Title:

A multimodal approach to explore the correlation between neuroinflammation and functional connectivity reorganization in patients with multiple sclerosis: a ^{11}C-PBR28 PET – resting-state functional MRI study.

Background:

Multiple Sclerosis (MS) is the most prevalent chronic inflammatory disease of the central nervous system and a leading cause of disability in young adults (Reich *et al.*, 2018). It is considered a primarily inflammatory disorder in which a dynamic and complex interplay between the immune system, microglia, astrocytes and neurons determines focal and diffuse damage of both white and grey matter.

Inflammation is initially transient and followed by remyelination, with the early course of the disease mainly characterized by episodes of neurological dysfunction that usually recover, called relapses. Sustained inflammation is associated with extensive and chronic neurodegeneration and leads to progressive accumulation of disability over time (Compston and Coles, 2008).

In the white matter, areas of inflammation and demyelination are easily detected with conventional magnetic resonance imaging (MRI) with new lesions expressing focal inflammation, causing local breakdown of the blood–brain-barrier (BBB) and being characterized by the extrusion of gadolinium contrast agents across the altered BBB (Absinta *et al.*, 2016). Demyelination also involves gray matter with cortical and deep gray matter (DGM) lesions being less inflammatory than their white matter counterparts and causing substantially less permeability of the BBB (Peterson *et al.*, 2001; Reich *et al.*, 2018).

In response to inflammation an essential role is played by activated resident microglia and peripherally recruited macrophages which infiltrate active lesions and remove myelin debris and inflammatory by-products; however, post-mortem studies have shown microglial activation in the normal appearing white matter (NAWM) of patients with MS, representing the earliest stage of a newly forming lesion and/or a chronic activation with continue production of inflammatory products with subsequent detrimental effects (Airas *et al.*, 2015; Frischer *et al.*, 2015). Up to date, the actual role of microglia in MS, whether pathogenic or protective, is still controversial (Prinz *et al.*, 2011). It has been shown that in gray matter, microglia may limit damage through pruning of dysfunctional synapses that express classical complement cascade proteins. This pruning process may become pathologic if activated astrocytes promote aberrant expression of complement at synapses, thereby accelerating degeneration at synapses level (Liddelow *et al.*, 2017). Moreover, the importance of glial activation as secondary mechanisms of injury has been highlighted by the evidence of impaired axonal transport, mitochondrial dysfunction, and increased energy demands related to the upregulation of ion channels leading to axonal degeneration in chornically demyelinated lesions and to slow degeneration of progressive multiple sclerosis (Mahad *et al.*, 2015). There is a growing body of

evidence about the possibility of non invasively detecting in vivo the specific contribute to inflammation of activated migroglia and macrophages; specifically new generation tracers (i.e. ^{11}C-PBR28) are able to bind with high specificity the 18 kDa translocator protein (TSPO, previously known as peripheral benzodiazepine receptor), considered a marker of neuroinflammation since its expression is up-regulated in activated migroglia and macrophages. In particular, a widespread inflammation of the NAWM and of the GM (especially the thalamus) has been depicted being higher in patients with progressive forms of MS and correlating with measures of physical and cognitive disability (Kreisl *et al.*, 2010; Hannestad *et al.*, 2012; Park *et al.*, 2015; Herranz *et al.*, 2016).

Although we cannot directly visualized in vivo synapses function, resting-state functional MRI (rs-fMRI) is an advanced technique able to monitor oscillations in the blood oxygen level dependent (BOLD) signal across time, under non-arousal (resting) states. BOLD signal correlates with neuronal potential of action (Logothetis *et al.*, 2001), thus representing a non invasive and reliable surrogate marker of neuronal activity in vivo. Specifically, it assesses in vivo the synchronized activity of population of neurons even not structurally connected; the temporal correlation of these BOLD signal fluctuations across various brain regions may reflect the resting-state functional connectivity (rs-FC) between them, i.e., the organization in resting-state networks (RSN) (Fox and Raichle, 2007). Rs-fMRI has been extensively used to explore the brain plasticity and the functional reorganization in patients with MS, to adapt and contrast inflammation and structural damage. With a seed-based analysis, which requires an *a priori* hypothesis, disrupted rs-FC has been proved in different brain regions. In particular, rs-FC abnormalities of thalamo-cortical network have been demonstrated and correlated with clinical measures of physical and cognitive disability (Tona *et al.*, 2014; Schoonheim *et al.*, 2015). Independent component analysis (ICA) can be used to analyze whole-brain rs-FC and synchronous spontaneous activity to determine well-defined large-scale RSNs without *a priori* hypotheses (Beckmann *et al.*, 2005). This method have been already applied to study patients with MS showing extensive alteration both in the within- and in the between-network connectivity (Rocca *et al.*, 2018).

To the best of our knowledge, the relation between chronic and diffuse microglia and macrophages activation depicted with ^{11}C-PBR28 PET and reorganization of functional connectivity studied with rs-fMRI has not been yet explored in MS. We propose to analyze rs-fMRI in a population of patients with MS (relapsing-remitting and secondary-progressive) already studied with ^{11}C-PBR28 PET, with the general aim of correlating functional alterations with PET and clinical data, thus better clarify the effects of sustained and diffuse inflammation through activation of microglia and macrophages on brain functional connectivity and disability in MS.

Aims:

1) To assess thalamic resting-state functional connectivity alterations in MS patients compared to healthy subjects (HS) and explore correlations between functional connectivity alterations and ^{11}C-PBR28 PET uptake and clinical scores.
2) To assess within- and between-network functional connectivity alterations in MS patients compared to HS and explore correlations between functional connectivity alterations and ^{11}C-PBR28 PET uptake and clinical scores.

Research plan:

Participants:

A group of 25 patients with MS (11 RRMS, 14 SPMS) and 17 HS have been enrolled for the study and are available for the analysis. All subjects had been genotyped for the TSPO gene Ala147Thr polymorphism, which predicts binding affinity to ^{11}C-PBR28 (Owen *et al.*, 2012); only high- and mixed-affinity binders underwent study procedures.

Inclusion criteria were: age between 18 and 65 years, a diagnosis of clinically definite MS, education >8 years, absence of clinical relapse within 3 months, no use of corticosteroids within 1 month of study enrollment, and being on stable disease-modifying treatment or no treatment for at least 6 months. Exclusion criteria were: treatment with benzodiazepines and blood thinners, general PET/MRI contraindications, and major medical and/or psychiatric disorders. In MS, major depression was excluded using the Beck Depression Inventory–II (cutoff score>28).

In MS subjects, within 1 week from imaging procedures, neurological disability was assessed using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) and cognitive performance with the following tests: Symbol Digit Modalities Test (SDMT), Trail Making Test (Trails A and B), California Verbal Learning Test–II (CVLT-II), Brief Visuospatial Memory Test–Revised (BVMTR), and Wisconsin Card Sorting Test–64 Card Version (WCST). For each patient, a z score was calculated to assess information-processing speed (average SDMT and Trails A z scores), and executive (average WCST and Trails B z scores) and memory (average CVLT-II and BVMT-R z scores) functions.

Data Acquisition:

A multimodal PET-MRI study was performed on all the participants according to a standardized protocol on a 3-T scanner. Specifically, a 90-minute ^{11}C-PBR28 MR-PET scan on a Siemens (Erlangen, Germany) simultaneous MR-PET system, BrainPET, a brain PET scanner operating in the bore of a 3T whole-body MR system equipped with an 8-channel head coil (Catana *et al.*, 2010). All participants received an intravenous bolus injection of 11C-PBR28 produced in-house (Zürcher *et al.*, 2015). MRI scans acquired simultaneously during PET included: (1) multiple gradient echo 3-dimensional (3D) magnetization prepared rapid acquisition (ME-MPRAGE) images (1mm iso tropic voxels) (van der

Kouwe *et al.*, 2008) for cortical surface reconstruction, co-registration to PET/7T data, segmentation of deep GM, and generation of attenuation correction maps30; (2) conventional 3D fluid attenuated inversion recovery (FLAIR) images (1mm isotropic voxels) for WM lesion segmentation; (3) diffusion images (60 diffusion-encoding directions, b value53,000s/mm2, 8 volumes without diffusion weighting, 2.5mm isotropic voxels) for assessing microstructural integrity in the pseudo-reference region used for normalizing PET data; (4) resting-state functional MRI acquisition including 120 volumes of spin-echo echo-planar images acquired using standard parameters (TR = 3,000 ms, TE = 30 ms, 3-mm slice thickness, 50 contiguous axial sections, refocusing pulse = 89°, FOV = 192 mm, matrix = 64 × 64, acquisition time = 7 min). Before fMRI acquisition participants were instructed to lie down, awake, and with their eyes closed, in a fully relaxed condition. Within 1 week from MR-PET, MS subjects also underwent 7T MRI on a Siemens scanner using a 32- channel head coil.

Data analysis:

PET data have been already analyzed and results are available (Herranz *et al.*, 2016).

Preprocessing of structural and functional images was carried out using FSL version 5.0 (FMRIB's Software Library http://fsl.fmrib.ox.ac.uk/fsl) and homemade codes based on Bash and Matlab R2017b (https://it.mathworks.com).

Resting-State fMRI Processing: Single-subject preprocessing of rs-fMRI will be carried out using FEAT (FMRI Expert Analysis Tool) version 6.00. After exclusion of the first 3 volumes of 120 resting state BOLD volumes to obtain a steady state condition, motion correction using MCFLIRT (Smith, 2002) and spatial smoothing at 5 mm Full-Width-at-Half-Maximum Gaussian kernel will be applied. Motion-related independent components will be identified and removed from images via ICA-AROMA (Pruim *et al.*, 2015), gross drifts and physiological fluctuations will be avoided by applying band-pass filtering at [0.008–0.09] Hz. Artifacts cleaning will be performed by adding cerebrospinal fluid and white matter signals as non-interest covariates to the voxel-wise denoising. Registration onto standard MNI space of single subject functional images will be performed in a linear/non-linear two-step procedure. T1-weighted high-resolution images will be linearly registered onto MNI space (FLIRT) to create a matrix that will be further used as affine starting matrix in the non-linear transformation of functional images from subject to standard space (FNIRT, http://www.fmrib.ox.ac.uk/datasets/techrep/tr07ja2/tr07ja2.pdf).

Seed-based analysis: For the seed analysis, a single image of both thalami for each subject will be created and transformed to functional space by applying the affine registration matrix obtained during the registration step of rs-fMRI preprocessing. Individual seed-ROI masks of the thalami will be obtained from each subject's high-resolution T1-weighted structural scan by using FMRIB's Integrated Registration and Segmentation Tool (FIRST/FSL), an automatic subcortical segmentation program. Each image will be visually inspected in the coronal, axial and sagittal planes to ensure accuracy. Left

and right masks of thalamus will be merged to obtain a single bilateral mask. This mask will be registered to functional coordinate space and will be used to extract the related fMRI time course. Time-series will be averaged across all voxels for the seed-ROI and will be fed into the fMRI Expert Analysis Tool to produced individual participant-level correlation maps of all voxels positively or negatively correlated with the seed (first level analysis). Afterward, higher level (group level) analysis will be performed by using FMRIB's Local Analysis of Mixed Effects. The general linear model will be applied to test for group averages and differences between the 2 groups (patients and controls) by using a 2-sample unpaired *t* test. The Z-statistic images will be thresholded by using clusters determined by $Z > 2.3$, and a whole-brain family-wise-error-corrected cluster significance threshold of $P < .05$ will be applied to the superthreshold clusters. Age, sex, and total gray matter volumes were entered in the model as nuisance covariates. Anatomic localization of significant clusters will be established according to the Harvard-Oxford Structural Atlas, the Juelich histologic atlas, and the Oxford Thalamic Connectivity Probability Atlas included in the FSL.

Independent component analysis (ICA): Following preprocessing, multivariate group probability ICA based on FSL Melodic software will be used to perform a temporal concatenation of the spatial ICA maps across all the subjects (Beckmann and Smith, 2004). A high pass temporal filtering cutoff of 100 s will be applied. For the stats, variance-normalize time courses and automatic dimensionality estimation using temporal concatenated ICA will be used. For the post-stats, threshold IC maps with background images to mean high-resolution images will be used. RSNs will be selected by careful visual inspection and those components, representing well-known RSNs, will be considered further for dual regression. Dual regression analysis will be adopted to perform a voxel-wise comparison of group ICA by regressing the group ICA maps to an individual set of time series components and re-regressing them back into the subject spatial maps (Beckmann *et al.*, 2009). The subject-specific spatial maps will be used for the statistical analysis of within and between-network rsFC.

Motion Analysis : Head motion may substantially affect rsFC differences between individuals in the same population (Van Dijk *et al.*, 2010). Motion parameter analysis will be performed to assess differences between rsFC (Werner *et al.*, 2014). Absolute and relative displacement values will be obtained by using the McFLIRT tool in FSL and assessed across the groups by two-sample *t*-test.

Within-Network rsFC: Subject-specific spatial maps of the MS patients and HS will be obtained from the dual regression analysis and then compared. The spatial maps per component will be analyzed by means of a two-sample unpaired *t*-test, entering covariates of no interest (age, sex, and total gray matter volume) into the model. Statistical differences will be assessed by randomizing different components per subject using non-parametric permutations, incorporating a threshold-free cluster enhancement technique, and by performing 5,000 random permutations (Nichols and Holmes, 2002). For each RSN, we will compare the patient group with the control group by applying unpaired *t*-test.

Brain areas involved in abnormal RSNs will be identified on the basis of the Harvard-Oxford atlas (Desikan *et al.*, 2006). Possible correlations among within-network rsFC and clinical scores will be assessed in patients by using a general linear model implemented in FSL. The statistical threshold will be set at $p < 0.05$, family-wise error (FWE) corrected.

Between-Network rsFC: We will use the FSLNets toolbox (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSLNets) on the MATLAB platform. After normalization of the extracted time courses of all the RSNs identified in each subject, full and partial correlation matrices will be calculated between the RSNs. Between-group comparisons of time series correlations will be performed using the two-sample unpaired *t*-test. The relationship between clinical scores and between-network rsFC will be assessed using Spearman's rank correlation. The statistical significance threshold will be set at $p < 0.05$, FWE corrected.

Correlation between fMRI and PET parameters: Possible correlations between within-network rsFC and PET uptake in patients will be assessed by using a general linear model implemented in FSL using as covariate of non interest age, sex, binding affinity for ^{11}C-PBR28 and total grey matter volume and as covariate of interest PET uptake data (normalized standardized uptake values) and clinical measures. PET uptake data will be entered as variable of interest while age, sex, binding affinity, T2-lesion volume and total GM volume will be entered as nuissance variables. The statistical threshold will be set at $p < 0.05$, family-wise error (FWE) corrected. Lastly, in those regions in which RSNs abnormalities are related to inflammation, we will explore a correlation between rs-FC alterations and measures of physical disability and cognitive impairment.

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