects comparisons. Within MS patients, V_T^L , V_T^{PL} , and V_T^{NLLM} were separately compared to V_T^{NLHM} , by using two-tailed Wilcoxon Signed-Rank Tests. Significance for all tests was set at 0.05.

The relative increase (Δ) in ^{18}F -PBR111 uptake in lesions, perilesional, and NLLM volumes vs NLHM, was computed as follows:

$$\Delta_L = (V_T^L - V_T^{NLHM}) / V_T^{NLHM}$$

$$\Delta_{PL} = (V_T^{PL} - V_T^{NLHM}) / V_T^{NLHM}$$

$$\Delta_{NLLM} = (V_T^{NLLM} - V_T^{NLHM}) / V_T^{NLHM}$$

Correlations with clinical measures. The relationship between V_T^{WM} and disease duration was studied by means of Spearman's partial rank correlation. The partial rank correlation (ρ) coefficient was computed while correcting for the effect of age. I explored the relationship between disease severity and relative increase in TSPO density within demyelinating lesions, by studying the Spearman's rank coefficient of correlation (ρ) between MSSS score and Δ_L . For this analysis, I considered all T2-FLAIR lesions for each patient as a single VOI.

Partial Volume Correction. In order to estimate the partial volume effect on the outcome measure V_T , a voxel-region based algorithm for Partial Volume Correction (PVC), consisting in Local Regression Analysis (LoReAn) (Coello et al., 2013), has been applied as an exploratory tool to the PET brain volumes of one MS patient (case 9, Table 4.1). ^{18}F -PBR111 V_T was estimated after PVC in the two largest lesional volumes and related perilesions, as well as in the NLHM white matter area of the same patient.

The variability across individual lesions, within each MS patient, was expressed using the coefficient of variation (C_V) in V_T^L across individual lesions, as follows:

$$C_V = \text{Standard Deviation}(V_T^L) / \text{Mean}(V_T^L)$$

Test-retest variability of V_T^{WM} was studied in four healthy volunteers and one MS patient, and was expressed as the absolute difference between the two consecutive days scans divided by the mean of the two scans. For the MS patient who was studied twice in consecutive days, I assessed test-retest variability of individual V_T^L and V_T^{PL} . Additionally, I calculated test-retest variability of individual *normalised* lesional and perilesional Distribution Volume Ratio (DVR) by normalising the corresponding V_T values by V_T^{NLHM} . The DVR was computed as follows:

$$DVR_L = V_T^L / V_T^{NLHM}$$

$$DVR_{PL} = V_T^{PL} / V_T^{NLHM}$$

4.3 Results

The demographic and clinical characteristics of study participants are presented in Table 4.1. MS patients (10 F, 1 M) and healthy controls (5 F, 6 M) were matched for genotype (7 HABs, 2 MABs, and 2 LABs in each group) and had similar age distributions [healthy controls, 51 years (28 – 65); (median (range)) and MS patients, 42 years (28 – 59); Wilcoxon Rank $p=0.80$].

4.3.1 Between Subjects contrasts

A significant effect of *TSPO* genotype on white matter binding of ^{18}F -PBR111 was observed for the whole population [V_T^{WM} , HABs 3.53 (2.12 – 5.40) (median (range)); MAB 3.00 (2.50 – 4.43); and LAB 1.47 (1.18-1.70); Kruskal-Wallis $p<0.01$].

Whole white matter: a trend towards lower whole white matter binding was found in healthy controls compared to MS patients [V_T^{WM} , healthy controls 2.50 (1.18 – 5.23); MS patients 3.53 (1.62 – 4.55); Wilcoxon Rank $p=0.06$] (Figure 4.2). In MS patients, the V_T^{WM} correlated with disease duration in HABs (Figure 4.3) (Spearman's $\rho=0.86$; $p=0.03$, corrected for age). The relationship between V_T^{WM} and disease duration was not assessed in the MAB and LAB groups separately, as each of these groups had only 2 subjects per group.

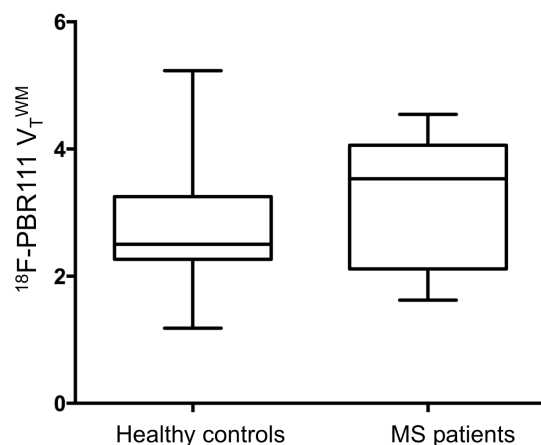


Figure 4.2 ^{18}F -PBR111 uptake in the whole white matter

^{18}F -PBR111 V_T in the whole white matter of MS patients and genotype- and age-matched healthy control subjects. The line in the middle of the boxes are median values, while the hinges represent the 25th and the 75th percentile respectively. Whiskers represent the maximum and minimum values. The contrast between MS patients and healthy controls showed a trend for higher ^{18}F -PBR111 V_T^{WM} in MS patients (Wilcoxon Rank $p=0.062$).

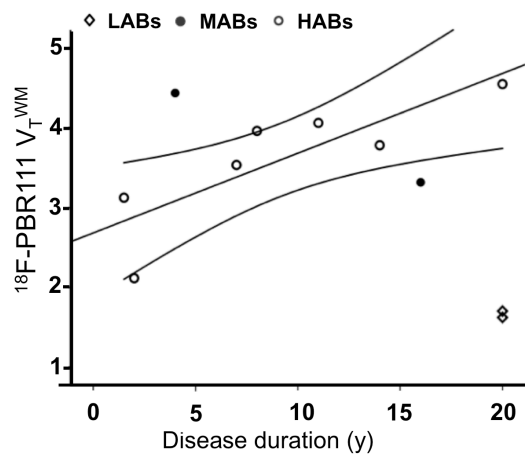


Figure 4.3 Relationship between $^{18}\text{F-PBR111}$ uptake in the whole white matter and disease duration

Relationship between whole white matter $^{18}\text{F-PBR111 } V_T$ and disease duration in MS patients for patients with different rs6971 genotypes (Spearman's partial correlation in HABs: $\rho=0.86$; $p=0.03$, corrected for age).

MRI Segmentation-Based VOIs. One hundred sixty-three T2-FLAIR lesions were identified in the patients [median total lesion volume per patient, 12708 mm³ (range, 392-32672 mm³)]. 96/163 individual lesions were larger than 100 voxels in volume (median number of lesions larger than 100 voxels per patient, 8; range, 1-17).

Both V_T^L (median, 3.88; range, 1.44 – 5.50) and V_T^{PL} (median, 3.61; range: 1.59 – 4.66) were greater than the V_T^{WM} V_T^{WM} from healthy controls (median, 2.50; range, 1.18 – 5.23; Wilcoxon Rank $p=0.02$ and $p=0.03$, respectively for V_T^L and V_T^{PL} ; Figure 3). Direct contrasts between healthy controls V_T^{WM} and MS patients V_T^{NLHM} and V_T^{NLLM} V_T^{NLLM} demonstrated trends to greater binding in the patients' VOIs (Wilcoxon Rank $p=0.09$ and $p=0.06$, respectively; Figure 4.4 and 4.5).

I noted a particularly low V_T for one of the patients within the HAB group, across all VOIs, as illustrated in Figure 4.5 (MS patients, case 11). The V_T was significantly lower than that of all other MS patients in the HAB group, and this appeared unexpected. There were no demographic or clinical features that would clearly differentiate this patient from others in our population, other than a relatively short duration of disease (2 years). To rule out any problems in the V_T quantification, I carefully inspected the fit of the TACs across VOIs, the plasma SUV and the parent fraction data to identify any clue that could have caused an abnormally low V_T value in this HAB patient. However, no abnormality was identified and the patient was included in the analysis despite being an outlier.

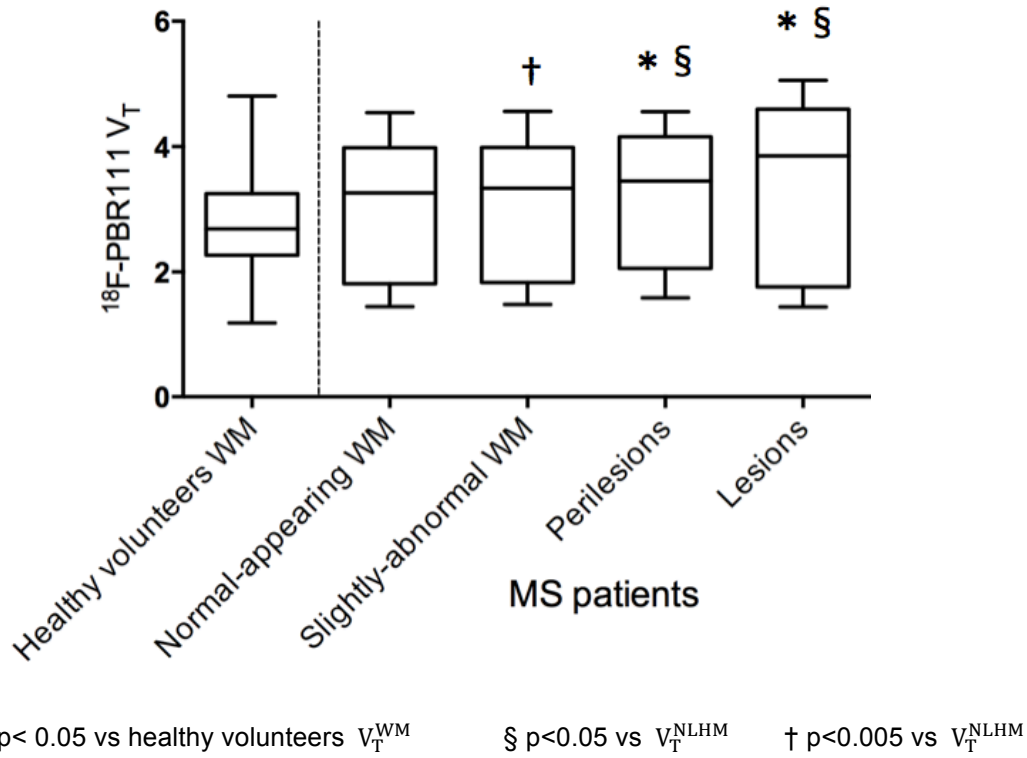


Figure 4.4 ^{18}F -PBR111 uptake in healthy volunteers and across MS patients VOIs

The line in the middle of the boxes are median values, while the hinges represent the 25th and the 75th percentile respectively. Whiskers represent the maximum and minimum values.

Between-groups contrasts showed: MS patients' ^{18}F -PBR111 V_T^{L} and $V_T^{\text{PL}} >$ healthy volunteers' V_T^{WM} (Wilcoxon Rank $p < 0.05$). Within MS patients contrasts showed: $V_T^{\text{L}} > V_T^{\text{NLHM}}$ and $V_T^{\text{PL}} > V_T^{\text{NLHM}}$ (Wilcoxon Rank $p < 0.05$), and $V_T^{\text{NLLM}} > V_T^{\text{NLHM}}$ (Wilcoxon Rank $p < 0.005$).

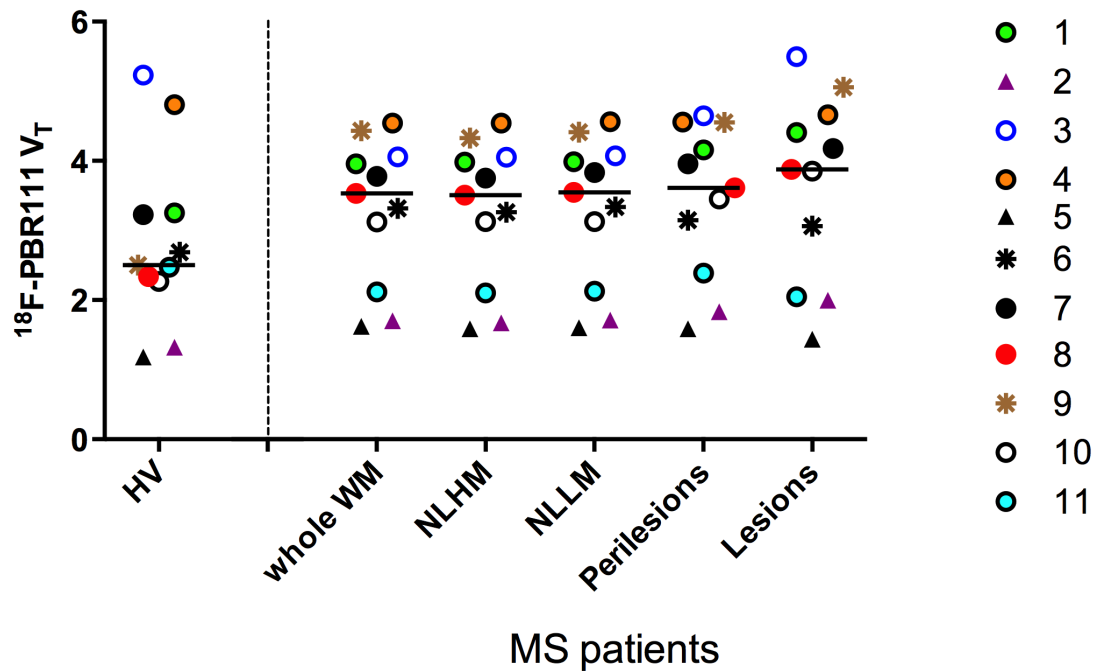


Figure 4.5 Individual ^{18}F -PBR111 uptake across white matter VOIs

Symbols represent separate subjects. For lesional and perilesional VOIs, each symbol represents the mean ^{18}F -PBR111 uptake for each patient across all lesions and perilesional volumes, respectively. In the legend, each symbol is numbered according to the cases' numbers listed in Table 4.1. The shapes of the symbols indicate the binding affinity classes: triangles for LABs, stars for MABs, and circles for HABs.

Gadolinium-enhancing lesions. The volume of the two Gadolinium-enhancing lesions was 16 mm^3 (lesion 1) and 8 mm^3 (lesion 2) respectively. There was a 23% increase in ^{18}F -PBR111 V_T relative to NLHM volume in lesion 1, while in lesion 2 there was no change in ^{18}F -PBR111 V_T relative to NLHM volume. In perilesional volumes, the increase in ^{18}F -PBR111 V_T relative to NLHM volume was 10% and 1%, for lesion 1 and lesion 2 respectively. The volume of these lesions was very small, especially for lesion 2 where the volume corresponded to only one voxel in a $2 \times 2 \times 2 \text{ mm}$ space.

4.3.2 Within Subjects contrasts

Relative ^{18}F -PBR111 V_T values for individual lesions and perilesional volumes, and in the NLHM and NLLM voxels, are presented separately for each MS patient (see Figure 4.6). The median C_V in V_T^L across individual lesions within the patients was 15.2% (range, 9 - 25%).

Within MS patients, I found an increased V_T in lesional (Δ_L , 10.7% (-9.5 – 35.6) [median (range)]; Wilcoxon Rank $p=0.03$), perilesional (Δ_{PL} 5.2% (-3.5 – 14.6); Wilcoxon Rank $p=0.01$) and NLLM volumes (Δ_{NLLM} 1.2% (0 – 2.4); Wilcoxon Rank $p=0.004$) relative to the V_T in NLHM volumes. The increased V_T in NLLM volumes was very small but consistent across all subjects. The maximum ^{18}F -PBR111 uptake increase relative to NLHM volumes was higher in lesions (89%) than in the perilesional volumes (54%). 60% of lesions and 67% of perilesional volumes had higher ^{18}F -PBR111 V_T relative to NLHM.

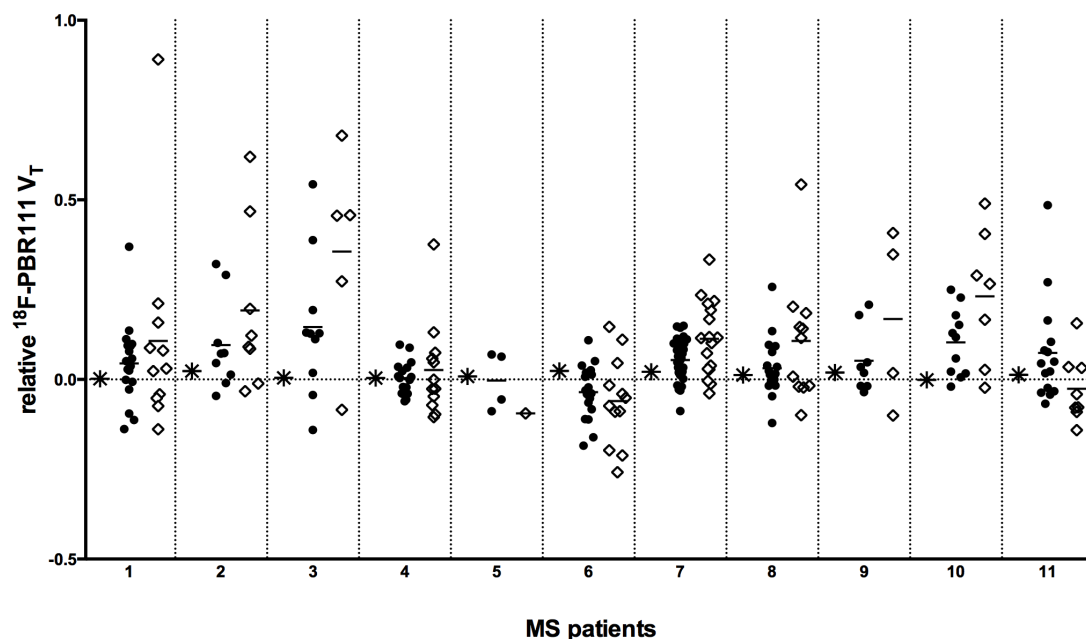


Figure 4.6 Relative $^{18}\text{F-PBR111}$ uptake across lesions, perilesional volumes and non-lesional white matter with low MTR

Relative $^{18}\text{F-PBR111}$ uptake (relative to the NLHM white matter) in NLLM white matter, in individual T2-FLAIR lesions and in perilesional volumes for the MS patients studied. The ordinant represents the relative difference in $^{18}\text{F-PBR111 } V_T$ in lesions ($\diamond$), perilesional volumes $\bullet$, and in NLLM white matter ($*$) relative to normal-appearing white matter (NLHM). On the abscissa, MS patients (see Table 4.1) are separately indicated.

Partial Volume correction: The relative increase in ^{18}F -PBR111 V_T in the two lesions (case no. 9, Table 4.1) examined after PVC were higher than pre-PVC (post-PVC Δ_L , 59.8% and 102.5%; pre-PVC Δ_L , 34.7% and 40.7%, respectively). The relative increase in ^{18}F -PBR111 V_T vs NLHM in perilesional volumes was not meaningfully altered by PVC (post-PVC Δ_{PL} , 18.9 % and 17.5%; pre-PVC Δ_{PL} : 17.9% and 20.8%, respectively).

Figure 4.7 (A-C) overlays locally thresholded ^{18}F -PBR111 V_T increases on T2-FLAIR hyperintense volumes in two patients with different clinical characteristics. In a patient who had experienced high disease activity (5 relapses in the last 2 years; Table 4.1, case 9), T2-FLAIR hyperintense lesion areas correspond to areas of increased ^{18}F -PBR111 signal. Conversely, in a patient with a more benign disease course and with no history of new neurological symptoms reported over the past 2 years (Table 4.1, case 4), focal areas of increased radiotracer binding correspond poorly with T2-hyperintense regions of white matter (Figure 4.7, D-F).

Disease severity, as assessed from MSSS scores, correlated with the mean ^{18}F -PBR111 V_T increase in lesions for each patient (expressed relative to that in individual NLHM white matter) (Spearman's $\rho=0.62$; $p=0.05$; Figure 4.8). The relationship of MSSS scores to ^{18}F -PBR111 V_T^{NLHM} and to the lesion load in the white matter was also examined, and I found no significant correlation to any of these two variables (Spearman's $\rho=-0.3$, $p=0.38$ and $\rho=-0.39$, $p=0.26$, respectively).

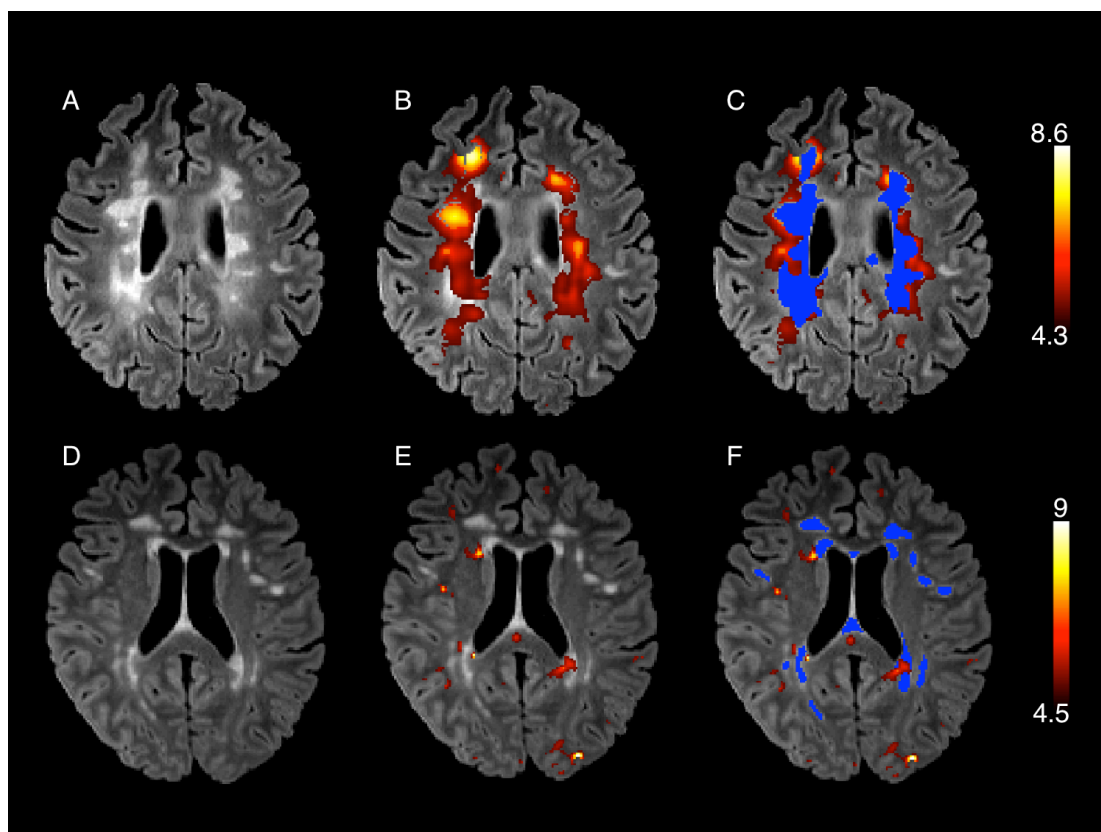


Figure 4.7 **Overlap between T2 FLAIR lesions and ^{18}F -PBR111 V_T**

A, D) T2-FLAIR images **B, E)** ^{18}F -PBR111 V_T parametric maps, overlaid (in warm colors) on T2-FLAIR images, and **C, F)** overlap between T2-FLAIR lesions (marked in blue) and ^{18}F -PBR111 V_T (in warm colors) from two illustrative patients. The lower threshold for V_T parametric maps corresponds to the value of ^{18}F -PBR111 V_T in the NLHM volume for each of the two patients. The upper threshold is twice the V_T in the NLHM.

The upper row illustrates a patient with recent active disease (Table 4.1, case 9). Here, T2-FLAIR hyperintense lesional areas correspond to areas of increased ^{18}F -PBR111 signal. The lower row illustrates a patient (Table 4.1, case 4) with a relatively benign disease course showing focal regions of increased ^{18}F -PBR111 V_T that correspond poorly to T2-FLAIR hyperintense areas.

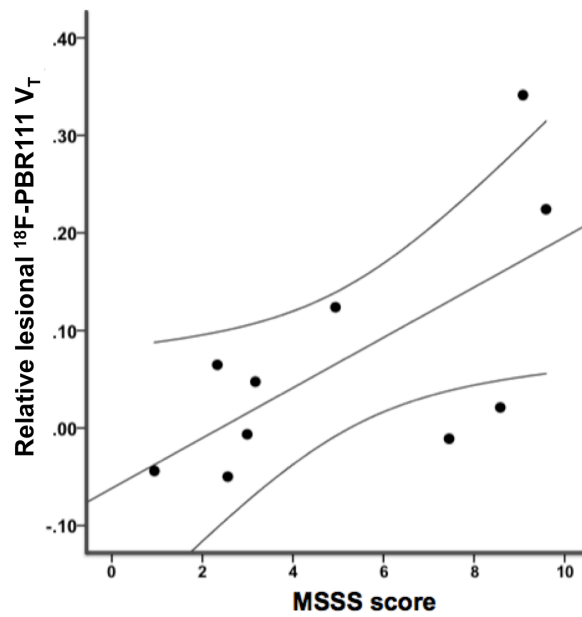


Figure 4.8 Relationship between lesional increase in ^{18}F -PBR111 uptake and disease severity

Positive relationship between MSSS scores and ^{18}F -PBR111 uptake in lesions (expressed relative to that in normal-appearing white matter) (Spearman's $\rho=0.62$; $p=0.05$).

4.3.3 *Test-Retest reproducibility*

The test-retest variability in ^{18}F -PBR111 V_T^{WM} across subjects (four healthy volunteers, one MS patient) was 23% (12% - 55%) [median (range)]. The test-retest variabilities in ^{18}F -PBR111 V_T^{L} and V_T^{PL} across the 18 T2-hyperintense lesion volumes identified in the MS patient who was studied twice on consecutive days were 25% (12% - 57%) and 27% (17% - 36%), respectively, while the median test-retest variabilities for the normalised DVR^{L} and DVR^{PL} were 8% (range 1%-31%) and 4% (range 0%-9%), respectively.

4.4 Discussion

TSPO PET can be used to assess the innate immune response in patients with MS *in vivo*, although methodological and technical challenges have limited its wide application. I have illustrated how corrected, quantitative measures with a 2nd generation TSPO radioligand, ¹⁸F-PBR111, promise new insights concerning clinical-pathological correlations relevant to disease progression. I found increased ¹⁸F-PBR111 V_T in the white matter lesional and perilesional volumes of MS patients compared to healthy volunteers. ¹⁸F-PBR111 V_T was higher in lesions, perilesional and non-lesional volumes with decreased MTR in MS patients relative to the normal-appearing white matter of the same subjects. Moreover, relative ¹⁸F-PBR111 V_T increase in the MS lesions was positively correlated with disease severity, adding to recent *in vivo* data consistent with a role for the innate immune response in the progression of neurodegeneration in MS (Giannetti et al., 2014).

Post-mortem autoradiographic studies in brains of patients with MS have consistently demonstrated that increased uptake of TSPO-targeted radioligands co-localizes with markers of activated microglia (Banati et al., 2000; Owen et al., 2010; Venneti et al., 2008; Vowinckel et al., 1997). An immunohistochemical analysis of post-mortem MS brain tissue was largely consistent with these findings, suggesting that most cells expressing TSPO in acute MS lesions were macrophages or microglia (Cosenza-Nashat et al., 2009), although it should be cautioned that antibody-based localization of the TSPO peptide and expression of the binding domain for the TSPO radioligands need not be the same. In a relapsing-remitting EAE model (Mattner et al., 2013), increased ¹⁸F-PBR111 uptake co-localizes with activated microglia and macrophages and parallels the temporal dynamics of their up regulation during experimentally

induced relapses.

I observed an increased ^{18}F -PBR111 uptake in approximately two-thirds of MRI-defined lesions and perilesional volumes in MS patients relative to their normal appearing white matter (NLHM). *Ex vivo* pathology studies show that acute, active lesions are characterised by a hypercellular inflammatory core, marked by prominent lymphocyte infiltration with high density of activated microglia and macrophages distributed evenly throughout the lesion (Trapp et al., 1999). Case 9 (Figure 4.7, top row) illustrates the strong co-localisation of increased ^{18}F -PBR111 V_T with T2-hyperintense lesions in active disease. In chronic active lesions, microglia are increased relative to distant normal white matter tissue and are more concentrated at the lesion edge than within the lesion. The hypercellular margin, characterised by a high density of activated microglia surrounding demyelinating plaques, is a consistent finding across neuropathological studies (Boyle and McGeer, 1990; Lucchinetti et al., 2000; Marik et al., 2007).

By contrast, approximately one third of the MRI-defined lesions and perilesional volumes were associated with ^{18}F -PBR111 uptake similar to, or lower than, that of the normal appearing NLHM white matter. Case 4 (Figure 4.7, bottom row) is an illustrative example of poor correspondence between areas of increased ^{18}F -PBR111 V_T and T2 FLAIR hyperintensities. I speculate that these volumes represent chronic inactive lesions that are hypocellular or have enlarged extracellular spaces leading to a relatively low density of all cells, including microglia. My findings of regional variation thus are consistent with post-mortem pathology observations in MS patients.

The median within-subject C_V in lesional ^{18}F -PBR111 uptake was above 15%. This indicates a moderately high variability of the observed ^{18}F -PBR111 signal between

the T2-hyperintense lesions even within a single MS patient, and further highlights that T2-hyperintense MRI contrast change reflects a wide range of neuropathology in MS lesions non-specifically (Bruck and Stadelmann, 2005).

An elegant study by Moll *et al.* (2011), using combined post-mortem pathology and MR imaging, reported that regions appearing normal on T2-weighted MR, but displaying reduced MTR, were associated with microglial activation and axonal degeneration. By applying the same image segmentation approach as Moll *et al.*, I observed a consistently, albeit very small, increased ^{18}F -PBR111 uptake in regions with reduced MTR (NLLM) across my study group. Nevertheless, there must be caution in interpreting this observation, as it is not clear what could be the true biological significance of a relative increase as small as that observed in the NLLM volume (1.2%).

Focal areas of activated microglia identified neuropathologically in white matter areas without apparent loss of myelin (Henderson *et al.*, 2009; Marik *et al.*, 2007) may represent areas at risk for the development of acute inflammatory lesions. They have been described previously as “pre-active lesions” (De Groot *et al.*, 2001) or regions of chronic microglial activation that may contribute independently to progressive neurodegeneration (Smith and Lassmann, 2002). Alternatively, white matter microglial activation in the absence of inflammatory demyelination may represent secondary reactive changes, perhaps associated with Wallerian degeneration (Singh *et al.*, 2013). Direct tests of these alternative hypotheses now seem possible using serial MRI observations to follow the course of these white matter changes identified by TSPO PET.

Although this work has gone further than previous studies in using a second

generation TSPO radioligand for MRI image-correlated quantitative analyses, my findings are in general agreement with those of some TSPO-targeted PET studies in MS. Previous work using ^{11}C -(R)-PK11195 demonstrated increased uptake to correspond to white matter Gadolinium-enhancing lesions (Banati et al., 2000; Debruyne et al., 2003; Vowinckel et al., 1997). One early study suggested the presence of focal areas of increased uptake in normal appearing white (and grey) matter (Banati et al., 2000). This was confirmed by a recent study by Rissanen and coworkers in secondary progressive MS, that found increased ^{11}C -(R)-PK11195 uptake in periventricular areas and in the normal appearing white matter, relative to healthy controls (Rissanen et al., 2014). However, patterns of ligand uptake in T2-weighted hyperintensities, and correlations between ^{11}C -(R)-PK11195 uptake and disease duration and disability have been inconsistent in previous studies (Banati et al., 2000; Debruyne et al., 2003; Versijpt et al., 2005). This could reflect differences in patient populations, differences in the proportion of specific (displaceable) signal between ^{18}F -PBR111 and ^{11}C -(R)-PK11195 or accurate modeling, which is particularly challenging for the lower affinity ^{11}C -(R)-PK11195 (Giannetti et al., 2014; Yaqub et al., 2012).

This is the first study using a 2nd generation TSPO ligand to successfully detect significant differences in radioligand uptake between MS patients and healthy controls. The lack of differences seen in previous studies may be explained by failure to control for the variance introduced by the rs6971 TSPO SNP (Oh et al., 2011; Takano et al., 2013).

There are a number of limitations of my analysis. I studied only a small number of patients. Further characterisation of the heterogeneity of the disease is needed. The use of disease-modifying treatments in the majority of MS patients studied may have

influenced the ^{18}F -PBR111 signal. A study by Ratchford and colleagues showed a 3.2% reduction in ^{11}C -(R)-PK11195 in relapsing-remitting MS patients after 1-year treatment with glatiramer acetate, for example (Ratchford et al., 2012). This estimate of treatment effect is much lower than the differences in binding I found between healthy volunteers and patients overall, however (approximately 40% in the whole white matter). However, in future work, it will be important to investigate the effects of various MS treatments on TSPO specific binding.

MS patients and healthy controls were not matched for gender, although to my knowledge there have been no reports of a gender effect on TSPO binding in humans. Studies in rodents suggested a higher number of microglia and astrocytes in adult females (Mouton et al., 2002; Schwarz et al., 2012), so I cannot exclude the possibility that a higher prevalence of women in the MS group may have contributed to the higher ^{18}F -PBR111 V_T in patients.

Finally, increased TSPO is seen not just in activated microglia, but also in rare, activated astrocytes (Cosenza-Nashat et al., 2009) and in lymphocytes (Berkovich et al., 1993), as well as in brain vascular endothelia (Turkheimer et al., 2007). The interpretation of increased ^{18}F -PBR111 V_T reported here as arising largely from activated macrophages or microglia is based on prior neuropathological studies demonstrating large numbers of these cells post-mortem in the regions studied and on the relatively high binding to them relative to other inflammatory cells (Boyle and McGeer, 1990; Lucchinetti et al., 2000; Marik et al., 2007; Trapp et al., 1999). A contribution from other inflammatory cell types cannot be excluded, but binding (B_{max}) to lymphocytes is relatively much lower (Canat et al., 1993).

I found considerable variability in ^{18}F -PBR111 V_T between subjects, independent of

their TSPO genotypes. The positive correlation between age and binding may explain some of this (Guo et al., 2013), but other factors also must contribute. The assessment of test-retest reproducibility of V_T demonstrated moderate measurement variance. One contribution to this can arise from identification of the true parent plasma input function. TSPO is abundant in the body and blood. Variation in the proportions of any displaceable (specific) and non-displaceable (non-specific) binding signals in plasma and tissue can also lead to variability. Consistent with these hypotheses, I found that the test-retest reproducibility of lesional and perilesional signal in MS was significantly improved after normalisation by correction for normal-appearing white matter signal with normal MTR. Normalisation of focal signal intensity in T2-MRI lesions by correction for NLHM values may be a particularly useful approach for increased precision of assessments in longitudinal studies.

The extent of inter-subject variability despite correction for genotype, and the relatively large overlap of ^{18}F -PBR111 signal between control and patient populations are consistent with data obtained with other 2nd generation TSPO radioligands in MS and other neuroinflammatory diseases (Kreisl et al., 2013b; Oh et al., 2011; Takano et al., 2013). This may indicate that clinical applications of TSPO PET imaging will require large sample sizes to detect increases in microglia activation in patient populations. In longitudinal applications, the use of normalisation (as suggested above) would reduce test-retest variability, increasing precision of the assessments, but may potentially lead to underestimation of changes in binding over time. All together these are limitations that may blunt the sensitivity of TSPO PET for detection of neuroinflammatory processes in vivo.

A number of methodological aspects are worth considering. Partial volume effect may have confounded accurate measurement of binding and led to the underestimation of

increases of TSPO binding in lesional VOIs. This should influence estimates in smaller regions to the greatest extent, but I have found no evidence that smaller volumes were associated with lower increases, suggesting that this was not a dominant effect. Furthermore, PVC applied to two large lesional volumes as an exploratory tool, suggested that tissue differences may be underestimated due to volume averaging. In fact, the relative increase in ^{18}F -PBR111 uptake in lesions were enhanced after PVC. However, as PVC did not affect the relative increases in perilesional volumes, it is unlikely that my observation of increased TSPO uptake in perilesional white matter was artifactually confounded by partial volume effects.

As ^{18}F -PBR111 V_T reflects both displaceable (presumed specific for TSPO) and non-displaceable (non-specific or off-target binding) binding, the true differences in inflammatory response between VOIs may be underestimated. I estimate that the proportion of ^{18}F -PBR111 V_T corresponding to specific signal ranges between 30 and 70% across genotypically defined binding affinity classes (Guo et al., 2013). The observed increases in lesion associated signal, (ranging between 15 - 50% relative to the normal-appearing tissue) therefore suggests an increase in microglia-related TSPO binding of between 20 - 80% across different lesions.

While a 2TC kinetic model has been found to fit the data well, we have observed that V_T increases slightly with time for reasons not yet well understood (Guo et al., 2013). This could have been due to some small, irreversible contribution, e.g., from slow entry of a radiolabelled metabolite into the CNS and its non-specific association. I attempted to control for this in my analysis by applying a correction factor to the values obtained with shorter scan durations intended to minimize biases due to underestimation of the signal. If the statistical analysis were run without the three subjects with shorter scan duration, I would have observed significant higher V_T in all

MS patients' ROIs (including NLHM and NLLM) relative to healthy controls V_T^{WM} (all $p < 0.05$). The correlation between MSSS severity and ^{18}F -PBR111 V_T increase in lesions would have remained significant ($r = 0.76$; $p < 0.05$) while the partial correlation between disease duration and V_T^{WM} in HABs would have lost significance ($r = 0.88$; $p = 0.12$), however only 5 subjects would have been left for this analysis.

Finally, it is worth mentioning that two MS patients were on natalizumab treatment at the time of the ^{18}F -PBR111 scan (Table 4.1). Natalizumab treatment reduces the relapse rate in MS patients more effectively than other available treatments (Mellergard et al., 2010; Stuve and Bennett, 2007). I hypothesize that treatment with natalizumab in those two patients may have reduced ^{18}F -PBR111 binding to a greater extent than was apparent in other patients treated with interferon- β , however further research is necessary to differentiate the effects on TSPO specific binding of specific disease-modifying treatments for MS.

In conclusion, this study demonstrates that quantitative TSPO PET with a 2nd generation radioligand is sensitive to an element of the inflammatory response in MS not apparent on MRI and so otherwise occult *in vivo*. It highlights opportunities for the integration of MRI and molecular imaging for understanding the evolution of the dynamic neuropathology of MS.

Chapter 5. Assessment of hippocampal ^{18}F -PBR111 binding and its relationship to depressive symptoms in patients with Multiple Sclerosis

5.1 Introduction

MS is associated with a higher prevalence of depression relative to that of the general population (Kessler et al., 2003), and the prevalence of depression in MS exceeds that associated with having one or more other long-term disabling conditions (Patten et al., 2003).

A causal association between the activation of innate immunity and the development of depression has been proposed. The action on the brain of pro-inflammatory mediators, such as cytokines (for example interleukin- 1α and β , interleukin-6, tumor necrosis factor- α) leads to a potent modulation of affect, resulting in “sickness behaviour” and, in vulnerable individuals, to the development of depressive symptoms (Dantzer et al., 2008). It is therefore possible that the increased prevalence of depressive symptoms in MS patients is a direct consequence of the chronic neuroinflammatory injury characteristic of MS pathology.

MRI studies have reported associations between depressive symptoms in MS patients and various parameters, such as the T1-weighted or T2-weighted lesion load, the localization of lesions to areas important for mood disorders, the extent of brain atrophy, and abnormalities in diffusion parameters. However, each of these associations accounted for only a small amount of total variance in depressive symptoms and specific associations have not been consistent across studies (Feinstein et al., 2014). As structural MRI-based alterations reflect a wide range of

neuropathological abnormalities, these studies also provide a limited insight into mechanisms underlying contributions of neuroinflammation to the pathophysiology of depression in MS.

Convergent lines of research implicate the hippocampus in the pathogenesis of depression (MacQueen and Frodl, 2011). Hippocampal atrophy is a well-characterised finding in patients with Major Depressive Disorder (MDD) and has been proposed to predispose to MDD (Cole et al., 2011). Hippocampal volume loss and altered morphology have been found to associate with depressive symptoms of MS patients (Gold et al., 2010; Gold et al., 2014; Kiy et al., 2011).

The hippocampus (HC), well recognised as involved in the modulation of affect, is highly susceptible to neuroinflammation and has the highest concentration of brain receptors for the pro-inflammatory cytokine interleukin-1 (Parnet et al., 2002). Post-mortem and imaging studies have found evidence of extensive demyelination, microglia activation, neuronal loss (Geurts et al., 2007; Papadopoulos et al., 2009) and atrophy in the hippocampus of MS patients (Sicotte et al., 2008). Hippocampal neuroinflammation in the rodent experimental allergic encephalomyelitis model is associated with dysfunctional neurogenesis (Giannakopoulou et al., 2013) and hippocampal neurogenesis may be critical to recovery from depression (Dranovsky and Hen, 2006).

Here I describe the use of TSPO-targeted ^{18}F -PBR111 PET as a measure of activated microglia to characterise hippocampal neuroinflammation in MS patients and to study the relationship between this hippocampal neuroinflammation and depressive symptoms. I have evaluated the distribution of the ^{18}F -PBR111 PET signal in the cortical grey matter and the hippocampus and other limbic regions in brains of MS

patients and healthy controls. I then tested for differences between MS patients and healthy controls in the binding ratio (the DVR) of hippocampus, and two other brain regions (thalamus and brainstem), to that of the general cortical regions. All comparisons were controlled for age and the rs6971 polymorphism that determines binding affinity of 2nd generation TSPO radioligands to TSPO (Guo et al., 2013). Finally I assessed the relationship of relative hippocampal ¹⁸F-PBR111 binding to the symptoms and diagnosis of depression in the MS patients, controlling for age and disability.

5.2 Methods

5.2.1 Study design

After baseline screening for eligibility, the MS patients and healthy controls returned to the research centre for an MRI/¹⁸F-PBR111 PET scan visit within approximately one week (see chapter 2 for details). All study subjects underwent a full medical, neurological and psychiatric examination at screening. This included administration of a structured diagnostic clinical interview based on criteria in the *Diagnostic and Statistics Manual of Mental Disorders, Fourth Edition, Text Revision* (DSM-IV TR) (American Psychiatric Association., 2000), and the administration of a self-reported clinical questionnaire for assessment of depressive symptoms (see clinical assessments section for details). All subjects were genotyped for the rs6971 SNP.

5.2.2 Study sample

Eleven patients with relapsing remitting MS [age, 45.1 ± 8.9 years (mean $\pm$ SD); 10 females] and 22 healthy controls (age, 49.8 ± 11.1 years; 14 females) were studied. The self-report questionnaire for depression was not administered to 7 of the healthy controls. Further details on subjects, including recruitment strategies, eligibility criteria, ethics approval, and full clinical assessments are provided in Chapter 2. The clinical and demographic characteristics of subjects, as well as the concomitant disease-modifying treatment used, are listed in Table 4.1. Pharmacological treatments prescribed for the MS patients had not been changed for at least 3 months prior to scanning. No subject received steroid treatment in the month before the PET scans.

6/11 MS patients were on antidepressant medications. Of these, four patients were treated with Selective Serotonin Reuptake Inhibitors (citalopram, n=2; sertraline n=1;

fluoxetine, n=1), one patient with mirtazapine, while another patient was receiving a tricyclic antidepressant (amitriptyline).

Subjects were stratified into three groups on the basis of their TSPO rs6971 SNP genotype: HABs, MABs, or LABs (Owen et al., 2012).

Case	Age	Gender	TSPO gene group	Disease duration (years)	EDSS	BDI	MDE
HV1	45	F	MAB			6	None
HV2	36	F	MAB			0	None
HV3	33	F	HAB			9	None
HV4	52	F	LAB			0	None
HV5	61	F	MAB			0	None
HV6	42	F	HAB			0	None
HV7	52	F	HAB			1	None
HV8	43	M	MAB			0	None
HV9	50	F	HAB			4	None
HV10	28	M	HAB			1	None
HV11	52	F	HAB			0	None
HV12	51	M	LAB			0	None
HV13	65	M	LAB			8	None
HV14	28	M	HAB			2	None
HV15	57	M	MAB				None
HV16	59	F	HAB				None
HV17	59	M	HAB				None
HV18	62	M	HAB				None
HV19	60	F	MAB				None
HV20	60	F	MAB				None
HV21	56	F	MAB				None
HV22	44	F	LAB				None
MS1	48	F	HAB	8	6.5	23	Current
MS2	39	F	LAB	20	4	9	Recent
MS3	40	F	HAB	11	4	24	Current
MS4	55	F	HAB	20	2	5	None
MS5	53	F	LAB	20	7	30	Current
MS6	50	F	HAB	2	4	10	Current
MS7	59	F	MAB	16	3	8	Recent
MS8	41	F	HAB	14	1.5	3	None
MS9	28	M	HAB	7	2	14	Recent
MS10	41	F	MAB	4	5.5	7	None
MS11	42	F	HAB	1.5	6	19	Current

Table 5.1 Clinical and demographic characteristics of study participants

HC: healthy controls MS: multiple sclerosis patient; HAB: high affinity binder; MAB: mixed affinity binder; LAB: low affinity binder; BDI: Beck depressive inventory; EDSS: expanded disability status scale; MDE; major depressive episode; TSPO: 18kDa Translocator Protein.

5.2.3 *Psychiatric diagnosis and clinical assessments*

Diagnoses of Major Depressive Episode (MDE) were made at screening visit by an experienced psychiatrist. For this purpose the Mini Neuropsychiatric Interview (MINI) (Sheehan et al., 1998), based on DSM-IV diagnostic criteria, was used. Current and recent (within the past six months) MDEs were recorded. The Beck Depressive Inventory-II (BDI-II) (Beck et al., 1996) was used for assessment of depressive symptoms. The BDI-II is a multiple-choice self-report scale consisting in 21 items. Patients are asked to rate how they have been feeling for the past two weeks. For each item, scores range between 0 and 3. Disability was assessed at screening by an experienced neurologist using the EDSS.

5.2.4 *Definition of Volumes of Interest*

Three anatomically-defined brain areas were evaluated: the hippocampus, my *a priori* chosen VOI, plus the thalamus and brainstem as control VOIs to test whether any observed increase in TSPO was hippocampus-specific or global. The choice of thalamus and brainstem as control VOIs was based on their potential for accurate segmentation and high TSPO signal. The brainstem volume included medulla, pons, and midbrain. A VOI encompassing the entire cortical grey matter was used as a pseudo-reference region (see Figure 5.1 and text below for details).

The amygdala is also an important region for the pathophysiology of affective disorders, as it has a crucial role in the modulation of fear and defensive behaviours and aversive responses to threat. However, in my pilot investigation I focused my interest on the hippocampus for its known involvement in MS pathology, and for its susceptibility to neuroinflammation. Also, the amygdala doesn't provide a particular advantage in terms of potential for accurate segmentation and robust TSPO signal,

hence was not chosen as control VOI.

Although brain regions such as the insula, cingulate cortex, and frontal cortex are also very interesting, considering their known role in the pathophysiology of depression, they were not included in my analysis. In fact, these neocortical areas are part of the cortical gray matter volume that I used as a pseudo-reference region, hence those regions were not appropriate VOIs for the calculation of the DVR. For exploratory purposes, the differences between MS patients and healthy controls in ^{18}F -PBR111 V_T were investigated and reported in the results section.

My pre-chosen VOIs (hippocampus, thalamus and brainstem) and the cortical grey matter were defined with the ICBM152 template (<http://www2.bic.mni.mcgill.ca>), derived from an average of MRI brain scans from the Montreal Neurological Institute. The template was nonlinearly warped to the high-resolution T1-MRI of each individual, with Statistical Parametric Mapping 5 (SPM5; Wellcome Trust Centre for Neuroimaging). The deformation parameters derived were then applied to a corresponding anatomical atlas, the Clinical Imaging Center atlas (Tziortzi et al., 2011) to bring this into the space of the individual subject. Finally, the MRI image, VOIs, and warped anatomical atlas were resampled to match the PET image resolution. From the atlas, the three VOIs were automatically defined with SPM5 (Figure 5.1 A). Due to defluorination of the metabolite (Fookes et al., 2008) ^{18}F -PBR111 defluorinates *in vivo* resulting in a relatively high signal in the bones of the skull (Carson et al., 2003; Guo et al., 2013) The VOI for the cortical grey matter was therefore eroded of 5 mm away from the skull to avoid “spill-in” from uptake of ^{18}F -fluoride by bone of the skull (Figure 5.1 B).

Each VOI then was applied to the dynamic PET data to generate regional TACs.

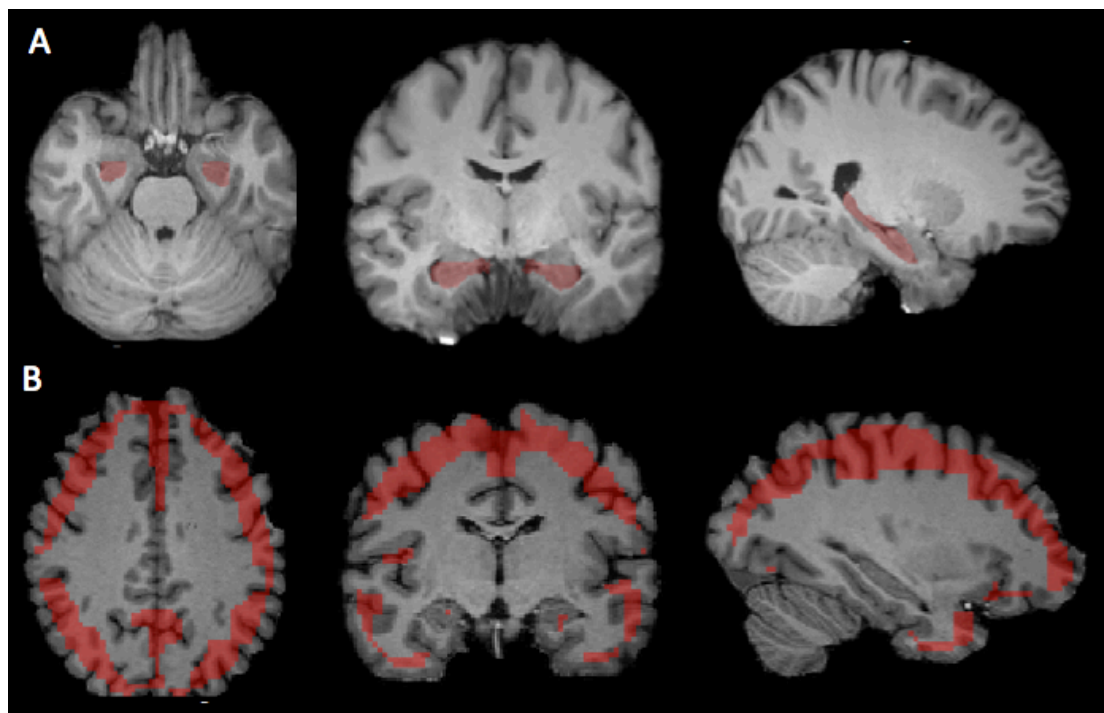


Figure 5.1 Definition of Volumes of interest The top row (A) illustrates the definition of the hippocampus using the CIC atlas. The bottom row (B) illustrates the definition of the VOI for the cortical grey matter used as a pseudo-reference region for the calculation of DVR. The VOI deriving from the anatomical atlas was eroded to avoid “spill in” from skull bone uptake of ^{18}F -fluoride produced by metabolism of the labeled PBR111.

5.2.5 *Quantification of TSPO signal*

^{18}F -PBR111 V_T was quantified using a 2TC kinetic model with metabolite-corrected arterial input function. Details on ^{18}F -PBR111 V_T quantification have been provided in Chapters 2 and 3. For the study of cortical and limbic distribution of ^{18}F -PBR111 signal, the Logan graphical method (Logan et al., 1990) was used to estimate the V_T of each VOI and further applied at the voxel level to produce parametric V_T maps.

I used regional relative ^{18}F -PBR111 binding as an index of the regional density of activated microglia. I calculated the DVR, which was defined the ratio of ^{18}F -PBR111 V_T in a VOI of interest to the ^{18}F -PBR111 V_T across the entire cortical grey matter. The use of DVR reduces variability in modeling the brain signal arising from imprecision in measurement of blood concentrations by normalising to a reference tissue uptake. By dosing so, DVR expresses only a relative signal and global differences in the specific binding component cannot be detected. However, my analysis was testing for differences in the regional distribution of activated microglia, which was assumed to be heterogeneous, rather than for absolute measures of signal in any region. The elimination of global differences in the ^{18}F -PBR111 specific signal also reduces the between-subject variability associated with the rs6971 polymorphism of the TSPO gene (Guo et al., 2013). Use of a region that is recognized to bind TSPO radiotracers and in which MS associated increases in binding are observed must be taken into account when assessing the magnitude and sign of the DVR for a region of interest.

5.2.6 Statistical analysis

Between-groups differences in BDI scores were tested using Mann-Whitney *U* Test for independent samples. The correlations between BDI scores, age, EDSS scores, and disease duration were studied using Spearman's non-parametric correlation.

To study the distribution of ^{18}F -PBR111 specific signal in cortical and limbic regions of healthy controls, I normalised voxel-level V_T values for the mean uptake in the cortical grey matter (Cort GM), to obtain parametric maps of *z* scores. Voxel-wise *Z* scores maps were computed as follows:

$$(1) z \text{ score}(x) = [V_T \text{ value}(x) - \text{mean (Cort GM)}] / \text{SD (Cort GM)},$$

where *x* represents each voxel of the parametric map.

Between-groups differences in ^{18}F -PBR111 DVR in the hippocampus, thalamus and brainstem were tested using an ANCOVA with ^{18}F -PBR111 DVR in the VOIs as dependent variables, controlling for age and rs6971 genotype. Differences in ^{18}F -PBR111 V_T in the 'reference' region, the cortical grey matter were also tested. The relationship between ^{18}F -PBR111 DVR in the hippocampus and BDI scores was tested in MS patients only, using Pearson's partial correlation, while controlling for age, EDSS scores, and disease duration. Statistical analyses were done using SPSS 20.0. To assess the proportion of variance in BDI scores explained by hippocampal ^{18}F -PBR111 binding, I first used a linear regression model with BDI scores as dependent variable and hippocampal ^{18}F -PBR111 DVR as an explanatory variable. Subsequently a second linear regression model was used using BDI scores as dependent variable, and hippocampal ^{18}F -PBR111 DVR, age, disease duration and EDSS scores as explanatory variables.

5.3 Results

5.3.1 MDE diagnosis and depressive symptoms in Multiple Sclerosis patients

5/11 MS patients (45%) met DSM-IV criteria for current diagnosis of MDE, while in 3 cases (27%) patients met criteria for a past MDE (within last 6 months), but did not have active symptoms at the time of examination. None of healthy volunteers had current or past MDE. MS patients had significantly higher BDI scores compared to healthy controls (MS, 10 (3-30) [median (range)]; HV, 0 (0-9); $Z=4.4$; $p<0.0001$).

BDI scores were significantly correlated to EDSS scores (Pearson's $r=0.72$; $p=0.013$) for MS patients, although there was no correlation between BDI scores and age or between BDI scores and duration of disease.

5.3.2 Distribution of ^{18}F -PBR111 uptake

Averaged ^{18}F -PBR111 signal (V_T) for healthy controls was distributed throughout the brain (see Chapter 3). Areas with higher ^{18}F -PBR111 signal, indicative of higher TSPO density, included periaqueductal grey, thalami, hippocampi, amygdala, cingulate gyrus and insula.

Figure 5.2 displays normalised z-score parametric maps from ^{18}F -PBR111 V_T for comparison of the distributions of ^{18}F -PBR111 signal in the cortical and limbic grey matter in healthy controls and MS patients. In both groups, the hippocampal signal was higher than average cortical grey matter signal. The group map for MS patients also highlights increased relative signal in the insula bilaterally. Since the insula is included in the pseudo reference region used for the calculation of the DVR, it was not possible to assess between groups differences in the insula using the DVR.

However, no increase in the insular V_T was observed in the MS patients relative to controls in my exploratory analysis.

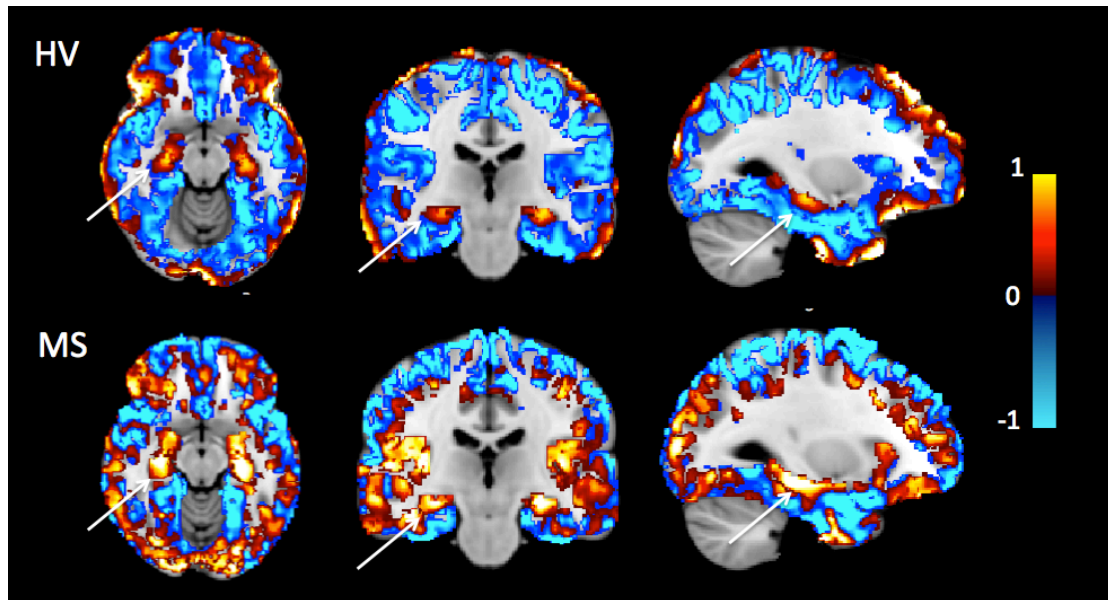


Figure 5.2 Distribution of cortical ^{18}F -PBR111 signal in healthy volunteers and MS patients

Parametric maps of normalised ^{18}F -PBR111 V_T (z scores) in the cortical and limbic grey matter areas, in healthy volunteers (top row), and MS patients (bottom row), in axial, coronal and sagittal views. Values higher than 1 (hot colours) indicate a signal higher than average cortical grey matter. Values lower than 1 (cool colours) indicate a signal lower than average cortical grey matter. Arrows illustrate the location of the hippocampus

5.3.3 *Between Subjects contrasts of ^{18}F -PBR111 DVR*

^{18}F -PBR111 DVR was higher in the hippocampus of MS patients relative to healthy controls [MS patients 1.08 ± 0.04 (mean $\pm$ SD); healthy controls 1.03 ± 0.09 ; $t = 2.49$; $p=0.018$] (Figure 5.3). The difference remained significant after correction for age and rs6971 genotype ($F=5.73$; $p=0.024$). By contrast, ^{18}F -PBR111 DVR in the thalamus and brainstem did not differ significantly between MS patients and healthy controls (Thalamus, MS patients 1.09 ± 0.06 , HVs 1.06 ± 0.07 ; N.s. Brainstem, MS patients 1.11 ± 0.14 , healthy controls 1.09 ± 0.11 ; N.s.) (Figure 5.3). The V_T in the cortical grey matter was similar between MS patients and healthy controls after correction for age and rs6971 genotype ($F=0.01$; $p=0.987$). My exploratory analysis also revealed that there was no difference in ^{18}F -PBR111 V_T in the insula, cingulate cortex, and frontal cortex between MS patients and controls (all $p>0.9$, correcting for age and rs6971 genotype).

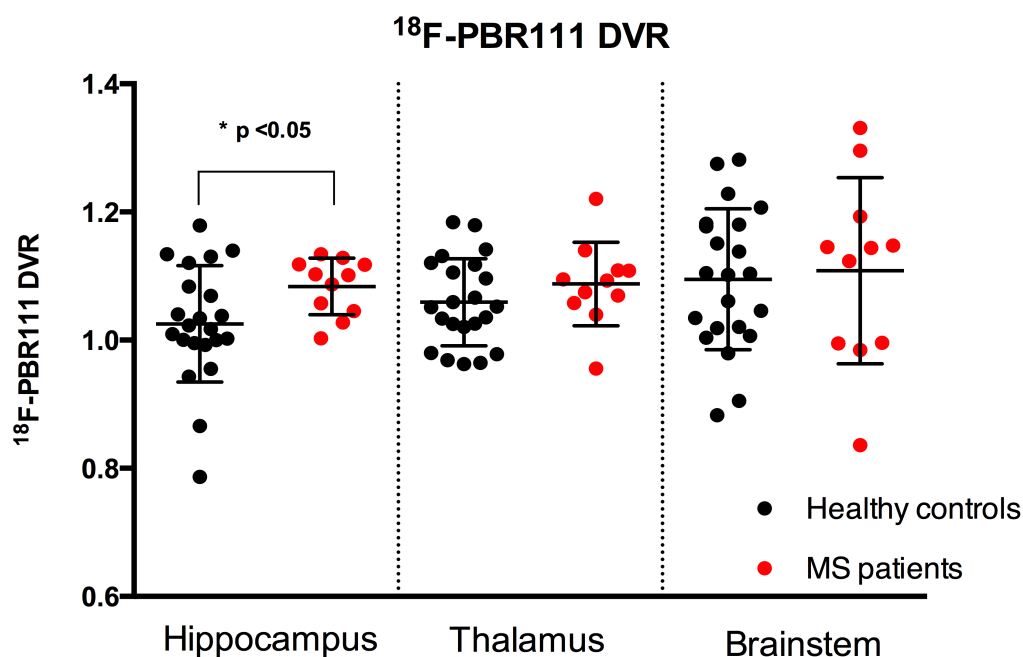


Figure 5.3 Comparison of ^{18}F -PBR111 DVR between groups in the VOIs

The Distribution Volume Ratio (DVR) of ^{18}F -PBR111 DVR was higher in MS patients (red) than healthy controls (black) in the hippocampus but not in the thalamus or brainstem. Each dot represents individual subjects' DVR values. The horizontal lines represent group's mean and standard deviation.

5.3.4 Relationship between ^{18}F -PBR111 DVR and MDE diagnosis

A current MDE diagnosis for MS patients was associated with a higher hippocampal ^{18}F -PBR111 DVR than in the group of MS patient with no history or with recent but resolved MDE (current MDE 1.11 ± 0.018 , no history and resolved MDE 1.06 ± 0.047 ; $t=2.48$; $p=0.044$; Figure 5.4). The hippocampal ^{18}F -PBR111 DVR values of the three MS patients with no history of MDE diagnosis (range, 1.028 – 1.057) were within the 95% confidence interval for the mean of ^{18}F -PBR111 DVR of healthy volunteers (95% CI, 0.98 – 1.066).

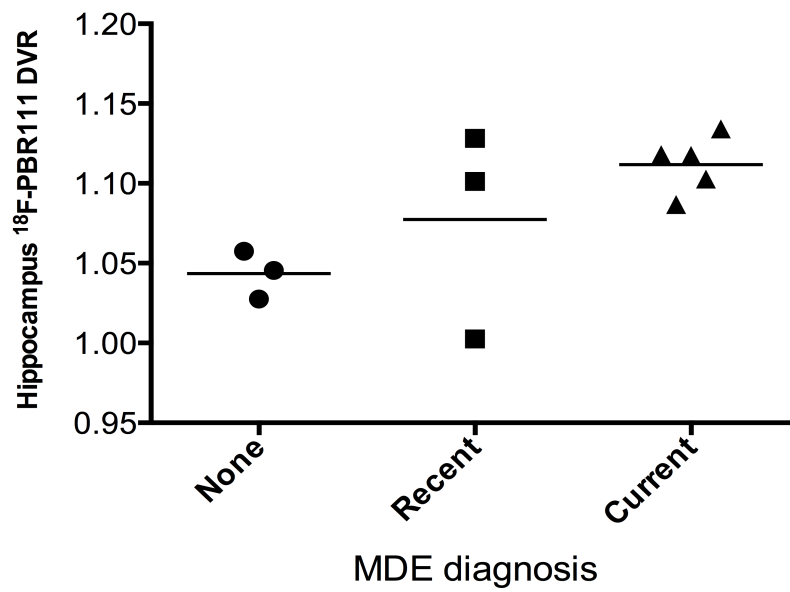


Figure 5.4 Relationship between MDE Diagnosis and hippocampal ^{18}F -PBR111 DVR in MS patients

Recent MDE diagnosis refers to the occurrence of a MDE in the last 6 months that has resolved at the time of scanning.

5.3.5 Relationship between ^{18}F -PBR111 DVR and depressive symptoms

I found a positive correlation in MS patients between ^{18}F -PBR111 DVR in the hippocampus and BDI scores (Pearson's $r=0.63$; $p=0.037$). The strength of the independent relationship was stronger with control for age, disease duration, and EDSS scores (Pearson's partial correlation $r=0.863$; $p=0.006$) (Figure 5.5). The correlation of BDI scores was specific for ^{18}F -PBR111 DVR in the hippocampus; there was no correlation between BDI scores and ^{18}F -PBR111 DVR in the thalamus (Pearson's $r=0.17$, N.s.) or brainstem (Pearson's $r=0.29$, N.s.).

Linear regression analysis showed that while a model with hippocampal ^{18}F -PBR111 DVR alone as a regressor explained 33% of the BDI score variance, a model that also included hippocampal ^{18}F -PBR111 DVR, age, disease duration and EDSS scores as regressors, explained 82% of the BDI score variance.

Figure 5.5 Relationship between hippocampal ^{18}F -PBR111 DVR and BDI scores

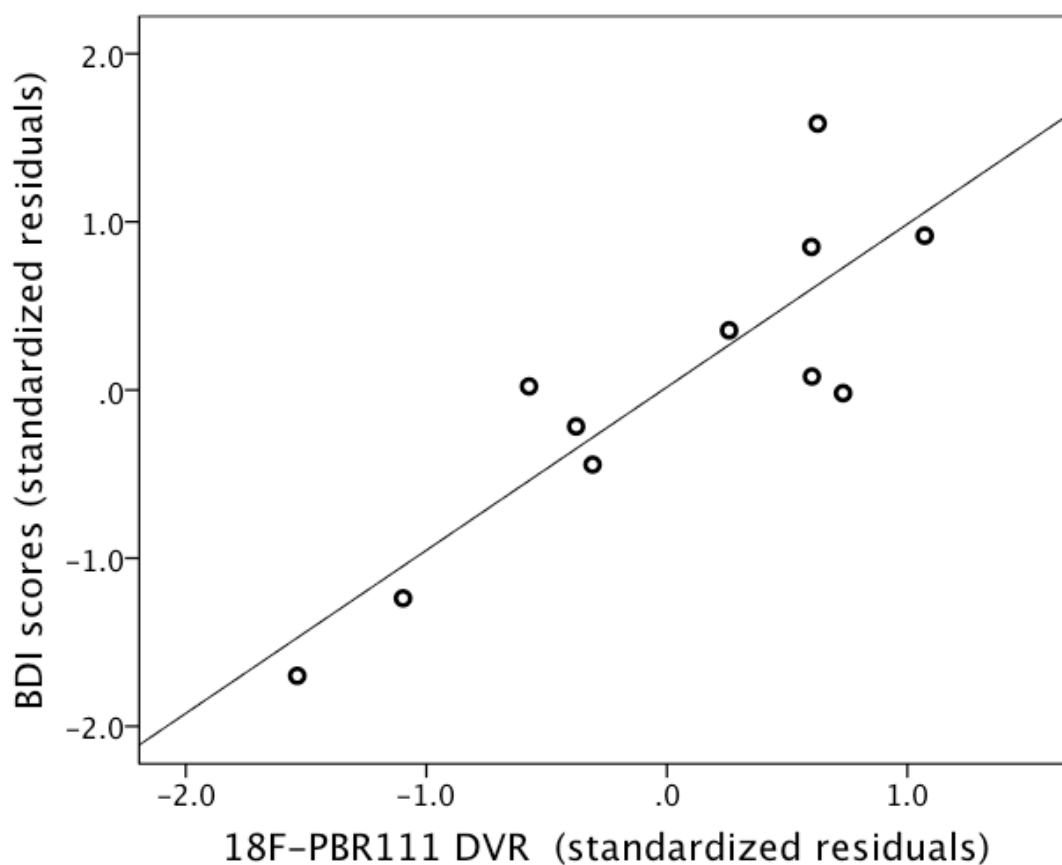


Figure 5.5 Relationship between hippocampal ^{18}F -PBR111 DVR and BDI scores

Partial regression plot illustrating the correlation between Distribution Volume Ratio of ^{18}F -PBR111 in the hippocampus and BDI scores in MS patients ($n=11$), after correcting for age, duration of disease and EDSS scores. Values represent standardized residuals of the dependent variable (BDI scores) and ^{18}F -PBR111 DVR when both these variables are regressed on the rest of the independent variables (age, duration of disease, and EDSS scores). BDI scores are represented on the ordinate, while ^{18}F -PBR111 DVR are represented on the abscissa.

5.4 Discussion

I presented the use of the novel TSPO-targeting PET radiotracer ^{18}F -PBR111 to assess hippocampal microglial inflammation in patients with relapsing-remitting MS, and to investigate whether depressive symptoms are associated with hippocampal inflammation.

PET imaging of the TSPO has long been used as a proxy of microglia activation. Specific ^{18}F -PBR111 signal co-localises with markers of activated microglia in rodents (Mattner et al., 2013) and, in MS patients *in vivo*, is increased in brain areas that T2-weighted MRI identifies as lesions (Colasanti et al., 2014) (see Chapter 4). My finding of a relatively high ^{18}F -PBR111 binding in the hippocampus is consistent with previous studies showing that the hippocampus is one of the grey matter regions with the highest density of microglia and TSPO binding in healthy subjects (Gulyas et al., 2009; Lawson et al., 1990; Mittelbronn et al., 2001).

I found additionally increased ^{18}F -PBR111 binding in the hippocampus of MS patients relative to healthy volunteers. There was no difference in the cortical signal normalised radiotracer binding in the thalamus and brainstem, two other large and TSPO-dense regions. This observation is consistent with previous data identifying a substantial disease burden in the hippocampus. Post-mortem neuropathology identifies hippocampal demyelination in brains of 50-80% of MS patients (Geurts et al., 2007; Papadopoulos et al., 2009). Activated microglia appear most abundant in actively demyelinating lesions or on the edges of more chronically demyelinated lesions. Also, post-mortem studies indicated that MS-related hippocampal damage increases with increasing disease duration, which would suggest that depressive symptoms are more common in secondary progressive MS and worsen with

progression. This prediction contrasts with the higher reported prevalence of depression amongst patients with relapsing remitting disease relative to secondary progressive MS (Feinstein et al., 2014). The presence of higher microglial activation in active lesions, characteristics of the relapsing remitting phase, could be a potential explanation for these apparently discrepant findings.

The most important finding of my study was that hippocampal, but not thalamic or brainstem, ^{18}F -PBR111 binding was highly correlated to self-reported depressive symptoms of patients. Also, hippocampal ^{18}F -PBR111 binding was highest in MS patients meeting the criteria for current MDE. Most of the subjects had a lifetime diagnosis of depression, and more than half of the MS patients studied were depressed at the time of the study, in line with the very high prevalence rates of depression reported in hospital-based clinics (Feinstein et al., 2014).

A link between hippocampal pathology and depressive symptoms in MS patients had been suggested previously by structural neuroimaging studies. Gold and coworkers reported an association of depressive symptoms with reduced dentate gyrus volume and higher cortisol levels (Gold et al., 2010). Kiy and coworkers used temporal horn volumetry as an indirect assessment of hippocampal volume in MS patients, and found depressive symptoms to be associated with smaller hippocampi (Kiy et al., 2011). In a follow up paper, Gold et al. described an association between hippocampal shape changes in MS patients and the affective component of their depressive symptomatology (Gold et al., 2014). My study's findings provide further insight into the relationship between hippocampal pathology and depressive symptoms in MS. They suggest that the action of innate immunity mediators at hippocampal level plays a significant role in the pathophysiology of the affective dysregulation associated with MS.

A role for neuroinflammation in the pathogenesis of depression has been postulated more generally. Epidemiologic studies have shown high comorbidity of depression with autoimmune and chronic inflammatory diseases, experimental studies have described cytokine-induced sickness behavior, and clinical observations have highlighted that immune activation therapies can cause clinically significant depression, and that pro-inflammatory cytokine levels are raised in the plasma of patients presenting with depression (Miller et al., 2009; Wager-Smith and Markou, 2011). Further evidence in direct support of a neuroinflammatory model of depression has been provided by studies using *in vivo* assessment of microglia activation with TSPO-targeted PET. An increased binding of first generation TSPO tracer ^{11}C -(R)-PK11195 was reported in hippocampus of euthymic bipolar disorder patients (Haarman et al., 2014). A recent study reported a global increase of ^{18}F -FEPPA V_T in the brain of patients with depression during a MDE (Setiawan et al., 2014). However, Hannestad and coworkers did not find differences in ^{11}C -PBR28 uptake between patients with mild-to-moderate depression and healthy volunteers (Hannestad et al., 2013).

Various mechanisms could underlie the association between hippocampal microglia activation and depressive symptoms observed in my study. Microglia-derived cytokines enhance enzymatic activity of indoleamine 2,3 dioxygenase (IDO), which could cause relative depletion of the serotonin precursor tryptophan and a reduction in the availability of serotonin for *de novo* synthesis, as well as the generation of neuroactive tryptophan metabolites that affect glutamatergic signaling at NMDA receptor (Dantzer et al., 2008). Pro-inflammatory cytokines also can activate hypothalamus-pituitary-adrenal (HPA) axis leading to excessive production of the

neuroactive peptide CRH, which in turn might precipitate depressive symptoms (Holsboer, 1999).

This analysis revealed an association between depressive symptoms and ^{18}F -PBR111 in the hippocampus, but not in the thalamus and brainstem. Some observations specifically implicate the hippocampus as a candidate region for the action of microglia-produced pro-inflammatory cytokines in mediating depressive symptoms: the highest IL-1 receptor density is observed in the hippocampus (Parnet et al., 2002) and LPS-induced depressive-like behavior is associated with abnormal cellular activity in specific brain structures such as hippocampus, extended amygdala, and hypothalamus (Frenois et al., 2007).

The cross-sectional nature of my study prevents us from making confident inferences of causality. Formally, it is difficult to establish whether hippocampal microglia activation causes depressive symptoms or vice versa. I cannot exclude the latter possibility; exposure of rodents to chronic stress and social isolation, for example, has been shown to cause proliferation, activation, and priming of hippocampal microglia (Walker et al., 2013). It is possible that MS patients, who are in an immunologically “primed” state (Ramaglia et al., 2012), may be more likely to manifest such a response. However, the relative anatomical specificity of the microglial response associated with BDI is striking. This important question needs further, longitudinal study, e.g., in populations treated with immunomodulators independent of any use of anti-depressants to evaluate the independent impact of a reduction of microglial activation on affective symptoms.

Some methodological limitations need to be considered. The sample of MS patients was small. A larger population is needed to evaluate the generalizability of my

observations. Most of MS patients studied were on disease modifying treatment. It is likely that this would have an effect, even if indirect, to reduce microglial activation and thus may have reduced the magnitude of differences that I observed (Ratchford et al., 2012). The interaction between disease modifying therapy, inflammation and depression is complex, as some disease modifying therapies, such as interferon- β , are themselves associated with depression. All of the patients with prominent depressive symptoms also were on antidepressant treatments that could have further confounded the relationship between depressive symptomatology and hippocampal inflammation, by influencing the expression of depressive symptoms, the activation of microglia or both. In future work, a longitudinal design will be needed to define independent contributions of these factors. Finally, I used the DVR as the outcome parameter to decrease variability of measurements and thus make such a modestly powered pilot study possible. Use of a normalised measure may have masked global differences in microglial activation, potentially contributing to an artifactually greater focality to microglial activation associated with depression. Alternatively, I cannot exclude that the observed between-groups difference could have been driven by a lower ^{18}F -PBR111 binding in the cortical grey matter (the pseudo-reference region) in MS patients, although this direction of effects (decreased microglial activation in associated with depression) would be counter to expectations based on other observations (Dantzer et al., 2008; Miller et al., 2009; Setiawan et al., 2014). Moreover, cortical grey matter ^{18}F -PBR111 V_T and the thalamic and brainstem ^{18}F -PBR111 DVR were similar across groups.

Despite the several factors that might have confounded detection of an association, the results are consistent with my hypothesis that microglial activation in the hippocampus is associated with depressive symptoms in patients with MS. The

strong association suggests that it is a major factor and, in conjunction with other observations cited above, I believe that it may be causal. In the next chapter I will develop additional evidence supporting this hypothesis by investigating neural correlates of both hippocampal neuroinflammation and depression in MS patients using resting state fMRI functional connectivity.

Chapter 6. Relationship of hippocampal 18-F PBR111 binding and depression to resting-state hippocampal functional connectivity in Multiple Sclerosis

6.1 Introduction

The previous chapter reports the association between hippocampal neuroinflammation assessed with TSPO–targeted PBR111 PET and depressive symptoms in relapsing remitting MS patients. I hypothesise that it is the disruption of hippocampal functions caused by direct or indirect actions of activated microglia that leads to depressive symptoms.

Beside its well-characterised role in encoding and retrieval of declarative memories and spatial navigation, there is increasing evidence that the hippocampus plays an important role in the pathogenesis of depression. Hippocampal atrophy is a consistently reported finding in depressed individuals, and has been suggested to predispose to the development of depression (Cole et al., 2011). Cognitive impairment in depressed individuals notably involved measures of memory functions that are heavily dependent on hippocampal function (Zakzanis et al., 1998). Connectivity studies have identified the hippocampus as one of the components of a network that is dysregulated in depressed individuals, and there are converging lines of evidence that the alterations in regulatory functions of the hippocampus, for example its modulation of the HPA stress hormone axis or regulation of the function of prefrontal, ventral tegmental and striatal areas, contribute to the development of depressive symptoms [for review see MacQueen and Frodl (2011)].

Resting state fMRI allows the study of functional connectivity using the analysis of

temporal correlations in spontaneous changes of the BOLD signal in the unstimulated, resting brain. Resting-state functional connectivity can be used to assess neural functional impairments (Barkhof et al., 2014).

This study characterised the functional connectivity of the hippocampus in healthy controls and MS patients using seed-based approach. I have tested for differences in the pattern of hippocampal functional connectivity between MS patients and healthy controls. Subsequently, I investigated whether the strength of hippocampal functional connectivities varies with the expression of depressive symptoms or with hippocampal microglia activation in MS patients as measured with ^{18}F -PBR111 PET.

6.2 Methods

6.2.1 *Study sample and design*

Thirteen MS patients and 15 healthy controls underwent resting state fMRI and were included in the present analysis. This includes the 11 MS patients whose data was included in the previous chapter and 15/22 of the healthy controls described there. Demographic characteristics of the study sample are presented in Table 5.1. Details on subjects, including recruitment strategies, eligibility criteria, ethics approval, and full clinical assessments, as well as concomitant treatment have been previously reported.

6.2.2 *fMRI protocol*

Subjects underwent fMRI scanning on the same day as PET scanning, approximately 1 hour before the beginning of the PET scan. MR data was acquired on a 3T Siemens Verio (Siemens Healthcare, Erlangen Germany) clinical MRI scanner equipped with a 32-channel phased-array head coil. Resting state fMRI data was acquired with a double-echo gradient echo EPI sequence, acquiring 150 volumes in 5m:06s. Each volume comprised 35 3-mm thick slices in a 225mm FOV, 64x64 matrix size, echo times at 13 and 31ms, parallel imaging factor of 2, and a TR of 2 seconds.

6.2.3 *Resting-state functional connectivity analysis*

Each subject's T1 anatomical images were stripped of their non-brain tissue using the brain extraction tool (BET) v2.1 (fractional intensity threshold=0.5, robust brain centre estimation). A bilateral hippocampal VOI was defined in standardised stereotactic (MNI152) space. The entire hippocampus was defined using a standard anatomical atlas (the CIC Atlas, thresholded at 0.5). This template hippocampal

region was then registered into each subject's functional data space using FLIRT (FSL's Linear Registration Tool). Time-series data were then extracted from the functional data using the transformed subject-specific hippocampal VOI as a mask image.

The fMRI Expert Analysis Tool (FEAT) v6.00 was used for pre-processing, general linear model (GLM) analysis and anatomical registration of the fMRI data. Pre-processing included temporal high-pass filtering (0.01 Hz), head-motion correction, and spatial smoothing with a 5mm FWHM (full-width half-maximum) Gaussian kernel. First-level analyses were conducted on each individual subject/scan by entering the extracted hippocampal time-course as a regressor, together with head-motion parameters as regressors of no interest. No haemodynamic modelling was added to the hippocampal regressor, as it already represents haemodynamic data. Contrasts simply modeled the positive and negative effects of the hippocampal time-series regressor. The resulting statistical images were registered to the subject's main anatomical image (acquired during the same session) and subsequently non-linearly warped to the template T1 anatomical image in standard MNI-space.

Group-level analyses were also conducted using FEAT, and used a mixed-model (FLAME-1) approach to test between-groups differences in order to model both within and between-subject variance. Age and gender were included in this model as additional regressors of no interest.

The analysis of correlations of hippocampal functional connectivity to depressive symptoms and hippocampal PET signal was exclusively done in MS patients. ^{18}F -PBR111 DVR and BDI scores were included as regressors of interest in the group-level model; nuisance regressors were added in the group-level model and these

included age, disease duration, and EDSS. In order to maximise sensitivity, fixed-effects models were used for the analysis of correlations in MS patients. This method in general limits the ability of the findings to be generalised to a population, but is appropriate for examination of effects in samples of particular patient groups, as used here (Friston et al., 1999).

All statistical images from the group models were thresholded at $p < 0.05$ (or $z = 2.3$; cluster-corrected for multiple comparisons).

6.3 Results

6.3.1 *Distribution of hippocampal resting-state functional connectivity*

In the healthy volunteers, regions showing significant positive FC with hippocampal seed were located in the limbic system (parahippocampal gyrus, amygdala, and insula), basal ganglia (putamen and caudate), thalamus, temporal lobe (superior/middle/inferior temporal gyri), medial and inferior prefrontal cortex (anterior cingulate cortex, subgenual cingulate, inferior frontal gyrus, orbital gyrus), parietal lobe (posterior cingulate, precuneus, angular gyrus, postcentral gyrus), occipital lobe and the cerebellum (Figure 6.1A). A similar pattern of functional connectivity was found in the MS patient group (Fig. 6.1B).

Whole-brain analyses of functional connectivity to the hippocampus did not reveal any significant group differences between MS patients and healthy controls.

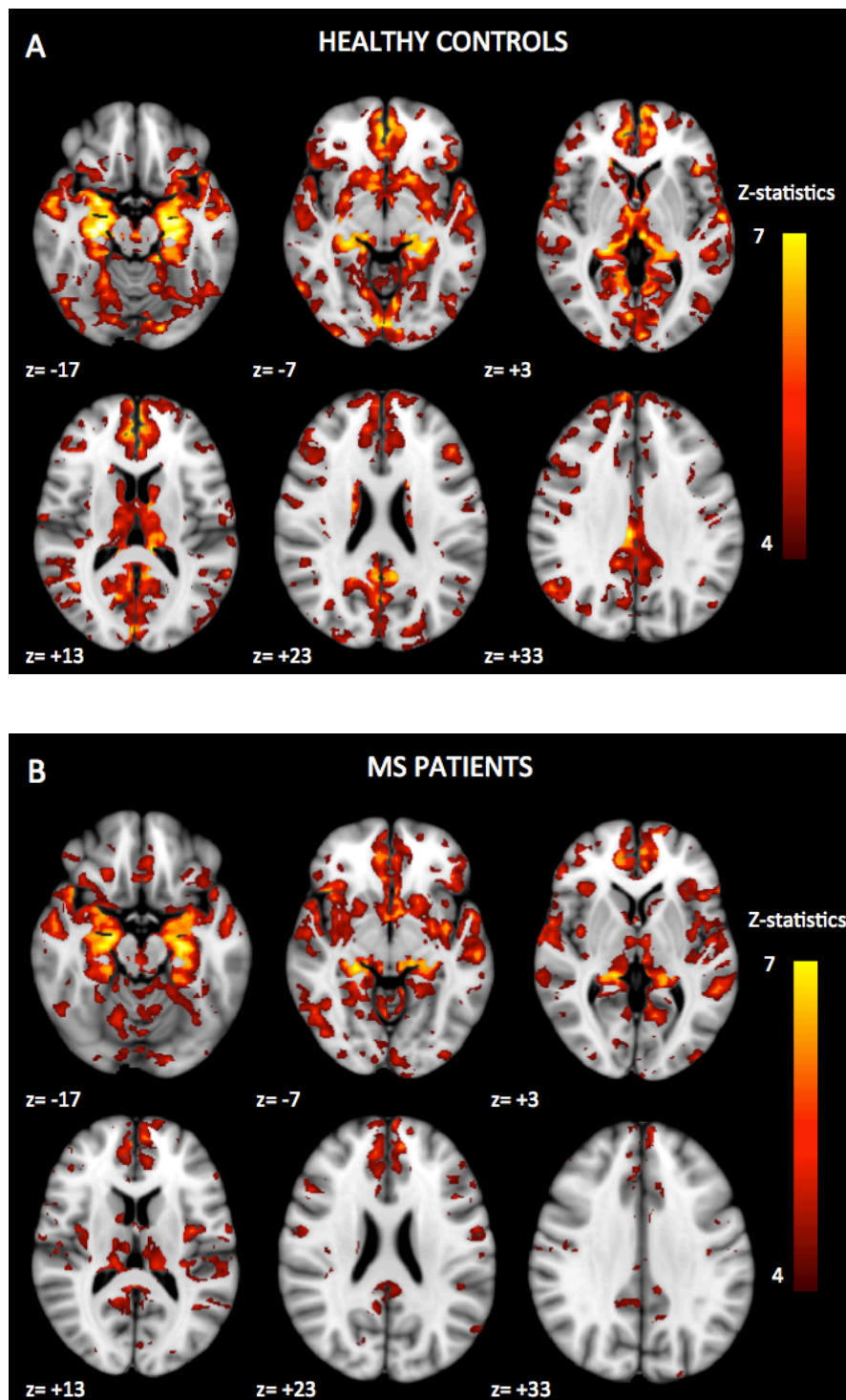


Figure 6.1 Patterns of significant positive functional connectivity with the hippocampal seed in healthy controls and MS patients

The threshold was set at $Z=4$, $p<0.05$ (corrected). The right side of the brain is to the left in the figure (images in radiological orientation).

6.3.2 *Relationships of functional connectivity to ^{18}F -PBR111 DVR and depressive symptoms*

The relationship of hippocampal functional connectivity to ^{18}F -PBR111 binding and to depressive symptoms was examined only in the MS patient group.

Whole brain analysis using hippocampal ^{18}F -PBR111 DVR as a regressor of interest, and controlling for age, disease duration, and EDSS scores by using them as additional regressors, revealed that hippocampal TSPO signal was positively correlated with the strength of functional connectivity between hippocampus and regions in the prefrontal cortex (anterior cingulate cortex, subgenual cingulate, inferior frontal gyrus, orbital gyrus), precentral gyrus and in the parietal (posterior cingulate, precuneus, angular gyrus, postcentral gyrus) and occipital cortices. Negative correlations were found in the insula, temporal lobe and basal ganglia (ventral striatum, putamen, caudate). Figure 6.2A illustrates the localization of clusters showing significant correlations between ^{18}F -PBR111 DVR and strength of functional connectivity to the hippocampus.

A whole brain analysis using BDI as a regressor of interest, and controlling for age, disease duration, and EDSS scores, revealed that a pattern of positive correlations between strength of functional connectivity to the hippocampus and depressive symptoms similar to that of the correlations with ^{18}F -PBR111 binding. BDI scores were positively correlated with the strength of functional connectivity between hippocampus and loci in the medial prefrontal cortex, cingulate cortex, subgenual, orbital, parahippocampal and lingual gyri, the posterior cingulate, precuneus and the occipital cortex. Negative correlations were observed in the frontal pole, frontal orbital cortex, putamen, insula, middle temporal gyrus, and lingual gyrus. Figure 6.2B

illustrates the localization of clusters with significant correlation between BDI scores and strength of functional connectivity to the hippocampus.

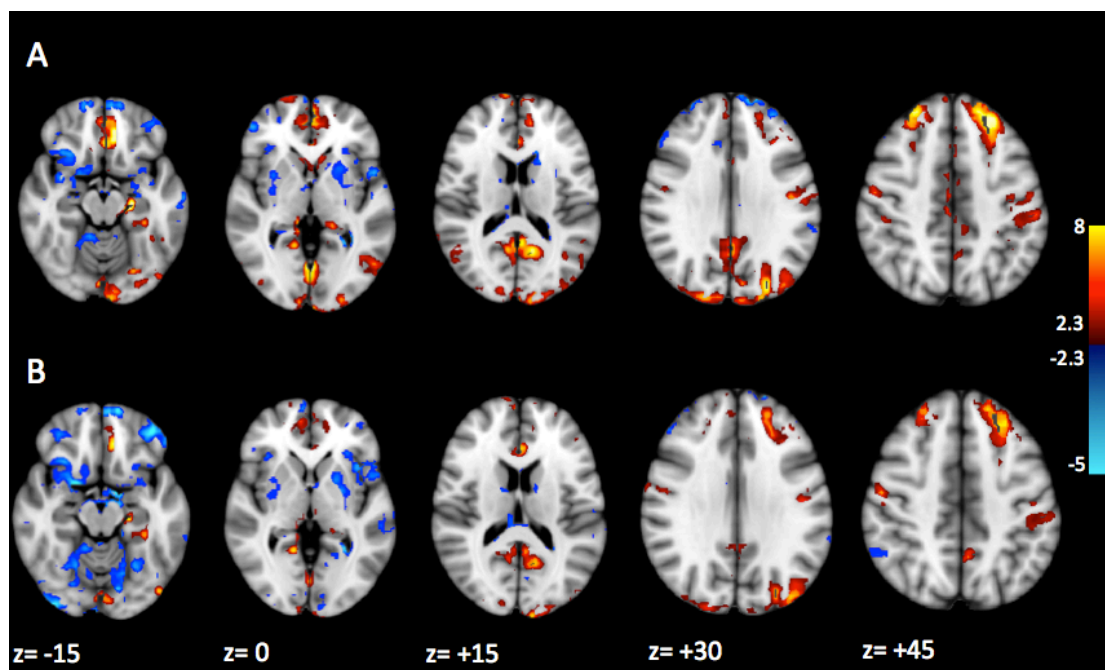


Figure 6.2 Whole brain map of correlations of hippocampal functional connectivity to hippocampal ^{18}F -PBR111 binding and depressive symptoms in MS patients

A) Loci with significant correlation between ^{18}F -PBR111 DVR and strength of functional connectivity to the hippocampus. B) Loci with significant correlation between BDI scores and strength of functional connectivity to the hippocampus. Threshold was set at $Z=2.3$, $p=0.05$. Loci with positive correlations are displayed in red-to-yellow, loci with negative correlations are displayed in blue-to-cyan.

Cluster Index	N. Voxels	Z-MAX	x	y	z
Positive correlations					
1	11948	9.81	2	-58	8
		9.06	2	-82	-10
		8.42	-2	-76	-8
		8.18	12	-32	-4
		7.79	-16	-96	24
		7.46	-4	-52	12
2	10958	8.27	-28	34	42
		7.99	0	64	10
		7.98	-8	38	-14
		7.74	-8	40	-10
		7.72	-22	44	42
		7.55	-8	34	-14
3	965	8.91	-22	-20	-18
		7.42	-20	-24	-12
		6.02	-32	-34	-18
		5.46	-18	-36	-2
Negative correlations					
4	19611	7.02	20	4	-16
		6.98	18	0	-20
		6.71	-16	-76	-52

Table 6.1 Anatomical clusters of correlations between hippocampal ^{18}F -PBR111 DVR and functional connectivity

The table includes statistical summaries of main anatomical clusters in Figure 6.2 reporting positive and negative correlations between ^{18}F -PBR111 DVR and functional connectivity of the hippocampus. For each cluster I report the total number of voxels (N. voxels) that pass significance, and the Z-statistics (Z-Max) and the Montreal Neurological Institute coordinates of the main peaks within the cluster.

Cluster Index	N. Voxels	Z-MAX	x	y	z
Positive correlations					
1	5685	7.17	0	-58	8
		7.06	-4	-54	8
		6.94	-6	-86	-20
		6.83	12	-32	-8
		6.8	0	-84	-10
		6.72	14	-52	2
2	2583	6.69	-10	36	-16
		6.58	2	64	12
		6.22	-16	68	10
		5.95	0	58	4
		5.81	-2	30	16
		5.52	26	42	44
3	2274	7.43	-28	34	42
		7.2	-18	48	42
		7.01	-20	44	40
		6.56	-20	38	42
		6.13	-22	46	32
		5.62	-26	28	46
4	988	5.35	-30	-26	52
		5.13	-46	-18	38
5	797	8.53	-22	-20	-18
		5.83	-20	-26	-10
		5.4	-40	-36	-24
		5.36	-34	-36	-16
		5.33	-40	-38	-32
		5.2	-38	-38	-28
6	515	5.16	26	-26	52
		5.13	52	-12	44
Negative correlations					
7	24631	8.17	20	4	-16
		8.06	-32	-80	-46
		7.9	-16	-80	-52
		7.19	38	-78	-46
8	471	6.34	-12	62	-16
		5.87	-4	62	-12
		5.66	6	34	-24
		5.5	-6	62	-16
		5.42	-6	54	-22

Table 6.2 Anatomical clusters of correlations between BDI scores and functional connectivity

The table includes statistical summaries of main anatomical clusters in Figure 6.2

reporting positive and negative correlations between BDI scores and functional connectivity of the hippocampus. For each cluster I report the total number of voxels (N. voxels), the Z-statistics (Z-Max) and the Montreal Neurological Institute coordinates of the main peaks within the cluster.

6.3.3 *Inter-relationships between ^{18}F -PBR111 DVR, functional connectivity, and depressive symptoms*

There was considerable overlap between loci with correlation of hippocampal functional connectivity to ^{18}F -PBR111 DVR and those with correlation of hippocampal functional connectivity to depressive symptoms. Figure 6.3 illustrates a whole brain “conjunction” map displaying loci where hippocampal functional connectivity was correlated to both ^{18}F -PBR111 binding and BDI scores. Loci with positive correlations were localised in the frontal lobe (subgenual cortex, anterior cingulate, superior and middle frontal gyri), parietal lobe (precuneus, posterior cingulate), and occipital lobe. Loci with negative correlations included insula, frontal lobe areas, and putamen

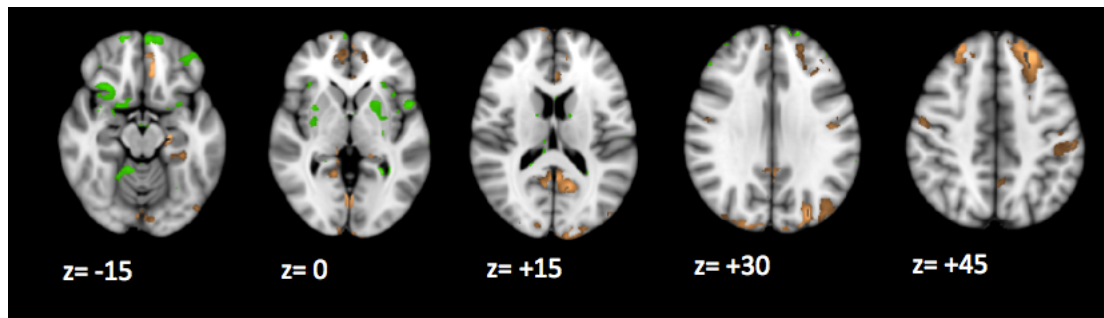


Figure 6.3 Conjunction map of correlations of hippocampal functional connectivity to both hippocampal ^{18}F -PBR111 binding and depressive symptoms in MS patients

Loci displaying significant correlations between strength of hippocampal functional connectivity and both ^{18}F -PBR111 DVR and BDI scores. Loci with positive correlations are coloured in copper, while negative correlations are displayed in green.

I further explored the relationships between the measures in prefrontal cortical volumes. From the “conjunction” map of significant positive correlations of hippocampal functional connectivity to both ^{18}F -PBR111 DVR and BDI scores, a volume in the prefrontal cortex (Brodmann areas 11, 12, 25, corresponding to orbital gyrus and subgenual cingulate) was defined (Figure 6.4). The average values of functional connectivity to the hippocampus within this volume were computed across subjects and plotted against both ^{18}F -PBR111 DVR and BDI scores to illustrate the relationship between the three variables, functional connectivity to the hippocampus, ^{18}F -PBR111 DVR and BDI scores in this locus (Figure 6.4). Controlling for age, disease duration, and EDSS scores, there was a moderately strong correlation between the strength of functional connectivity to the hippocampus and BDI scores (Partial correlation $r=0.62$) and between the strength of functional connectivity to the hippocampus and ^{18}F -PBR111 DVR (Partial correlation $r=0.63$).

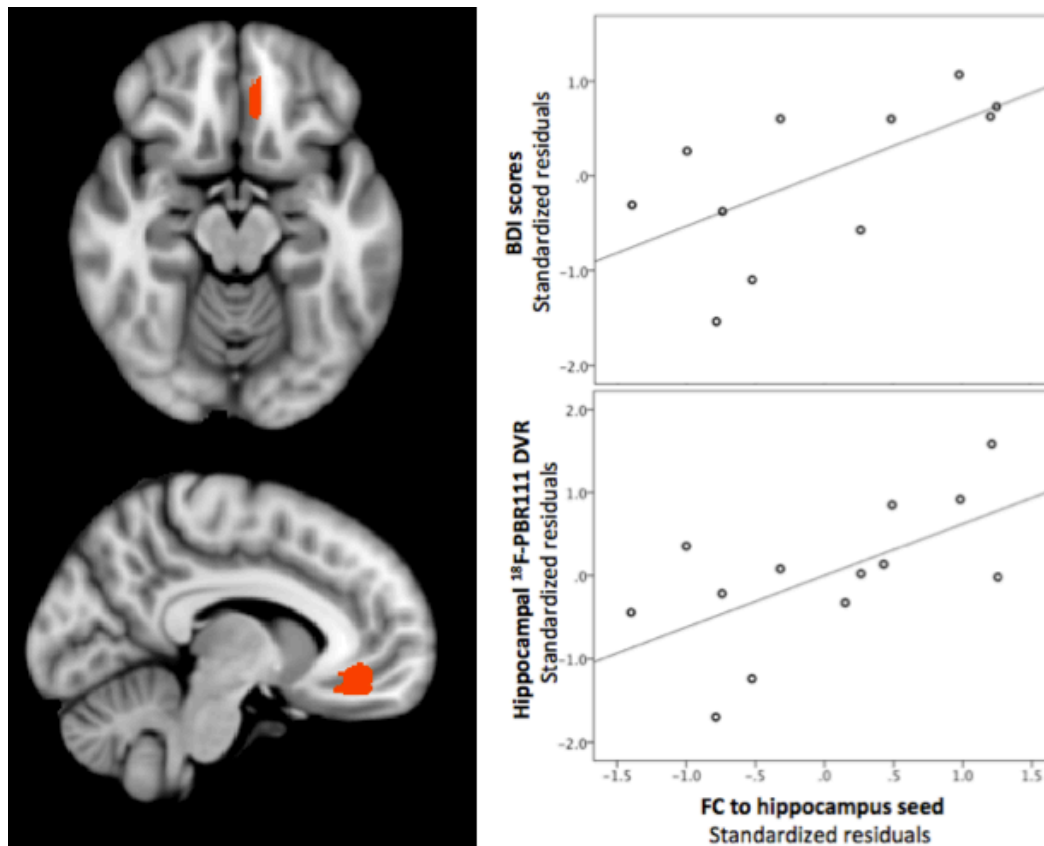


Figure 6.4 Relationship between hippocampal functional connectivity, hippocampal ^{18}F -PBR111 DVR and BDI scores

An area of the prefrontal cortex chosen for illustration purposes. The scatterplots illustrate the high correlation of these three variables to each other: hippocampal functional connectivity, hippocampal ^{18}F -PBR111 DVR, and BDI scores. Values represent standardized residuals of the dependent variables (BDI scores and ^{18}F -PBR111 DVR, respectively) and standardized residuals of functional connectivity of the hippocampus, when all these variables are regressed on the rest of the independent variables (age, duration of disease, and EDSS scores).

6.4 Discussion

I evaluated resting-state functional connectivity of the hippocampus in MS patients and healthy volunteers, in order to test for a mechanistic basis for the underlying the reported association (Chapter 5) between hippocampal neuroinflammation and depressive symptoms in MS patients. I described the relationship between the strengths of functional connectivity of the hippocampus with the whole brain and measures of hippocampal neuroinflammation and depressive symptoms in MS patients.

The patterns of observed functional connectivity of the hippocampal are consistent with previous reports. The hippocampus is anatomically connected to the parahippocampal gyrus, amygdala, prefrontal cortex, thalamus, and basal ganglia (Phillips et al., 2003; Rosene and Van Hoesen, 1977). My findings are in line with those of other studies of functional connectivity of the hippocampus, that reported functional connectivity of the hippocampus to thalamus, prefrontal cortex, medial parietal lobe, posterior cingulate, observed at rest and during execution of memory tasks (Cao et al., 2012; Ranganath et al., 2005; Stein et al., 2000; Zarei et al., 2012).

I did not observe significant differences in functional connectivity of the hippocampus between the healthy subjects and the MS patients. Differences in functional connectivity in MS patients relative to healthy subjects have been previously reported (Barkhof et al., 2014). It is likely that my study was underpowered to detect any true difference, particularly given the heterogeneity amongst the MS patients that was highlighted by my parametric analysis. Previous literature also is consistent with population heterogeneity. Contrasting results have been obtained. For example, while Roosendaal et al. reported reduced resting state hippocampal functional

connectivity to anterior cingulate, thalamus, and prefrontal cortex (Roosendaal et al., 2010), Loitfelder and coworkers found no differences in resting-state functional connectivity of hippocampus to anterior cingulate cortex between MS patients and healthy controls (Loitfelder et al., 2012). Finally, therapeutic interventions that have been reported to affect the strength of hippocampal functional connectivity also may have confounded my results (Leavitt et al., 2014; Prakash et al., 2011).

The most important observation of this study is the correlation of TSPO signal, indicative of microglia activation, to functional connectivity between the hippocampus and several brain areas. A very similar pattern of positive and negative correlations was found between BDI scores and hippocampal functional connectivity. Although hippocampal ^{18}F -PBR111 DVR and BDI scores are themselves highly correlated, the overlap of the precise spatial patterns for correlations of the two variables with functional connectivity is striking. A possible interpretation of these results is that modulation of hippocampal connectivities by microglial inflammation is a contributing cause of depressive symptoms in patients with MS.

The functional connections between hippocampus and the above-mentioned regions are supported by theoretical models of depression. Neuroimaging, neuropathological, and lesion analysis data suggest implicate the hippocampus in an extended anatomical network formed by the neural projections of the subgenual and other areas of the orbitomedial prefrontal cortex, together with other regions such as amygdala, posterior cingulate cortex, ventral striatum and thalamus. Impaired functions of key nodes within this network could dysregulate emotional expression and give rise to the clinical signs and symptoms of depression (Drevets et al., 2008; Mayberg, 1997).

Although my study was unable to directly test whether microglia activation in the

hippocampus affects its function, preclinical evidence supports this hypothesis. In EAE, hippocampal neuroinflammation alters adult neurogenesis (Giannakopoulou et al., 2013) and causes alterations of GABAergic transmission (Nistico et al., 2013). This effect is likely to be mediated by activated microglia as incubation of hippocampal slices from healthy mice with activated microglia causes similar alterations of GABAergic transmission (Nistico et al., 2013).

My study has been an exploratory pilot. The sample size was small. In consequence, a fixed effect model was used for my correlation analysis, but this means that generalization of my findings to the wider MS population must be made cautiously. The small size of the population precluded exploration of the impacts of potential confounding factors such as gender, use of concomitant medications, global and regional brain atrophy, localization of lesions and alteration in structural connectivity in patients. I also have focused my interest only on hippocampal functional connectivity because of the prominence of the microglial activation signal in this region (see Chapter 5), but it cannot be excluded that hippocampal neuroinflammation is associated with alterations in functional connectivity in regions other than the hippocampus.

To my knowledge, this is the first study using a combination of PET imaging of microglia and fMRI to investigate clinical-pathology correlations in vivo in MS patients. While conclusions are preliminary, they further support the hypothesis of a pathogenic role for hippocampal microglial activation in the genesis of depression in MS.

Chapter 7. Summary, conclusive remarks and future directions

The studies reported in the present thesis aimed to evaluate the potential of a 2nd generation TSPO PET radiotracer as a tool to assess neuroinflammation in the brain of patients with MS for the study of clinical-pathological correlations *in vivo*.

As illustrated in Chapter 3, it was demonstrated that ¹⁸F-PBR111 yields a measurable signal, which is influenced by rs6971 polymorphism of the TSPO gene, known to determine the affinity of 2nd generation radioligands to TSPO, and age. These findings confirmed the usefulness of ¹⁸F-PBR111 for PET imaging of TSPO in human subjects. The observations reported in Chapter 3 also served as guidance for the analyses presented in subsequent chapters. Specifically, the data highlighted the importance of genotypic stratification of subjects in order to improve both accuracy and precision of measurements. Other observations, with high relevance from a methodological perspective, included the unexpectedly high variability of ¹⁸F-PBR111 signal across healthy subjects, despite correction for rs6971 SNP and age and, and the finding of significant signal in the skull, probably from ¹⁸F released with metabolism of the radiotracer, that limits the accuracy of measurements in the cortical grey matter.

My second analysis, reported in Chapter 4, examined ¹⁸F-PBR111 uptake in the white matter of MS patients. The focus on white matter was based upon the notion that activation of microglia is a prominent feature in the MS white matter. My objective was to test whether the *in vivo* signal obtained with ¹⁸F-PBR111 PET in MS

corresponded to previous neuropathological observations. I segmented the white matter into lesional, peri-lesional, “slightly abnormal” and non-lesional brain tissue, based on MRI-based measures, and demonstrated that ^{18}F -PBR111 signal in MRI-defined lesional areas was the highest throughout the MS patients’ brain, and was higher relative to that of healthy controls’ white matter. These findings, including the observation of high variability between lesions even within the same patient, are consistent with neuropathological observations of lesion heterogeneity and suggest that the measurement of the relative TSPO signal increase in MRI-defined lesional areas could be used to estimate the extent of focal inflammatory activity individually across various white matter lesions. Additionally, an important finding of my analyses was the observation of an association between the rate of development of disability and the average increase in ^{18}F -PBR111 signal across lesions, relative to the normal-appearing white matter, supporting a role for the innate immune response in the progression of neurodegeneration in MS.

In the third and last set of analyses, including the use of resting-state fMRI, I examined the association of hippocampal microglial activation with depressive symptoms in MS patients and to the strength of functional connectivity of the hippocampus to the rest of the brain. The hippocampus is a region vulnerable to inflammation, is one of the specific sites of MS pathology, and is involved in the pathophysiology of depression. MS patients displayed an increased hippocampal ^{18}F -PBR111 signal, relative to healthy controls, which was associated with their depressive symptoms. Using a multimodal neuroimaging approach, I demonstrated that the strength of functional connectivity of the hippocampus to specific areas in the frontal and parietal lobes, was correlated to both depressive symptoms and hippocampal microglia activation measured with TSPO PET. These findings are

consistent with accumulating evidence suggesting a causal role for neuroinflammation in the pathogenesis of depression in MS.

This work demonstrated that the integration of TSPO-targeted PET with structural and functional MRI provides more specific information on the underlying dynamic neuropathology in the MS brain, relative to the use of MRI alone. I feel that my research addressed some important research questions and that my findings provided a novel insight into the methodology, the underlying mechanisms, and the utility of *in vivo* imaging of neuroinflammation in MS using a 2nd generation TSPO radioligand. However, various important research questions remain unanswered and warrant further investigation.

We don't know whether the signal obtained with TSPO PET imaging reflects a particular stage of activation of the microglia. Post-mortem studies using combined immunohistochemistry with markers of microglia activation and TSPO autoradiography are necessary to clarify this. In Chapter 4, I reported a higher ¹⁸F-PBR111 signal associated with MS lesions relative to other areas of the brain. Various cellular types may have contributed to the increased signal observed in lesions, including activated microglia, but also activated macrophages in and around the lesion, as well as in the perivascular spaces. It is difficult to ascertain what is the contribution of the signal coming from microglia activation alone, which limit our ability to interpret our findings with precision.

The results reported in Chapter 4 demonstrated that white matter ¹⁸F-PBR111 signal in patients with MS was associated with clinical indicators of illness, such as duration of disease and rate of progression of disability. In our subsequent analyses, presented

in Chapter 5, we found an association between ^{18}F -PBR111 DVR in the hippocampal grey matter to the severity of depressive symptoms. It must therefore be considered the possibility that the levels of depression in MS patients may affect the relationship between TSPO signal white matter and clinical variables (disease duration and MSSS) reported in Chapter 4. We have not tested directly this possibility in our dataset, as our small sample size would contraindicate the inclusion of too many control variables in the correlation analyses. However, the same association between depression and ^{18}F -PBR111 signal, was not found for thalamic or brainstem gray matter DVR, or for cortical V_T , suggesting that it was specific for the hippocampus. It is therefore unlikely that the association between clinical indicators of disease and TSPO signal in the white matter, is influenced by the levels of depression of MS patients. It is also unlikely that the presence of depressive symptoms in MS patients have biased the study of differences in TSPO signal between MS patients and healthy controls, contributing to the increased ^{18}F -PBR111 signal observed in MS patients. Increased microglia activation in the brain of MS patients is a robust and well-characterised neuropathological finding, while there is very little evidence in post-mortem studies suggesting a significantly increased density of activated microglia due to depression. Similarly, it is unlikely that the presence of antidepressant treatment biased the results, since I am not aware of any evidence specifically supporting a significant effect of antidepressants in moderating levels of microglia activation.

A high between-subjects variability of ^{18}F -PBR111 signal was found in healthy subjects, even after correction for genotype and age. The factors that determine this variability are unknown. It is most likely that biological factors play a dominant role, however the physiological mechanisms regulating the expression of TSPO are not well understood (Bae et al., 2014; Morohaku et al., 2014).

It is possible that the *in-vivo* TSPO signal in the human brain also reflects a “priming” response of microglia to various stimuli able to promote an inflammatory response, including systemic inflammation and stress. Systemic inflammation could be experimentally modeled using acute administration of *E. Coli* lipopolysaccharide (LPS) and a study in non-human primates suggest that an LPS challenge caused robust increase of TSPO PET signal as measured with ^{11}C -PBR28 (Hannestad et al., 2012). Stress is also a stimulus able to activate brain innate immune responses. Rodents studies have shown that acute and repeated stress cause activation and proliferation of microglia, shift microglial immunophenotype towards a pro-inflammatory profile and potentiate sickness behavioural responses to inflammatory challenges and other acute inflammatory insults (Nair and Bonneau, 2006; Sugama et al., 2007; Sugama et al., 2009; Sugama et al., 2011; Tynan et al., 2010). The hippocampus has been the area most frequently implicated in the inflammatory effects of stress. These effects are likely to be mediated by the stress hormones glucocorticoids, as direct glucocorticoid administration induces microglia proliferation, and inhibition of glucocorticoid signaling abolished the microglia response to acute stress (Frank et al., 2012; Nair and Bonneau, 2006). Furthermore, early autoradiography studies in rodents demonstrated that TSPO binding in the hippocampus is increased in response to acute stressful stimuli, such as acute swim stress (Avital et al., 2001), or administration of the anxiogenic beta-carboline (Drugan et al., 1995). I am currently setting up a PET study to assess the effect of acute and sub-acute glucocorticoid administration on TSPO signal in healthy subjects.

There have been various challenges during the course of my PhD project. The use of a very novel tracer, which had never been characterized in humans before, added complexity to my work. Basing an entire PhD thesis on the use of such a novel

radiotracer, without available human data, may have represented a high risk plan, that could have potentially jeopardized the success of my research project, should I had found that the characteristics of the tracer were unpromising for imaging purposes. If this would have been the case, my back-up plan was to switch to ^{11}C -PBR28, another 2nd generation TSPO tracer. ^{11}C -PBR28 had been developed in Imanova and was ready for clinical use simultaneously as ^{18}F -PBR111. ^{11}C -PBR28 had been previously characterized by various PET imaging research groups and its excellent imaging characteristics were well-known. As explained in the paragraph 1.5.1, one of the initial reasons for preferring ^{18}F -PBR111 over other tracers including ^{11}C -PBR28 was the relatively small difference in TSPO affinity of ^{18}F -PBR111 across TSPO binding groups. During the preparatory phase of my study, my colleague Dr Owen and coworkers at Imanova discovered the possibility of predicting TSPO binding status with 100% accuracy, by genotyping subjects for the rs6971 polymorphism of the *TSPO* gene. This discovery simplifies the setup and interpretation of studies using ^{11}C -PBR28 where the differences between binding groups is more marked, since it allows identification of the TSPO binding status at screening with a simple genetic test, therefore a study in MS patients using ^{11}C -PBR28 would have then been perfectly feasible.

Although the nature of my pilot studies was exploratory and provided preliminary rather than conclusive findings, the knowledge acquired with these studies indicated the suitability of ^{18}F -PBR111 for TSPO imaging research. Various other TSPO tracers, both using ^{18}F and ^{11}C radioisotopes, have been recently developed and appear to be also promising tracers. ^{11}C -PK11195 is still very broadly used in many PET centres, and has the advantage of producing a signal that is not affected by TSPO binding groups, which simplifies the recruitment of study subjects and the logistic of

the experiments. An important question relates to the ideal tracer to be used in future TSPO imaging studies. The studies presented in this thesis are unable to specifically answer that question as no direct comparisons between tracers have been tested. However, in my opinion various conclusions could be drawn indirectly from my studies, for guiding the choice of TSPO radiotracers in the future. First, I noted a very high between-subjects variability in the TSPO signal with ^{18}F -PBR111, which is similar to that reported with all other known TSPO tracers, including ^{11}C -PK11195 and ^{11}C -PBR28, when similar quantification methods are compared. This means that a large number of subjects is always required for cross-sectional investigations, and considering the costs and challenges of PET scanning, the possibility of combining data acquired in different PET centers, by using the same tracers and the same methodology, would be of utmost importance. If each of the many research groups specialized in PET imaging keeps using a different TSPO tracer, it is unlikely that meaningful clinical information will be acquired any time soon with TSPO PET imaging. Ideally, the perfect candidate tracer for broad use across PET centers will need to be not only an excellent tracer in terms of affinity to TSPO and suitability for accurate quantification, but also will need to 1) have a reliable and relatively simple radiosynthesis procedure; 2) use ^{18}F as radioisotope to facilitate use in centers where a cyclotron is not available; 3) not require pre-characterisation of TSPO binding group of subjects by genotyping as well as allow the possibility of obtaining measurable signals in LAB subjects. Unfortunately no tracer available at the moment has simultaneously all these characteristics. ^{18}F -PBR111 displays most, but not all of these features. I believe it is preferable than ^{11}C -PK11195 due to higher affinity to TSPO, and hence a better signal-to-noise ratio, which is reflected by the excellent quality and precision of parametric images obtained with ^{18}F -PBR111 (for example

Figure 4.7). Also, I think that ^{18}F -PBR111 is preferable relative to all ^{11}C tracers for its ^{18}F -label. It also has a reliable and robust radiosynthesis that hardly fails. It allows imaging of subjects with LAB binding status due to the limited difference between TSPO binding status groups. However, it has various limitations: the defluorination of the tracer to the skull prevents a precise measurement of signal from the superficial layers of the cortex, and the differences between binding groups still affect the *in-vivo* signal as demonstrated by our findings in Chapters 3 and 4. Finally, for various practical reasons, including the complexity of legal arrangements over its license for use in human subjects, its synthesis has not been implemented in many PET centers and is unlikely that any time soon it will be used as broadly as ^{11}C -PK11195 or ^{11}C -PBR28. In summary, I would prefer ^{18}F -PBR111 to other TSPO tracers for multi-center studies focusing on subcortical brain regions where inclusion of all subjects including LABs is important. On the other hand, I would select an alternative high affinity tracer, for example ^{11}C -PBR28, for studies looking at the cortical signal, where it would be possible to pre-select participants, for example for studies in healthy volunteers or for longitudinal within-subject observations. Overall, I think further research is still needed to identify an ideal TSPO radiotracer.

Other methodological challenges discovered in this thesis were the issues of time-stability of V_T (Chapter 3), suggesting that the duration of the scan may affect the estimation of the signal, and the moderate-poor within-subject variability of the signal observed in test-retest observations (Chapter 4). These characteristics are not specific to ^{18}F -PBR111, as they have been observed with most TSPO radiotracers tested so far when similar quantification methods were used (Coughlin et al., 2014; Endres et al., 2009; Fujimura et al., 2009; Fujita et al., 2008; Jucaite et al., 2012). My experimental

observations suggest possible ways to deal with these problems. With regard to the time-stability analysis, since the time-dependent increase in V_T is global, as described in Chapter 3, it is possible to reduce the scan duration to 60 minutes allowing for a consistent underestimation of the signal. It is important that for group comparisons, consistent reductions in scan duration are used across groups, assuming that the degree of underestimation is homogenous across different clinical populations.

With regard to the variability in test-retest measurements, we noticed that the use of a normalized measure, the DVR, was associated with a significantly improved test-retest reproducibility of the signal (Chapter 4), which makes this ligand perfectly suitable for longitudinal observations. Obviously the use of DVR also assumes that the predicted change in signal must be localized and not global, which may not always be the case.

To assess the source of the within-subject variability of TSPO signal, it would be crucial to assess the test-retest reproducibility of the specific binding of TSPO radioligands (BP_{ND}). An accurate quantification of TSPO specific signal *in vivo* is now possible by means of a “blocking” study, in which a TSPO PET radioligand is used in combination with the administration of a TSPO “cold” ligand that competes for the same binding site, allowing the determination of the “non-displaceable volume of distribution” (V_{ND}), and hence estimation of the specific TSPO signal (BP_{ND}). Separate assessment of the within-subject reproducibility of the TSPO specific signal could be estimated by a test-retest TSPO blocking reproducibility study.

Another important methodological remark is related to the implications that the blood brain barrier breakdown in acute MS lesions may have for accurate modeling of the tracer. In fact, kinetic modelling of PET tracers makes the assumption of passive diffusion of the tracer across an intact blood-brain barrier, which is not true for acute

MS lesions. A study using ^{11}C -PK11195 in traumatic brain injury (Folkersma et al., 2009), where blood brain barrier breakdown is also observed, concluded that the use of quantification methods using a reference region led to a systemic bias in measurement, relative to a model using plasma input function, and that this was most likely due to variability in kinetic parameters related to the delivery of the tracers from blood to the tissue. The use of a kinetic model with a plasma input function, as that used in my studies, is therefore recommended to ensure precision of measurements in studies in MS patients, where blood brain barrier breakdown is likely to occur.

My conclusive remarks are related to the potential usefulness of TSPO PET as a biomarker for drug efficacy or for patient stratification.

Longitudinal studies, in particular those testing the sensitivity of TSPO signal to the anti-inflammatory effects of immune-modulating treatments, are necessary to fully characterize the use of TSPO PET as an imaging marker of neuroinflammation in MS. TSPO PET could be a potentially useful biomarker of drug efficacy for early drug development and could shed light on the mechanisms underlying the effects of immune-modulation on clinical manifestations of MS, including depressive symptoms. However, without clear evidence from treatment studies in support to these hypothesis, it is impossible to estimate now the exact impact that TSPO PET imaging may have for development of new treatments in MS and for studying the response to treatment. I feel that this is a highly promising, albeit still very unexplored, application of TSPO imaging, and that the field should prioritize this particular research question. ^{18}F -PBR111 is a very good candidate for these types of studies, however other 2nd generation tracers may be also suitable. The main aspects

to consider for the optimization of the longitudinal studies are the need of a consensus between PET centres for identification of a “common” TSPO radioligand for multicentre use, and the need of careful characterization of test-retest variability of specific TSPO signal. Both these aspects have been discussed earlier in this chapter.

With regard to the potential usefulness for ^{18}F -PBR111, and more generally, TSPO imaging, for patient stratification, it must be acknowledged that TSPO PET has been used for more than 15 years, since the development of ^{11}C -PK11195, and it has not been able to provide a significant advancement in terms of clinical stratification of patients yet. In my opinion, it is unlikely that with ^{18}F -PBR111 the situation will dramatically improve. The variability between subjects remains high and is only partially explained by clinical variables. The effect size of differences between subjects is relatively small, and we also need to consider the extreme cost of PET imaging and the practical challenges of the radiosynthesis procedure, the need of arterial lines and of genetic stratification. The most compelling research question and unmet need with regard to patient stratification, is the ability of a biomarker to predict the progression of neurodegeneration of MS. With ^{18}F -PBR111, we did report an association between the rate of development of disability and the average increase in ^{18}F -PBR111 signal across white matter lesions, relative to the normal-appearing white matter, suggesting the utility of ^{18}F -PBR111 in monitoring disease progression. However, even more important would be the confirmation that TSPO imaging could be used to follow progressive neurodegeneration in secondary progressive patients, based on the known role of cortical microglial activation in driving disease progression. This had been suggested previously by Politis and coworkers, however for replication of this observation, it would be important to use a tracer without spill-over of signal from the skull due to defluorination, as this is known to bias the

precision of measurements of the cortical grey matter signal. Based on these considerations, I feel that an imminent application in the clinical routine of TSPO PET imaging, using ^{18}F -PBR111 or any of the other existing TSPO tracers, is unlikely. However, as described in details earlier, there are a number of questions that warrant further investigation. Therefore I feel it is still premature to draw a conclusive evaluation on the exact role of TSPO imaging for clinical application in MS.

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