

# **REDUCED DYNAMICS OF FUNCTIONAL CONNECTIVITY AND COGNITIVE IMPAIRMENT IN MULTIPLE SCLEROSIS**

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## Abstract

**Background.** In multiple sclerosis (MS), abnormalities of brain network dynamics and their relevance for cognitive impairment were never investigated.

**Objectives.** To assess dynamic resting state (RS) functional connectivity (FC) on 62 relapsing-remitting MS patients and 65 sex-matched healthy controls enrolled at seven European sites.

**Methods.** MS patients underwent clinical and cognitive evaluation. Between-group network FC differences were evaluated using a dynamic approach (based on sliding windows correlation analysis) and grouping correlation matrices into recurrent FC states.

**Results.** Dynamic FC analysis revealed, in healthy controls and MS patients, three recurrent FC states: two characterized by strong intra- and inter-network connectivity, and one characterized by weak inter-network connectivity (State3). Twenty-three MS patients were cognitively impaired (CI). Compared to cognitively preserved (CP), CI MS patients had reduced RS FC between sub-cortical and default-mode networks in the low-connectivity State3 and lower dwell time (i.e. time spent in a given state) in the high-connectivity State2. CI MS patients also exhibited a lower number and a less frequent switching between meta-states, as well as a smaller distance travelled through connectivity states.

**Conclusions.** Time-varying RS FC was markedly less dynamic in CI vs CP MS patients, suggesting that slow inter-network connectivity contribute to cognitive dysfunction in MS.

## Introduction

Cognitive impairment is a common and debilitating feature of multiple sclerosis (MS) occurring in 40-70% of patients<sup>1</sup> and has been associated with several MRI measures of brain white and grey matter damage.<sup>2</sup> Multiparametric MRI studies consistently demonstrated that in addition to structural abnormalities, changes to the recruitment and functional connectivity (FC) of several cognitive brain networks also help to explain the presence and severity of cognitive deficits.<sup>2</sup> However, current understanding of the role of functional abnormalities in cognitive performance of MS patients is still incomplete, mostly due to inconsistencies in the findings between several studies.<sup>3</sup> Interpretation of the results obtained using active cognitive fMRI tasks is influenced by the ability of patients to correctly perform the task, which is clearly reduced in patients with clinical and cognitive deficits. Resting state (RS) FC seems a promising approach, unbiased by the task, to estimate the role of functional reorganization. However, even using this strategy, conflicting results have been found. Some studies supported the hypothesis that increased RS FC of selected brain regions (or networks) is related to better cognitive performance,<sup>4-6</sup> while others showed the opposite, with increased RS FC in cognitively impaired patients.<sup>7-9</sup> Many clinical and methodological factors may help to explain these discrepancies, and have been taken into account in some recent, large-scale studies.<sup>3</sup>

One aspect that has not previously been considered is the assumption that brain FC is static across the whole duration of image acquisition (usually taking about 10 minutes), and thus the strength of the interaction between different brain regions is assumed to be constant over time. However, RS FC between regions changes dynamically over time.<sup>10, 11</sup> This led to a shift from measuring static to measuring time-varying (dynamic) FC between different brain regions.<sup>10</sup> Dynamic FC analysis allows capturing reoccurring patterns of interaction among intrinsic networks at rest.<sup>10, 11</sup> Studies assessing dynamic RS FC have shown the utility of this method in shedding light not only on the physiological processes in healthy individuals,<sup>11</sup> but also in diseased subjects, for diagnostic purposes<sup>12</sup> or to improve the understanding of their clinical manifestations.<sup>13</sup> Only a few studies have investigated RS FC dynamic properties in MS patients, describing trends towards abnormal connectivity dynamism in this population.<sup>14-16</sup>

In this work, we hypothesized that dynamic RS FC analysis may provide novel insights into the mechanisms associated with cognitive impairment in MS patients. Since cognitive impairment is mainly characterized by decreased information processing speed, our *a priori* hypothesis was that cognitively

impaired (CI) MS would show a slower inter-network connectivity than cognitively preserved (CP) MS. To test this hypothesis, we analyzed cognitive and dynamic RS fMRI data collected within a multicenter project by applying a recently developed method,<sup>11</sup> which is based on short time windowed correlations computed on time courses of spatial independent components, and clustering dynamic connectivity patterns using a k-means approach.

## **Materials and methods**

Ethical approval and patient consent. Approval was received from the local ethical standards committees on human experimentation and written informed consent was obtained from all subjects prior to study participation.

Subjects. Subjects were prospectively recruited from January 2009 to May 2012 as part of a project on imaging correlates of cognitive impairment in MS at seven European centers.

Inclusion criteria are reported in the Supplementary material. The final dataset included 62 relapsing-remitting MS patients (mean age=39.5 years, 40 women) and 65 healthy controls (Table 1).

Clinical and neuropsychological assessment. Within 48 hours from MRI, MS patients underwent a neurological evaluation with rating of the Expanded Disability Status Scale score and a neuropsychological assessment, performed at each participating site by experienced neurologists and neuropsychologists, unaware of the MRI results, using validated translations of the neuropsychological tests. Cognitive performance was assessed using the Brief Repeatable Battery of Neuropsychological Tests,<sup>1</sup> which includes the Selective Reminding Test (SRT) to assess verbal memory; the 10/36 Spatial Recall Test (10/36 SRT) to assess visuospatial memory; the Symbol Digit Modalities Test (SDMT) and the Paced Auditory Serial Addition Test (PASAT) 2'' and 3'' to assess attention and information processing speed; and the Word List Generation (WLG) test to assess verbal fluency. In addition, the Wisconsin Card Sorting Test was administered to evaluate executive function.<sup>17</sup> Patients with at least two abnormal tests ( $\geq 2$  SD below the normative value provided by Boringa et al.<sup>18</sup> for the Brief Repeatable Battery of Neuropsychological Tests and by Heaton et al.<sup>17</sup> for the Wisconsin Card Sorting Test) were considered CI.<sup>19</sup> Z-scores for each of the previous domains and a global Z-score of cognitive function (obtained by averaging Z-scores of all tests) were calculated.

MRI acquisition. Brain MRI was performed using 3.0 Tesla systems at all sites. A RS fMRI scan of the brain was collected using a repetition time of 3000 ms and a total acquisition time of 10 minutes at all participating centres. The remaining RS fMRI sequence parameters are described in details in the Supplementary material. A dual-echo and a 3D T1-weighted scan were also collected and used for brain lesion and atrophy analysis, whose results have been reported elsewhere.<sup>19, 20</sup>

Group independent component analysis and selection of networks of interest. Details of RS fMRI pre-processing are reported in the Supplementary material. Pre-processed data from healthy controls and MS patients were analyzed using spatial group independent component analysis (ICA), and the GIFT software,<sup>21</sup> following three main steps: (i) data reduction, (ii) group ICA (repeated 20 times in ICASSO to ensure stability), and (iii) back reconstruction. The number of independent group components (IC) was set to 100 to ensure comparability of dynamic FC analysis with previous studies.<sup>11, 22</sup> ICs visual inspection, a frequency analysis and a template-matching procedure (using the components provided by Allen et al.<sup>11</sup> as reference templates) allowed to remove components clearly related to motion and physiological artifacts, and the selection of 43 components of interest, which were assigned to the sub-cortical, auditory, somatomotor, visual, cognitive, default-mode and cerebellar networks (Figure 1).

Static functional network connectivity analysis. We computed static functional network connectivity (sFNC), defined as pairwise Pearson's correlation between the whole IC time courses, as a measure of average connectivity among ICs during the entire scan duration (see Supplementary material for details).

Dynamic functional network connectivity analysis. Dynamic FNC (dFNC) between two IC time courses was computed using a sliding window approach with a window size of 22×repetition time (66 s) in steps of 1×repetition time,<sup>11</sup> resulting in 178 inverse covariance matrices, as reported in details in the Supplementary material. DFNC Pearson's correlation matrices computed for each subject were *r*-to-*z* Fisher transformed.

Once obtained the sliding-window dFNC matrices, dynamic connectivity properties were assessed using two approaches: 1) hard clustering analysis, and 2) calculation of “fuzzy meta-states”.

*1. Hard clustering analysis.* Average recurring connectivity patterns were identified using a clustering technique based on a k-means algorithm.<sup>22</sup> The selected optimal number of centroid states (estimated using

the elbow criterion) was 3, as shown in Supplementary Figure 1. Average dwell times (the time spent in each FC state) and probability of transitioning from one state to another were also computed.<sup>11</sup>

2. *Fuzzy meta-state analysis*. Clustering analysis assumes that subjects are in a single connectivity “state” at each timepoint. A more flexible approach is to consider that multiple states (estimated by using temporal ICA) might be represented to varying degrees at the same timepoint. The contribution of each state for a specific time is characterized by a vector called “meta-state”.<sup>23</sup> Four global metrics can be associated with meta-states, and give an overall description of connectivity dynamics: a) the number of times that subjects switch from one meta-state to another; b) the number of distinct meta-states subjects occupy during their scans; c) the range of meta-states subjects occupy; and d) the overall distance traveled by each subject through the state space.<sup>23</sup>

Figure 2 shows a schematic summary of the analysis pipeline.

Statistical analysis. Between-group comparisons of demographic and clinical variables were performed using age-adjusted generalized linear random effect models accounting for clustering, using random intercepts and an unstructured covariance matrix.

*Between-group comparison of sFNC*. We used a MANCOVAN framework<sup>24</sup> to assess between-group sFNC differences. This method first performs a backward selection, by testing whether each factor in the model (in this case, age, site and group) explains variability in the multivariate response, and then proceeds to perform univariate tests only on factors retained in the reduced model.<sup>24</sup>

*Between-group comparison of dFNC*. Element-wise between-group differences in connectivity strength were tested using two sample t tests for non-paired data (as implemented in the GIFT dFNC toolbox).<sup>11</sup> Group differences in dwell times and global meta-state metrics were also evaluated using two sample t tests, while inter-site heterogeneity of these metrics was tested using ANOVA models on data from healthy controls.

The following pair-wise comparisons were performed for both sFNC and dFNC: healthy controls *vs* MS patients; healthy controls *vs* cognitively preserved (CP) MS; CP MS *vs* CI MS patients. Such comparisons were performed only on connections significantly correlated (or anticorrelated) at the one sample t test in at least one group.

In line with previous literature,<sup>25</sup> between-group connectivity differences were interpreted considering both the absolute strength and directionality of correlations. In other words, even if encoded by the same color from the two-sample t test, increments of positive correlations were considered as connectivity increases, while reductions of negative correlations were considered as connectivity decreases. The same strategy was applied for interpreting increments of negative correlations and decrements of positive correlations, respectively.

*Correlation analysis.* SFNC and dFNC correlation strengths, as well as global dynamic metrics, were correlated with clinical and structural MRI variables using the Spearman's rank correlation coefficient.

A p-value <0.05 was considered as statistically significant. Results were tested both correcting for the number of tested ICs using the false discovery rate approach<sup>26</sup> and, given the exploratory nature of this study, at uncorrected threshold.

Validation analysis. Dynamic RS FC techniques are relatively novel, and the probability of detecting FC dynamism in RS fMRI data acquired with a relatively long repetition time is still debated.<sup>27</sup> To test if our data were sensitive enough to detect RS FC dynamic properties, we performed a validation analysis against simulated data, as described in details in the Supplementary material.

## **Results**

Clinical/neuropsychological findings. Twenty-three (37%) MS patients were CI, with no difference in the distribution of CI and CP patients among sites. Nineteen CI MS patients (i.e., 83% of the CI group) showed impairment at the information processing speed, which was the most frequently involved domain. Compared to CP, CI MS patients were significantly older (Table 1). As described in details elsewhere<sup>19, 20</sup> and in line with previous literature,<sup>2</sup> T2 and T1 lesion volumes, as well as brain volumetric measures, were worse in CI vs CP MS patients. The mean motion detected during the RS fMRI examination (quantified using the framewise displacement) did not differ between healthy controls and MS patients (p=0.5), nor between CP and CI MS patients (p=0.2).

sFNC analysis. The comparison between MS patients and healthy controls showed reduced sFNC in MS vs controls of sub-cortical networks with cognitive, default-mode and visual networks; and increased within-

network sFNC in cognitive and default-mode networks of MS patients (Table 2). The increased sFNC within cognitive networks was also detected when comparing CP MS patients vs healthy controls ( $p=0.01$  to  $0.001$ ).

Compared to CP, CI MS patients showed reduced sFNC within the somatomotor network, between sub-cortical and auditory networks, and between visual and cognitive/default-mode networks. They also showed increased sFNC of the default-mode networks with the majority of the remaining networks (including somatomotor, sub-cortical and cognitive networks) (Table 2).

dFNC analysis. *Hard Clustering analysis.* Clustering analysis revealed 3 recurring FC states (Figure 3). States 1 and 2 were characterized by a strong intra- and inter-network connectivity (especially within and between visual, somatomotor, auditory and cognitive networks), while State 3 was characterized by an overall weak inter-network connectivity, with some stronger connections within visual, cognitive and default-mode networks. The main dFNC hard clustering metrics (including dwell times in the three recurring states and probability of transitioning from one state to another) were not heterogeneous across healthy controls acquired at different sites ( $p=0.3$  to  $0.8$ ).

Comparison between healthy controls and MS patients showed (Figure 3; Table 3):

- reduced RS FC in MS patients between sub-cortical networks and visual/cognitive networks in all states, and between visual and cognitive networks in State 2;
- increased RS FC in MS between sub-cortical and somatomotor networks in all states.

Dwell times did not differ between MS patients and healthy controls.

Compared to CP MS, CI MS patients showed:

- reduced RS FC between sub-cortical and default-mode networks in the low-connectivity State 3 (Table 3);
- lower dwell time in the high-connectivity State 2 (mean of CI MS patients= $10.6$  windows,  $SD=15.3$ ; mean of CP MS patients= $19.9$  windows,  $SD=17.7$ ;  $p=0.05$ ).

*Meta-state analysis.* The four global meta-state metrics were not significantly different between healthy controls and MS patients (Table 4) and were not significantly heterogeneous across healthy controls acquired at different sites ( $p=0.2$  to  $0.7$ ).

CI MS patients exhibited lower dynamic fluidity than CP MS, defined as a significantly lower number of meta-states and a less frequent switch between meta-states. Moreover, CI MS patients operated



over a more restricted dynamic range than CP MS, since the total distance travelled through connectivity states was significantly smaller in the first group (Table 4).

*Correlation analysis.* In CI MS patients, the decreased number of switches between meta-states and the smaller total distance travelled through connectivity states were significantly correlated with lower normalized brain volumes ( $r=0.35$ ,  $p=0.005$  and  $r=0.37$ ,  $p=0.002$ , respectively). No correlations were found between sFNC/dFNC measures and clinical, cognitive and lesional MRI variables.

Validation analysis. Supplementary results report the details of the validation analysis performed against simulated data. Briefly, we found that, despite the use of a relatively long repetition time to acquire our data, it was possible to detect significant dynamic RS FC properties in some connections involving the default-mode and cognitive networks, and, to a smaller extent, the somatomotor, cerebellar and subcortical networks.

## **Discussion**

The analysis of network dynamic FC received increasing attention and is showing great potential for exploring neurological conditions and mental disorders.<sup>10</sup> While these methods were extensively used in studies investigating neuropsychiatric diseases,<sup>13,22</sup> only a few studies have applied these techniques to explore static and dynamic RS FC brain abnormalities and their relationship with deficits in specific cognitive domains in MS patients.<sup>14-16</sup>

Our sFNC analysis showed that, compared to healthy controls, MS patients had distributed RS FC abnormalities within and between sub-cortical and cognitive/default-mode/visual networks, characterized by both decreased and increased sFNC. Concomitant increase and decrease of sFNC between different brain networks was also found when comparing CI and CP MS patients. Using the dynamic fMRI analysis, we detected recurring patterns of inter-network RS FNC in both healthy controls and MS patients, and showed that time-varying RS FC was markedly less dynamic in CI MS than in CP MS patients. Of note, the main dFNC metrics were not heterogeneous across sites, suggesting a limited impact of inter-site heterogeneity on our findings.

The heterogeneous pattern of sFNC abnormalities we found agrees with many studies on this topic, that consistently demonstrated abnormal RS FC within and between the main functionally-relevant brain

networks in MS. This abnormal network RS FC is thought to result from a disconnection related to the well-known disease-related structural damage. In line with these studies, we found both reduction<sup>3, 6, 9, 28</sup> and increase<sup>5, 7, 29</sup> of sFNC not only when analyzing the whole group of MS patients in comparison to healthy controls, but also when considering the presence or absence of cognitive impairment.<sup>5-7, 29-31</sup> Many factors may contribute to these heterogeneous abnormalities of sFNC in MS patients, thus explaining the discrepancies between studies,<sup>3, 5-7, 9, 28, 29</sup> including the presence and location of white and grey matter lesions, damage to the white matter pathways connecting regions within a network and damage to relevant relay-stations of a network. Furthermore, measuring brain RS FC using a static connectivity approach, which represents an average across different activity during an unconstrained RS,<sup>11</sup> may have provided only a limited view of the presence and clinical relevance of network abnormalities in these patients.

Explicit investigations of RS FC dynamics have unambiguously demonstrated the time-varying nature of both connectivity strength and directionality (i.e., positive or negative),<sup>32, 33</sup> suggesting that capturing this variability may engender new understanding of the FNC alterations found in neuropsychiatric diseases, such as Alzheimer's disease<sup>12</sup> and schizophrenia.<sup>22, 23</sup> Specifically, differences in dFNC previously observed in Alzheimer's disease have been explained by differences in dwell time in different default-mode network configurations, rather than steady state connectivity magnitude.<sup>12</sup> Compared to healthy subjects, patients with schizophrenia were characterized by higher occurrence of states of decreased RS FNC<sup>22</sup> and by more time spent in sparsely connected states.<sup>34</sup> Another study showed that time-varying whole-brain network connectivity patterns are markedly less dynamic in schizophrenia patients, particularly in patients with high levels of hallucinatory behavior.<sup>23</sup>

Given that dFNC provides measures of connectivity changes over time, rather than representing the mean FC over a relatively long period of time,<sup>10</sup> this approach might provide relevant pieces of information in the study of cognitive impairment in MS, which is typically characterized by decreased information processing speed, as it was the case for our patients.

The analysis of dFNC confirmed that different patterns of strong and weak inter-network connectivity reoccur in healthy controls and MS patients. Specifically, we identified 3 FNC States; one of them (State 3) was characterized by weak intra- and inter-network connectivity, the remaining two by high inter-network connectivity. The comparison between controls and MS patients showed reduced cortical sub-

cortical dFNC in MS patients in all states, and increased RS FC between sub-cortical and somatomotor networks.

Consistent with our working hypothesis of the importance of dFNC analysis for improving the understanding of the mechanisms related to cognitive impairment in MS patients, compared to CP patients, CI MS patients had reduced dFNC between sub-cortical and default-mode networks in the low-connectivity State 3 (a finding not detected by sFNC). They also experienced a significantly lower dwell time in State 2, meaning that they spent less time than CP MS patients in a high-connectivity state. This finding can be interpreted as a maladaptive mechanism contributing to cognitive dysfunction in these patients. The impact of abnormal network dynamics on cognitive impairment is also confirmed by the meta-state analysis, which showed that, compared to CP, CI MS patients had lower number of meta-states and less frequent switching between states, operating over a restricted dynamic range (less distance travelled through connectivity meta-states). The notion that reduced network dynamics and reduced time spent in a highly-connected state may help to distinguish patients with a given pathological condition and more severe symptoms is in line with some recent data from patients with schizophrenia<sup>34</sup> and with the preliminary results obtained by MS studies investigating cognitive deficits in single domains, including executive functions<sup>16</sup> and memory.<sup>15</sup>

Our study has some limitations. First, it is cross-sectional. Longitudinal studies are required to clarify the dynamic relationship between cognitive impairment and RS FC abnormalities in MS patients. Second, the use of a relatively long repetition time for RS fMRI acquisitions did allow to capture only a fraction of RS FC dynamism in our data. It is likely that future studies using optimized MR protocols with shorter repetition times will allow the detection of more dynamic states and a better characterization of dFNC abnormalities in MS patients. Third, despite the multicenter setting, the achieved small sample size was relatively small, limiting the general applicability of our findings, since results were partially obtained at uncorrected threshold. As a consequence, further studies, on larger datasets, are needed. Fourth, we limited our analysis to dFNC and cognitive impairment. Other aspects related to the complex clinical manifestations of MS (e.g., disability, fatigue, depression) should be explored using this approach. Fourth, measures of structural damage (lesions and atrophy), which was more severe in CI vs CP MS patients, may have influenced our RS FC results. However, previous studies showed that RS FC was able to explain CI in MS patients beyond structural damage.<sup>30</sup> Finally, we chose sliding windows to analyze network dynamic

connectivity. However, there is a rich set of tools that have been proposed to characterize the dynamic reconfiguration of brain connectivity over the past few years.<sup>35</sup> Therefore, our findings need to be replicated and confirmed using a different approach.

In conclusion, sFNC analysis showed the loss of functional connectivity between sub-cortical and cortical networks in our MS cohort. Using dFNC, we identified recurring patterns of strong and weak inter-network connectivity in both healthy controls and MS patients. Only with the dynamic approach we were able to detect reduced cortical-subcortical RS FC, a lower permanence in high-connectivity states and less dynamic RS FC configurations in CI compared to CP MS patients, suggesting that reduced inter-network connectivity dynamism is one of the mechanisms contributing to cognitive impairment in MS patients. The effect of treatment (particularly cognitive rehabilitation) on inter-network connectivity and whether this may improve cognitive performance should be investigated by future studies.

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**Conflicts of interest disclosure**

A. d'Ambrosio has nothing to disclose. P. Valsasina received speakers' honoraria from ExceMED. A. Gallo received honoraria for speaking and travel grants from Biogen, Sanofi-Aventis, Merck, Genzyme, Teva, and Novartis. N. De Stefano has received honoraria from Biogen-Idec, Genzyme, Merck Serono, Novartis, Roche and Teva for consulting services, speaking and travel support. He serves on advisory boards for Merck Serono, Novartis, Biogen-Idec, Roche, and Genzyme, he has received research grant support from the Italian MS Society. D. Pareto has received honoraria as speaker from Novartis and Genzyme. F. Barkhof acts as a consultant to Biogen-Idec, Janssen Alzheimer Immunotherapy, Bayer-Schering, Merck-Serono, Roche, Novartis, Genzyme, and Sanofi-aventis. He has received sponsorship from EU-H2020, NWO, SMSR, EU-FP7, TEVA, Novartis, and Toshiba. He is on the editorial board of Radiology, Brain, Neuroradiology, MSJ, and Neurology. O. Ciccarelli receives research grant support from the Multiple Sclerosis Society of Great Britain and Northern Ireland, the NIHR UCLH Biomedical Research Centre; she is a consultant for Teva, Roche, Novartis, Biogen, Genzyme and GE. She is an Associate Editor for Neurology, for which she receives an honorarium. C. Enzinger received funding for traveling and speaker honoraria from Biogen Idec, Bayer Schering Pharma, Merck Serono, Novartis, Genzyme and Teva Pharmaceutical Industries Ltd./sanofi-aventis; received research support from Merck Serono, Biogen Idec, and Teva Pharmaceutical Industries Ltd./sanofi-aventis; and serves on scientific advisory boards for Bayer Schering Pharma, Biogen Idec, Merck Serono, Novartis, Genzyme, Roche, and Teva Pharmaceutical Industries Ltd./sanofi- Aventis. G. Tedeschi received compensation for consulting services and/or speaking activities from Bayer Pharma, Biogen Idec, Merck, and Teva Pharmaceutical Industries and receives research support from Biogen Idec, Merck Serono, and Fondazione Italiana Sclerosi Multipla. M.L. Stromillo has nothing to disclose. M.J. Arévalo has nothing to disclose. H.E. Hulst has received compensation for consulting services and/or speaking activities from Merck Serono, Genzyme, Novartis, and Roche. N. Muhlert has nothing to disclose. M. Koini has nothing to disclose. M. Filippi is Editor-in-Chief of the Journal of Neurology; received compensation for consulting services and/or speaking activities from Biogen Idec, Merck-Serono, Novartis, Teva Pharmaceutical Industries; and receives research support from Biogen Idec, Merck-Serono, Novartis, Teva Pharmaceutical Industries, Roche, Italian Ministry of Health, Fondazione Italiana Sclerosi Multipla, and ARiSLA (Fondazione Italiana di Ricerca per la SLA). M.A. Rocca received speakers honoraria from Biogen Idec, Novartis, Genzyme, Sanofi-Aventis, Teva, Merck Serono, and Roche and receives research support from the Italian Ministry of Health and Fondazione Italiana Sclerosi Multipla.

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**Table 1.** Main demographic and clinical measures in healthy controls and patients with multiple sclerosis (MS), considered as a whole and divided according to the presence/absence of cognitive impairment. P values marked with\* survive to false discovery rate correction for multiple comparisons.

|  | <b>Healthy<br/>controls<br/>(N=65)</b> | <b>MS<br/>patients<br/>(N=62)</b> | <b>p<sup>a</sup></b> | <b>CP MS<br/>patients<br/>(N=39)</b> | <b>CI MS<br/>patients<br/>(N=23)</b> | <b>p<sup>a,b</sup></b>    |
|--|--|-----------------------------------|----------------------|--------------------------------------|--------------------------------------|---------------------------|
| <b>M/F</b>   | 27/38                                  | 22/40                             | 0.7                  | 15/24                                | 7/16                                 | 0.2 <sup>a</sup>          |
| <b>Mean age, years (SD)</b>                                      | 35.8<br>(9.4)                          | 39.5<br>(8.5)                     | <b>0.006</b>         | 37.2<br>(7.8)                        | 43.3<br>(8.3)                        | <b>0.007<sup>b*</sup></b> |
| <b>Median Expanded Disability<br/>Status Scale score (range)</b> | -                                      | 2.0<br>(0-6.0)                    | -                    | 1.5<br>(0-4.0)                       | 2.0<br>(1.0-6.0)                     | 0.3                       |
| <b>Mean disease duration, years (SD)</b>                         | -                                      | 8.2<br>(6.3)                      | -                    | 7.1<br>(4.8)                         | 9.9<br>(8.1)                         | 0.4                       |

<sup>a</sup> Age-adjusted generalised linear random effect models accounting for clustering; <sup>b</sup> *post-hoc* comparison between CP and CI MS patients.

Abbreviations: CP=cognitively preserved; CI=cognitively impaired; SD=standard deviation.



**Table 2.** Significant differences in static functional network connectivity strength (measured using Pearson's correlation coefficients) between healthy controls and patients with multiple sclerosis (MS), considered as a whole and divided according to the presence/absence of cognitive impairment. P values marked with\* survive false discovery rate correction for multiple comparisons.

| <b>Independent Components (and corresponding network)</b>             | <b>Connectivity strength in MS patients</b><br><br>(p value, one-sample t test) | <b>Connectivity strength in healthy controls</b><br><br>(p value, one-sample t test) | <b>p value (between-group comparison)</b> |
|---|---|--|---|
| <b>Reduced pair-wise RS sFNC in MS patients vs healthy controls</b>   |   |  |   |
| 9 (Subcortical) – 61 (Cognitive)                                      | 0.029 (n.s.)  | 0.104 (<0.001*)  | 0.007                                     |
| 26 (Subcortical) – 61 (Cognitive)                                     | 0.042 (0.04)  | 0.117 (<0.001*)  | 0.008                                     |
| 26 (Subcortical) – 25 (Cognitive)                                     | 0.011 (n.s.)  | 0.094 (<0.001*)  | 0.006                                     |
| 9 (Subcortical) – 40 (DMN)  | 0.002 (n.s.)  | 0.066 (<0.001*)  | 0.007                                     |
| 26 (Subcortical) – 45 (DMN)   | 0.068 (0.001*)  | 0.134 (<0.001*)  | 0.004                                     |
| 11 (Subcortical) – 33 (Visual)  | -0.086 (0.001*)   | -0.227 (<0.001*)   | 0.001*                                    |
| 38 (Subcortical) – 94 (Visual)  | 0.024 (n.s.)  | -0.052 (<0.001*)   | 0.006                                     |
| 62 (Cognitive) – 23 (Visual)  | -0.100 (<0.001*)  | -0.191 (<0.001*)   | 0.006                                     |
| 90 (Cognitive) – 23 (Visual)  | -0.164 (<0.001*)  | -0.255 (<0.001*)   | 0.005                                     |
| <b>Increased pair-wise RS sFNC in MS patients vs healthy controls</b> |   |  |   |
| 28 (Cognitive) – 52 (Cognitive)                                       | 0.161 (<0.001*)   | 0.059 (0.003*)   | 0.002                                     |
| 52 (Cognitive) – 90 (Cognitive)                                       | 0.186 (<0.001*)   | 0.089 (<0.001*)  | 0.006                                     |
| 5 (DMN) – 40 (DMN)  | 0.122 (<0.001*)   | 0.034 (n.s.)   | 0.008                                     |
| <b>Reduced pair-wise RS sFNC in CI vs CP MS patients</b>              |   |  |   |
| <b>Independent Components (and corresponding network)</b>             | <b>Connectivity strength in CI MS</b>   | <b>Connectivity strength in CP MS</b>  | <b>p Value</b>                            |
| 8 (Somatomotor) – 57 (Somatomotor)                                    | 0.019 (n.s.)  | 0.235 (<0.001*)  | <0.001*                                   |
| 8 (Somatomotor) – 92 (Somatomotor)                                    | -0.057 (n.s.)   | 0.124 (<0.001*)  | <0.001*                                   |
| 87 (Somatomotor) – 15 (DMN)   | 0.012 (n.s.)  | -0.140 (<0.001*)   | 0.005                                     |
| 38 (Subcortical) – 75 (Auditory)                                      | 0.025 (n.s.)  | 0.122 (<0.001*)  | 0.006                                     |
| 81 (Subcortical) – 20 (Auditory)                                      | 0.043 (n.s.)  | 0.233 (<0.001*)  | 0.003                                     |
| 52 (Cognitive) – 76 (Visual)  | 0.037 (n.s.)  | -0.074 (0.005*)  | 0.006                                     |
| 90 (Cognitive) – 24 (Visual)  | -0.029 (n.s.)   | -0.167 (<0.001*)   | 0.003                                     |
| 22 (DMN) – 84 (Visual)  | 0.011 (n.s.)  | -0.118 (<0.001*)   | 0.006                                     |
| <b>Increased pair-wise RS sFNC in CI vs CP MS patients</b>            |   |  |   |
| 61 (Cognitive) – 92 (Somatomotor)                                     | 0.175 (<0.001*)   | 0.054 (0.02*)  | 0.005                                     |
| 5 (DMN) – 62 (Cognitive)  | 0.181 (<0.001*)   | 0.054 (0.04)   | 0.005                                     |
| 4 (DMN) – 38 (Subcortical)  | -0.130 (<0.001*)  | -0.033 (n.s.)  | 0.005                                     |
| 15 (DMN) – 8 (Somatomotor)  | 0.175 (<0.001*)   | 0.069 (0.009*)   | 0.009                                     |

Abbreviations: MS=multiple sclerosis; CP=cognitively preserved; CI=cognitively impaired; RS=resting state; sFNC=static functional network connectivity; DMN=default-mode network.

**Table 3.** Significant differences in dynamic functional network connectivity strength (measured using Pearson’s correlation coefficients) between healthy controls and patients with multiple sclerosis (MS), considered as a whole and divided according to the presence/absence of cognitive impairment in each of the three connectivity states. P values marked with\* survive false discovery rate correction for multiple comparisons.

| <b>Independent Components<br/>(and corresponding network)</b>                  | <b>Connectivity strength<br/>in MS patients<br/><br/>(p value, one-sample<br/>t test)</b>  | <b>Connectivity<br/>strength in healthy<br/>controls<br/><br/>(p value, one-<br/>sample t test)</b> | <b>p value<br/><br/>(between-<br/>group<br/>comparison)</b> |
|--|--|---|---|
| <b>State 1: Reduced pair-wise RS dFNC in MS patients vs healthy controls</b>   |  |   |   |
| 11 (Subcortical) – 23 (Visual)   | 0.015 (n.s.)   | -0.147 (0.003*)   | 0.007   |
| 38 (Subcortical) – 94 (Visual)   | 0.056 (n.s.)   | -0.068 (0.04)   | 0.01  |
| 9 (Subcortical) – 28 (Cognitive)   | -0.002 (n.s.)  | 0.089 (0.004*)  | 0.02  |
| 38 (Subcortical) – 80 (Cognitive)  | -0.023 (n.s.)  | 0.089 (0.001*)  | 0.002*  |
| <b>State 1: Increased pair-wise RS dFNC in MS patients vs healthy controls</b> |  |   |   |
| 26 (Subcortical) – 70 (Somatomotor)  | -0.158 (<0.001*)   | -0.019 (n.s.)   | 0.01  |
| 26 (Subcortical) – 92 (Somatomotor)  | -0.133 (<0.001*)   | -0.039 (n.s.)   | 0.03  |
| <b>State 2: Reduced pair-wise RS dFNC in MS patients vs healthy controls</b>   |  |   |   |
| 11 (Subcortical) – 17 (Visual)   | -0.197 (<0.001*)   | -0.313 (<0.001*)  | 0.007*  |
| 11 (Subcortical) – 23 (Visual)   | -0.234 (<0.001*)   | -0.355 (<0.001*)  | 0.006*  |
| 11 (Subcortical) – 33 (Visual)   | -0.222 (<0.001*)   | -0.361 (<0.001*)  | 0.002*  |
| 24 (Visual) – 34 (Cognitive)   | 0.049 (n.s.)   | -0.088 (0.004*)   | 0.003*  |
| 11 (Subcortical) – 25 (Cognitive)  | 0.069 (0.01*)  | 0.175 (<0.001*)   | 0.004*  |
| 11 (Subcortical) – 28 (Cognitive)  | 0.102 (0.001*)   | 0.206 (<0.001*)   | 0.01  |
| 11 (Subcortical) – 90 (Cognitive)  | 0.092 (0.007*)   | 0.186 (<0.001*)   | 0.02  |
| <b>State 2: Increased pair-wise RS dFNC in MS patients vs healthy controls</b> |  |   |   |
| 26 (Subcortical) – 10 (Somatomotor)  | -0.133 (<0.001*)   | -0.057 (0.02)   | 0.02  |
| <b>State 3: Reduced pair-wise RS dFNC in MS patients vs healthy controls</b>   |  |   |   |
| 9 (Subcortical) – 84 (Visual)  | 0.002 (n.s.)   | -0.037 (0.002*)   | 0.03  |
| 9 (Subcortical) – 94 (Visual)  | 0.015 (n.s.)   | -0.035 (0.001*)   | 0.004   |
| 11 (Subcortical) – 94 (Visual)   | -0.055 (<0.001*)   | -0.096 (<0.001*)  | 0.02  |
| <b>State 3: Increased pair-wise RS dFNC in MS patients vs healthy controls</b> |  |   |   |
| 26 (Subcortical) – 10 (Somatomotor)  | -0.062 (<0.001*)   | -0.016 (n.s.)   | 0.01  |
| 26 (Subcortical) – 70 (Somatomotor)  | -0.052 (<0.001*)   | -0.009 (n.s.)   | 0.01  |
| <b>State 3: Reduced pair-wise RS dFNC in CI vs CP MS patients</b>              |  |   |   |
| <b>Independent Components<br/>(and corresponding network)</b>                  | <b>Connectivity strength<br/>in CI MS<br/><br/>(p value at the one-<br/>sample t test)</b> | <b>Connectivity<br/>strength in CP MS<br/>(p value at the one-<br/>sample t test)</b>               | <b>p value<br/><br/>(between-<br/>group<br/>comparison)</b> |
| 9 (Subcortical) – 22 (DMN)   | 0.023 (n.s.)   | 0.119 (<0.001*)   | 0.01  |
| 11 (Subcortical) – 5 (DMN)   | -0.022 (n.s.)  | 0.058 (0.002*)  | 0.005*  |

Abbreviations: CP=cognitively preserved; CI=cognitively impaired; RS=resting state; dFNC=dynamic functional network connectivity; DMN=default-mode network.

**Table 4.** Global measures of connectivity dynamics (reported as mean values and standard deviations) in healthy controls and patients with multiple sclerosis (MS), considered as a whole and divided according to the presence/absence of cognitive impairment. P values marked with\* survive false discovery rate correction for multiple comparisons.

|  | <b>Healthy controls</b> | <b>MS patients</b> | <b>p<sup>a</sup></b> | <b>CP MS patients</b> | <b>CI MS patients</b> | <b>p<sup>a</sup></b> |
|--|-------------------------|--------------------|----------------------|-----------------------|-----------------------|----------------------|
| <b>Number of meta-states (SD)</b>                            | 11.83<br>(4.5)          | 11.0<br>(3.7)      | 0.2                  | 11.84<br>(3.8)        | 9.56 (3.0)            | <b>0.02*</b>         |
| <b>Changes between meta-states (SD)</b>                      | 25.86<br>(7.8)          | 25.14<br>(6.7)     | 0.5                  | 26.53<br>(6.9)        | 22.78<br>(5.5)        | <b>0.03*</b>         |
| <b>Range of meta-states occupied (SD)</b>                    | 6.55<br>(1.1)           | 6.43<br>(1.2)      | 0.6                  | 6.61 (1.2)            | 6.13 (1.2)            | 0.12                 |
| <b>Total distance travelled through the state space (SD)</b> | 30.44<br>(8.5)          | 29.62<br>(7.8)     | 0.5                  | 31.05<br>(7.9)        | 27.21<br>(6.8)        | <b>0.03*</b>         |

<sup>a</sup>Two sample t test.

Abbreviations: SD=standard deviation; CP=cognitively preserved; CI=cognitively impaired.

## Figure legends

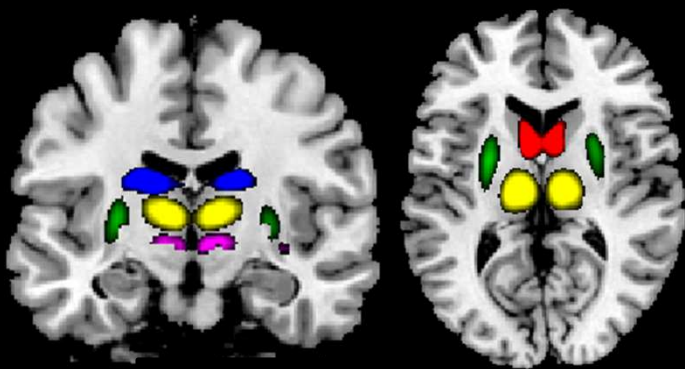
**Figure 1. Composite map of the 43 identified independent components (ICs) in all study subjects.** After the selection procedure (see text for further details), ICs were sorted into seven subcategories (sub-cortical, auditory, sensorimotor, visual, cognitive, default-mode and cerebellar networks). Five ICs were assigned to sub-cortical, 2 to auditory, 10 to visual, 7 to sensorimotor, 9 to cognitive, 8 to default-mode and 2 to cerebellar networks. Each color in the composite map corresponds to a different IC within a given subcategory. Images are presented in neurological convention.

**Figure 2. Summary of the post-processing pipeline used to assess dynamic functional network connectivity (dFNC).** A) Independent component analysis (ICA) identified spatial patterns and associated time courses of relevant functional networks in the data; B) network time courses underwent cross-correlation analysis by using a sliding windows approach, i.e., by calculating the correlation between time courses at all time points within a chosen window, and repeating the process by gradually moving the window through the scan length; C) hard clustering was applied to windowed correlation matrices to estimate transient, recurrent connectivity states, as well as between-group differences in connectivity strengths and dwell time in such states; D) fuzzy meta-state analysis was also applied to windowed correlation matrices to assess between-group differences in large-scale dFNC properties.

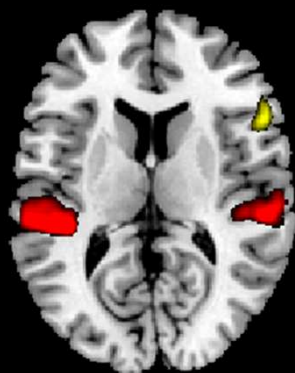
**Figure 3. Results of dynamic functional network connectivity (dFNC) analysis.** Recurring connectivity states in healthy controls (first row) and multiple sclerosis patients (second row). Thick black lines partition the dFNC matrices into the seven subcategories depicted in Figure 1 in the Supplementary material (subcortical, auditory, somatomotor, visual, cognitive, default and cerebellar). Average dFNC connectivity strength between each pair of independent components

(ICs) is color-coded according to the intensity bar reported on the right (red: positive associations between ICs, blue: negative associations between ICs). Third row: comparison of dFNC between healthy controls and multiple sclerosis patients ( $p < 0.05$ , uncorrected). Between-group differences are color-coded according to their p value (color intensity) and dFNC connectivity strength (red-yellow: lower positive dFNC [or higher negative dFNC] in multiple sclerosis vs healthy controls; blue-lightblue: higher positive dFNC [or lower negative dFNC] in multiple sclerosis vs healthy controls). Yellow boxes indicate between-group differences in dFNC (discussed in details in the text). List of abbreviations: MS=multiple sclerosis; HC=healthy controls.

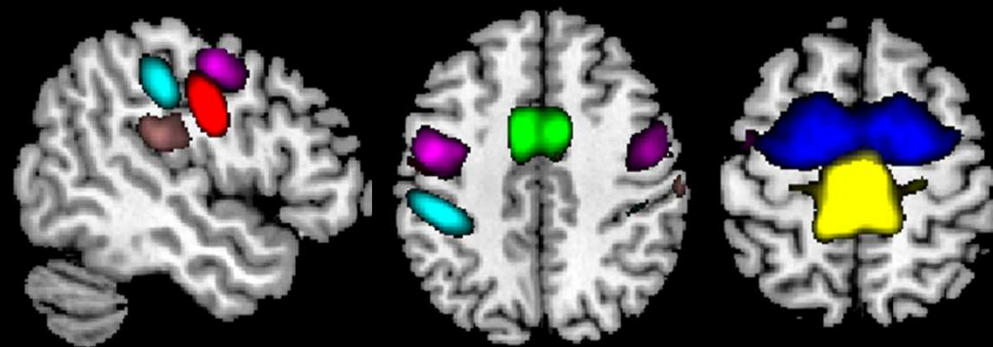
Subcortical networks (5)



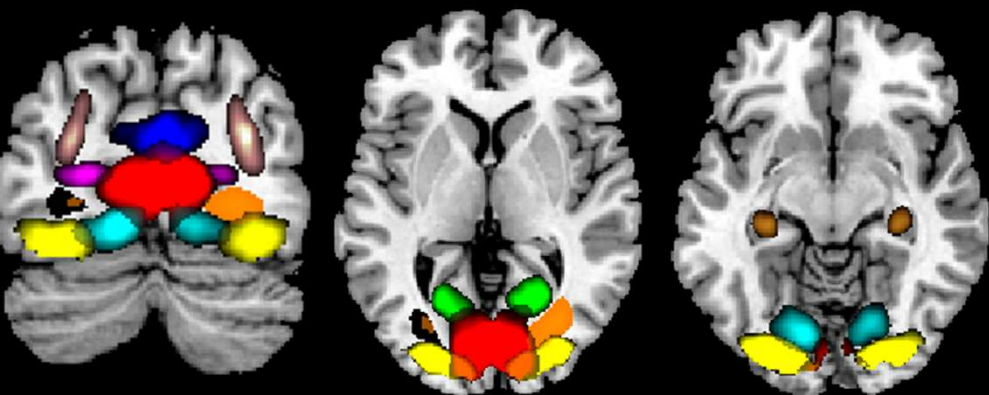
Auditory networks (2)



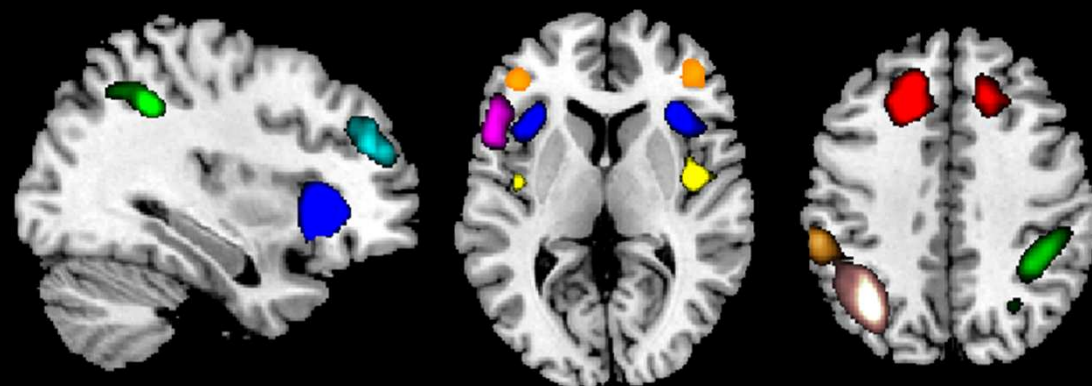
Sensorimotor networks (7)



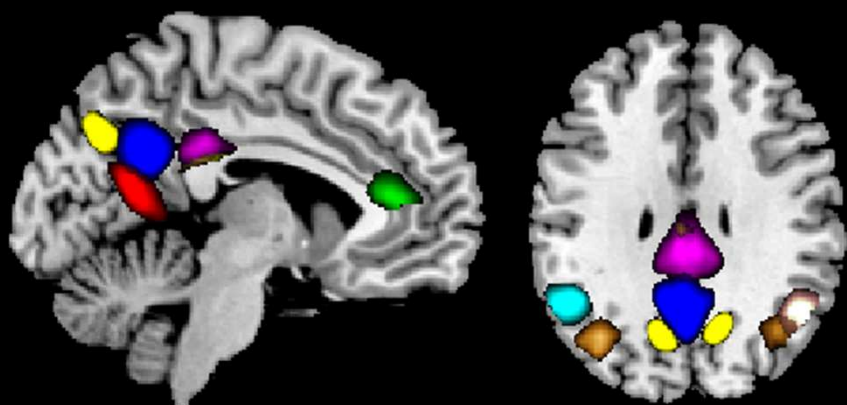
Visual networks (10)



Cognitive networks (9)



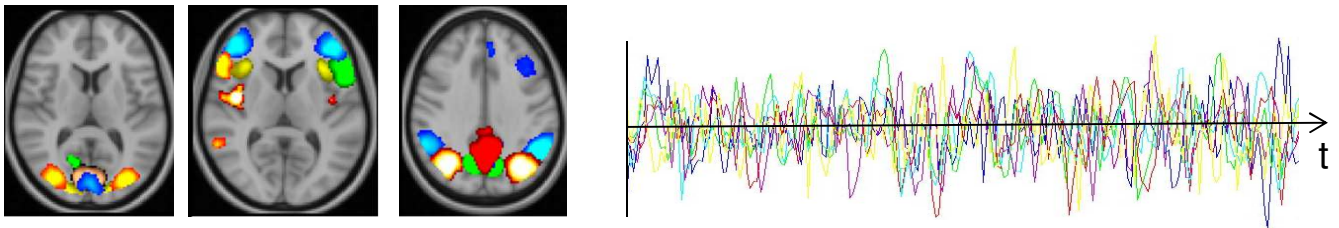
Default mode networks (8)



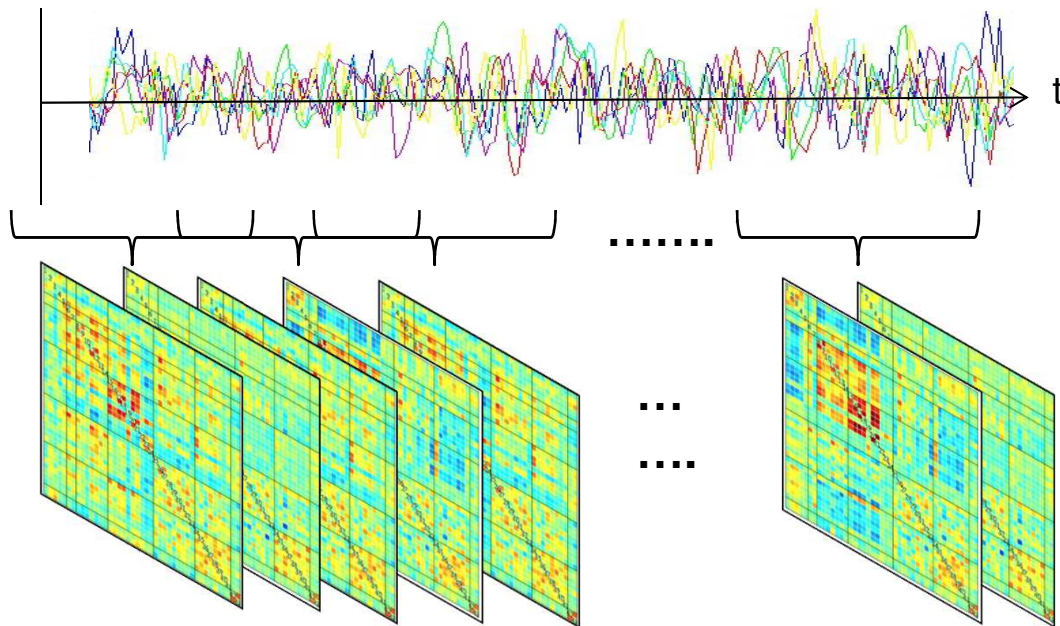
Cerebellar networks (2)



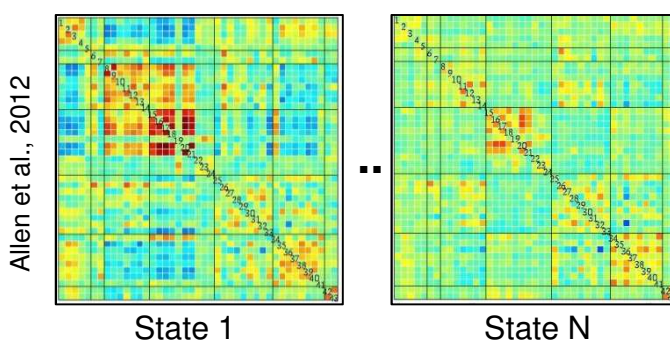
## A) ICA: spatial patterns and time series of functional networks



## B) Sliding windows: windowed correlation matrices

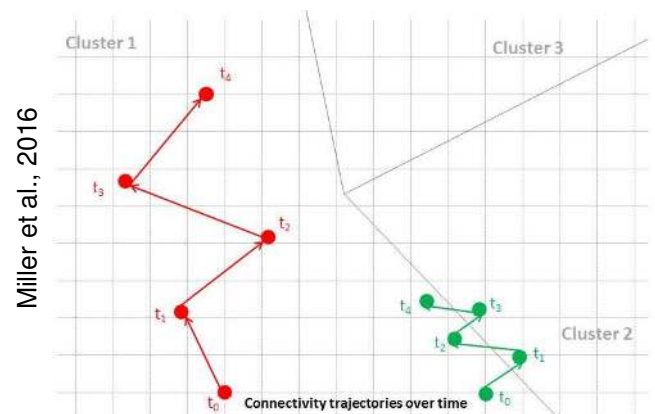


## C) Hard clustering



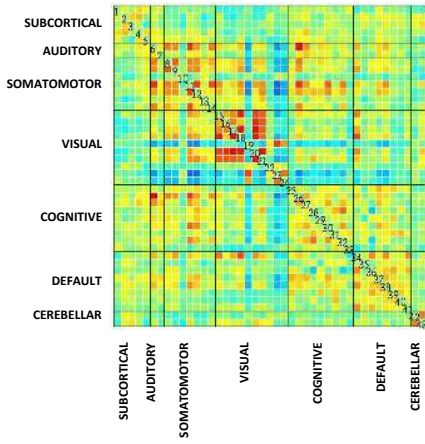
- Average recurrent states
- T sample statistic on cellwise correlations
- Statewise dwell time

## D) Fuzzy meta-states

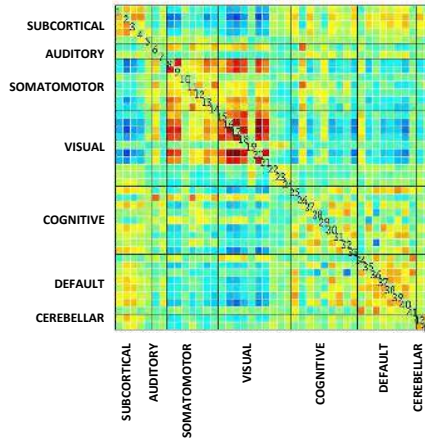


- Number of distinct meta-states
- Number of switches between meta-states
- Range of meta-states occupied
- Overall distance traveled

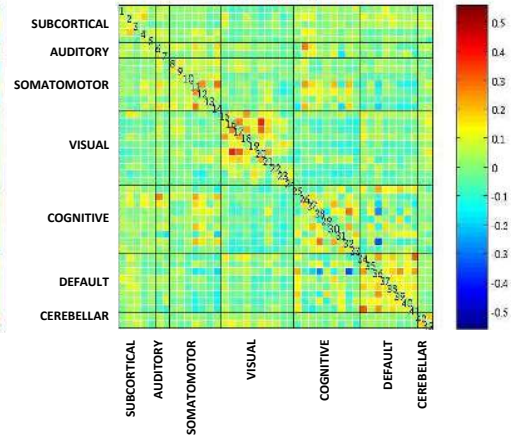
### State 1: HC



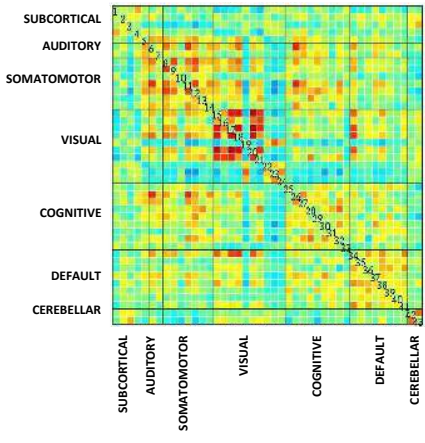
### State 2: HC



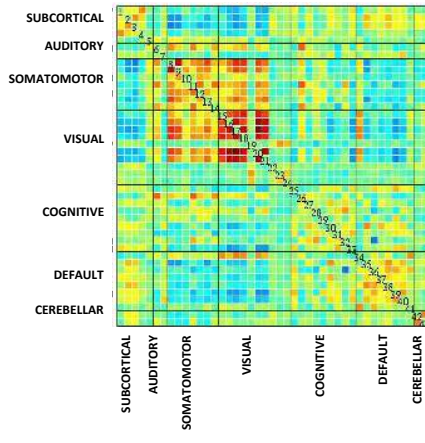
### State 3: HC



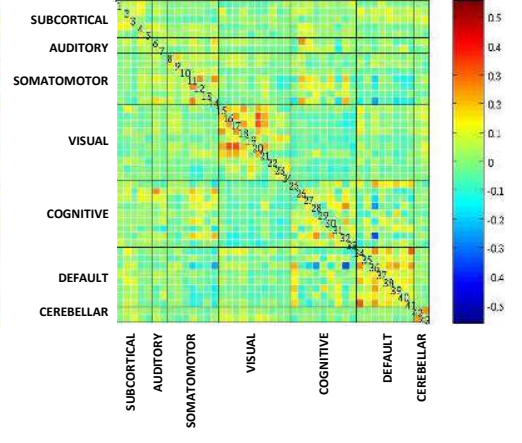
### State 1: MS



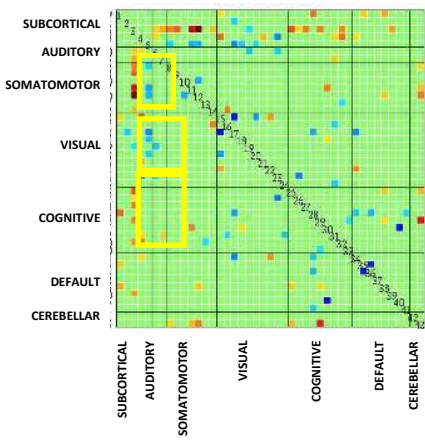
### State 2: MS



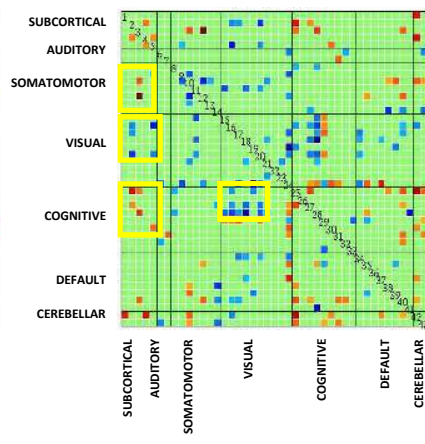
### State 3: MS



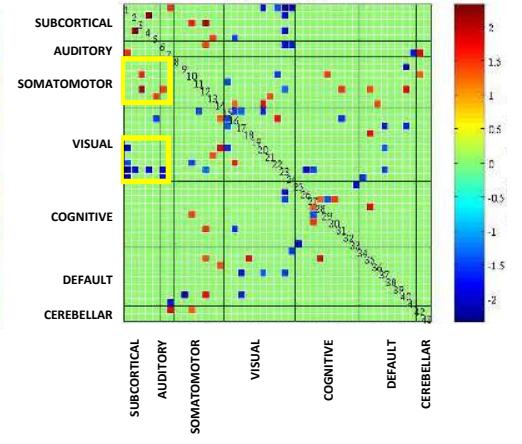
### State 1: MS vs HC



### State 2: MS vs HC



### State 3: MS vs HC





## **Supplementary material**

### **Methods**

Inclusion criteria. The inclusion criteria for this study were right-handedness and age between 20 and 65 years. In addition, patients had to have a diagnosis of relapsing-remitting multiple sclerosis (MS),<sup>1,2</sup> no relapse or corticosteroid treatment within the month prior to scanning, no therapy with muscle relaxants/psychoactive drugs, no history of substance abuse and no history of psychiatric conditions, including major depression.

MRI acquisition. Brain MRI was performed using a 3.0 Tesla system at each site. In all subjects, a resting state (RS) fMRI scan of the brain was collected using a T2\*-weighted echo planar imaging sequence with the following parameters: repetition time=3000 ms, echo time=35 ms, flip angle=90°, 30 contiguous, 4-mm thick axial slices, with a matrix size of 128×128 (apart from one center, where the matrix size was 64×64) and a field of view=240×240 mm. Total acquisition time of the RS fMRI sequence was 10 minutes. During RS fMRI scanning, subjects were instructed to keep their eyes closed, to remain motionless and not to think anything in particular. All subjects reported that they had not fallen asleep during scanning, according to a questionnaire delivered immediately after the MRI session.

RS fMRI pre-processing. RS fMRI data processing was performed using a combination of toolboxes, as previously suggested.<sup>3</sup> We performed rigid head motion correction using SPM12 (<http://www.fil.ion.ucl.ac.uk/spm/>) software. Then, RS fMRI data were despiked using the AFNI3s 3dDespike algorithm to mitigate the impact of outliers, and coregistered to the corresponding 3D T1-weighted scan. Using SPM12, data were subsequently warped to the Montreal Neurological Institute template and resampled to 2 mm<sup>3</sup> isotropic voxels. Instead of gaussian smoothing, we smoothed the data to 6 mm full width at half maximum using AFNI3s BlurToFWHM algorithm which performs smoothing using a conservative finite difference approximation to the diffusion equation. This approach is particularly useful in multicenter studies, since it has been shown to reduce scanner-specific variability in smoothness, providing “smoothness equivalence” to data across sites.<sup>4</sup>

Static functional network connectivity analysis. We computed static (or stationary) functional network connectivity (sFNC), defined as pairwise Pearson’s correlation between the whole independent component time courses, as a measure of average connectivity among different independent components during the

entire scan duration. Since correlation among brain networks is primarily shown to be driven by low frequency fluctuations in BOLD fMRI data,<sup>5</sup> we detrended (linear, quadratic and cubic) and band pass filtered the processed IC time courses between 0.01-0.15 Hz prior to computing FNC between independent components. Framewise displacement was included as a confounding covariate in the FNC calculation to mitigate the effect of micro head movements.<sup>6,7</sup> The mean sFNC matrix was computed over the main study groups.

Dynamic functional network connectivity analysis. Dynamic FNC (dFNC) between two independent component time courses was computed using a sliding window approach with a window size of  $22 \times$  repetition time (66 s) in steps of  $1 \times$  repetition time,<sup>3</sup> also including framewise displacement as a confounding covariate. A rectangular window of 22 time points convolved with Gaussian of  $\sigma=3 \times$  repetition time was used to obtain tapering along the edges. Covariance was estimated from the regularised inverse covariance matrix using the graphical LASSO framework, as described in details elsewhere.<sup>4</sup> We imposed an additional L1 norm constraint on the inverse covariance matrix to enforce sparsity, as described in detail elsewhere.<sup>8</sup> DFNC Pearson's correlation matrices computed for each subject were *r*-to-*z* Fisher transformed.

Validation analysis. DFNC techniques are relatively novel, and the probability of detecting FC dynamism in RS fMRI data acquired with a relatively long repetition time is still debated.<sup>9</sup> Therefore, to test if it was possible to capture FC dynamic properties in our data, we performed a validation analysis against simulated data, as suggested by Hindriks et al.<sup>9</sup> Briefly, we calculated two measures that quantify the degree of connectivity dynamism present in RS fMRI data: i) the standard deviation (SD) of sliding-window correlation time series; and ii) the  $\zeta$  metric proposed by Zalesky et al., 2014,<sup>10</sup> which essentially measures how long and large are excursions of correlation values, with respect to their median value, in sliding-windows correlation time series. These measures were calculated on real and simulated RS fMRI time series, built using the SimTB software (<http://mialab.mrn.org/software/simtb>).<sup>11</sup> Simulated data were constructed to have the same repetition time (i.e., 3000 ms), the same number of time points (i.e.,  $n=200$ ), the same number of independent components of interest (i.e.,  $n=43$ ) and the same scheme of positive/negative associations between independent component time courses as real RS fMRI data; however, they were forced to be static, since just one hard clustering connectivity State, constant across all time points, was allowed to be present.

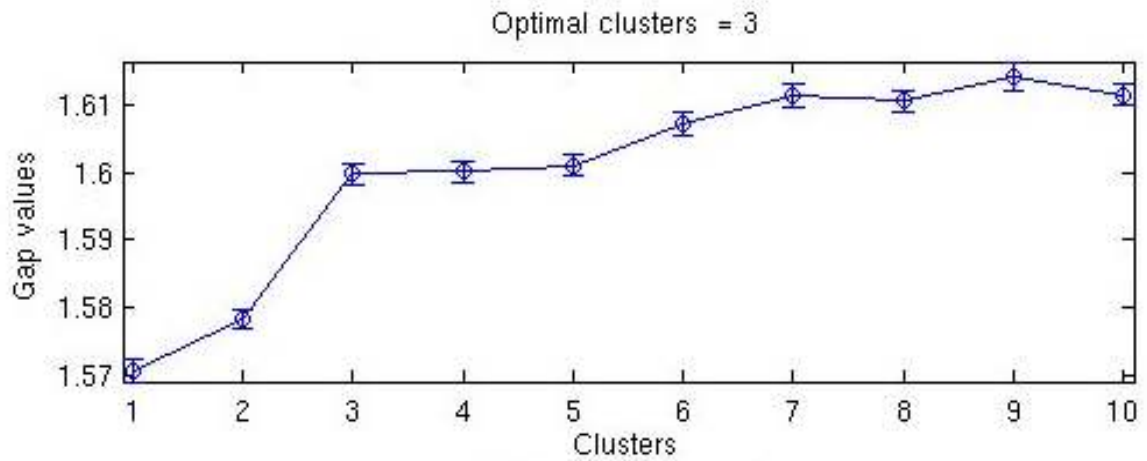
Two-sample t tests were used to compare SD and  $\zeta$  between real and simulated data, in order to test to what extent real data were significantly more dynamic than simulated data.

## **Results**

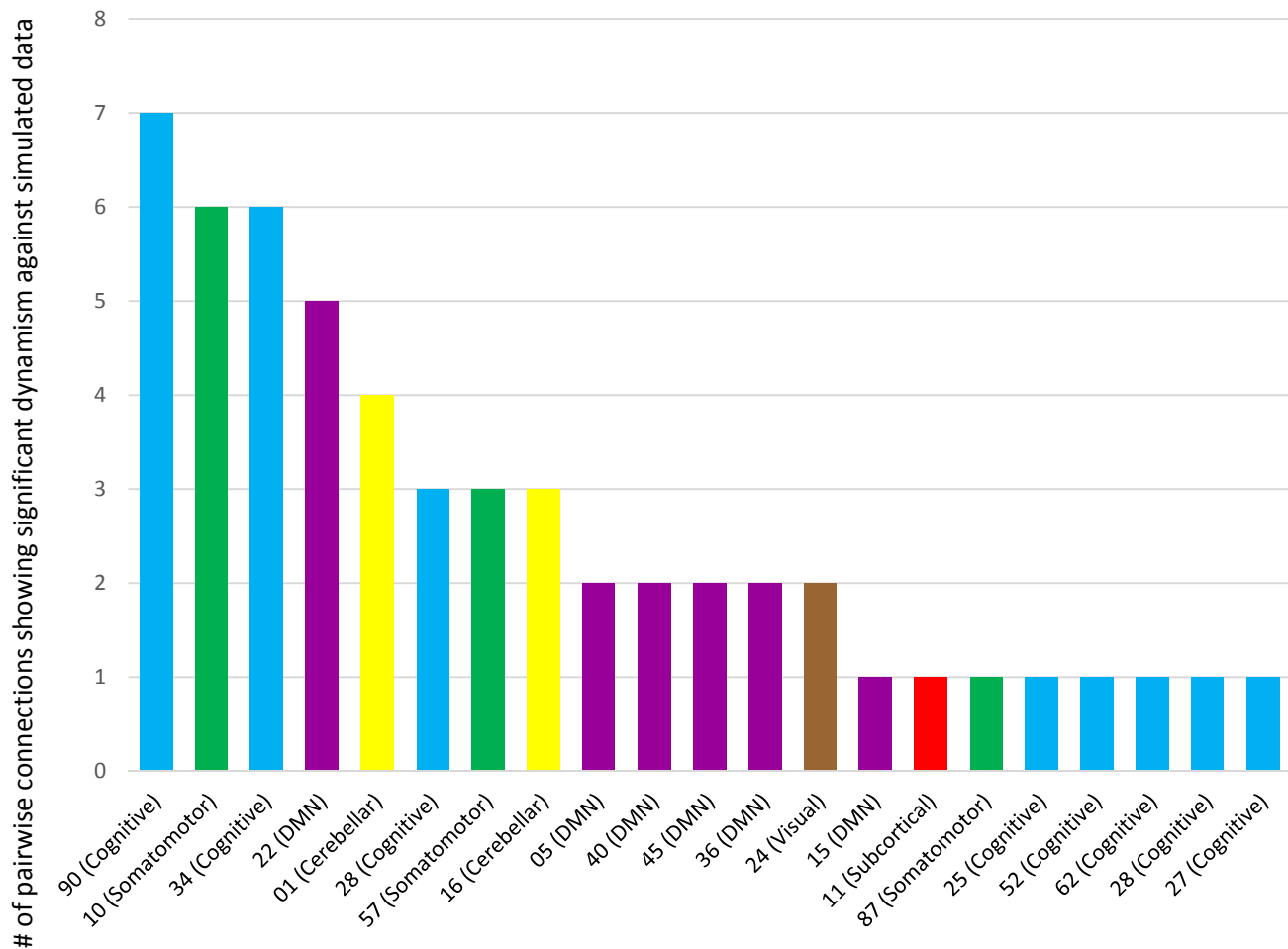
Validation analysis. SD did not differ between real and simulated sliding-window correlation time series ( $p=0.7$  to  $0.9$ ). However,  $\zeta$  was significantly different between real and simulated sliding-window correlation time series in 27 pairwise connections ( $p=0.03$  to  $0.05$ ). The most recurrent independent components involved in such connections, which are shown in Supplementary Figure 2, mainly belonged to the cognitive network (8 components) and to the default-mode network (6 components), while the somatomotor (3 components), cerebellar (2 components), subcortical (1 component) and visual networks (1 component) showed a relatively lower degree of dynamism. Interestingly, most of the independent components having significant dynamic properties against simulated data were showing dFNC differences between healthy controls and MS patients, as well as between cognitively impaired and preserved MS patients (Table 3).

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**Supplementary Figure 1.** Plot of the elbow criterion, produced by the dynamic functional network connectivity toolbox, suggesting  $n=3$  as optimal number of centroid states for the hard clustering dynamic functional network connectivity analysis. See text for further details.



**Supplementary Figure 2.** Bar charts representing the independent components (and the corresponding number of pairwise connections) having significant dynamic properties against simulated resting state fMRI data. Colors indicate the network associated to each independent component: blue=cognitive network; violet=default-mode network; green=somatomotor network; yellow=cerebellar network; brown=visual network; red=sub-cortical network.