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Ventral tegmental area disconnection contributes two years early to correctly classify patients converted to Alzheimer's disease: implications for treatment.

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Running head: Dopaminergic dysfunction in AD progression.

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ABSTRACT

Background: Recent cross-sectional studies highlighted the loss of dopaminergic neurons in the ventral tegmental area (VTA) as an early pathophysiological event in Alzheimer's disease (AD). **Objective:** In this study, we longitudinally investigated by resting-state fMRI (RS-fMRI) a cohort of patients with mild cognitive impairment (MCI) due to AD to evaluate the impact of VTA disconnection in predicting the conversion to AD. **Methods:** a cohort of 35 patients with MCI due to AD were recruited and followed-up for 24 months. They underwent cognitive evaluation and RS-fMRI to assess VTA connectivity at baseline and at follow-up. **Results:** At 24-month follow-up, 16 out of 35 patients converted to AD. Although converters and non-converters to AD did not differ in demographic and behavioral characteristics at baseline, the first group showed a significant reduction of VTA-driven connectivity in the posterior cingulate and precentral cortex. This pattern of additional disconnection in MCI-converters compared to non-converters remained substantially unchanged at 24-month follow-up. **Discussion**: This study reinforces the hypothesis of an early contribution of dopaminergic dysfunction to AD evolution by targeting the default-mode network. These results have potential implications for AD staging and prognosis and support new opportunities for therapeutic interventions to slow down disease progression.

Keywords: VTA, Resting-state MRI, functional connectivity, amnestic mild cognitive impairment, Alzheimer's Disease, dopaminergic system.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia [1] with a gradual clinical onset and it is characterised by a long phase of subclinical symptoms. Research in the last thirty years contributed in identifying a prodromal clinical condition, the so-called Mild Cognitive Impairment (MCI), which is associated with a higher risk for developing dementia [2]. MCI is characterised by the occurrence of various cognitive symptoms with memory impairment being the most common clinical presentation. The amnestic form of MCI (a-MCI) is associated with an increased risk of conversion to typical AD, while the non-amnestic forms, which are dominated by cognitive symptoms other than memory deficits, are more likely to convert to non-AD forms of dementia [3]. Even within the a-MCI classification, some people convert to AD in a short time while other patients remain stable for longer intervals, and providing patients and their carers with informative prognosis is extremely challenging. Previous studies based on different types of biomarkers have identified some genetic [4, 5], cognitive [6, 7], environmental [8-12], and neuroimaging predictors [13-16] of conversion to dementia. Degeneration in the cholinergic system, whose neurons are located in the basal forebrain and project to the hippocampus and frontal and cingulate cortices, is associated with memory dysfunctions and behavioural symptoms in patients with AD [17-19]. Cholinesterase inhibitors are currently used to treat patients with AD despite their moderate effect on clinical symptoms [20]. More recently, clinical and neurophysiological evidence revealed that dopaminergic dysfunction is also implicated in AD pathophysiology [21-29]. In a transgenic model of AD, Nobili et al. [30] demonstrated a progressive degeneration of neurons from the ventral tegmental area (VTA) (i.e., a dopaminergic-rich structure located in the midbrain) [31], which correlated behaviourally with reward and memory functions [30, 32]. Notably, the meso-cortico-limbic system, which is strictly involved in reward [33, 35], as well as in executive and memory functions [30, 36, 37] stems from dopamine neurons located in the VTA midbrain region. Importantly, this degeneration of dopaminergic-rich neurons was found to predate the deposition of beta-amyloid plaques and accumulation of brain atrophy in VTA projecting areas [30]. Structural

and functional alterations of VTA's neurons have been described also in humans. VTA size and its connectivity with the hippocampus was associated with measures of episodic memory in patients with AD and a-MCI [36] and in the pre-symptomatic older adults [37]. As detailed below, specific changes in functional connectivity were also reported between VTA and some critical nodes of the default mode network in patient with AD and a-MCI [38]. Taken altogether, these studies indicate a clear pathophysiological role of dopaminergic degeneration in AD pathophysiology with a clinical focus not only on cognitive but also behavioural symptoms.

Resting state functional MRI (rs-fMRI) is a robust approach that provides information on spontaneous fluctuations of brain activity *in vivo* [39, 40]. Based on this technique, functional connectivity maps of the brain can be estimated *in vivo* to assess changes in connectivity within and between networks. In previous rs-fMRI studies in AD, reductions of connectivity have been regarded as a surrogate measure of neuronal dysfunction/degeneration [9, 41]. In a recent work, Serra and co-Authors [38] demonstrated a pattern of disconnection between VTA and its projection areas that resembled the anatomical distribution of the default-mode network (DMN) in patients with AD and MCI. These changes were already present at the stage of MCI but more pronounced in AD patients, and were associated with the presence and severity of neuropsychiatric symptoms [38], suggesting a specific contribution of VTA dysfunction to DMN disruption across AD evolution. This hypothesis opens potentially relevant therapeutic implications, but needs to be confirmed in longitudinal studies.

Here, we aimed to test the hypothesis that VTA driven disconnection in a-MCI patients is associated with a higher risk of conversion to AD. A secondary aim was to assess whether such disconnection becomes progressively more severe over time. To this purpose we recruited a cohort of consecutive patients responding to the diagnosis of a-MCI due to AD [42], who were scanned twice, at baseline and 24-month follow-up, and clinically reclassified in MCI-converters and non-converters to AD.

2. Methods

2.1 Participants

A cohort of 45 participants were enrolled in the study. They were consecutively recruited over 24 months from the Specialist Dementia Clinic of Catholic University of Rome and from Santa Lucia Foundation, IRCCS, Rome. To be included in the study, they had to fulfil current diagnostic criteria for a-MCI due to AD [42] within the clinical categories of a-MCI single domain (a-MCI-SD), when presenting with an isolated deficit of long-term memory, or a-MCI multiple domain (a-MCI-MD), when presenting with long-term memory impairment associated to additional deficits in cognitive functions other than memory [3]. In either case, the essential inclusion criterion was the presence of subjective memory impairment at clinical onset, corroborated by an informed assistant. As detailed below, they had to perform below the cut-off of normality at least in one of the administered tests for episodic memory. In addition, patients classified as a-MCI-MD had to report pathological scores in at least one additional cognitive domain different from memory. By definition, all MCI patients had not to fulfil the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criteria for the diagnosis of major neurocognitive disorders [43]. Their MMSE score [44, 45] had to fall within the cut-off of normality ($\geq$23.8) and their Clinical Dementia Rating score (CDR) [46] had to be equal to 0.5. In order to assess the functional impairment of patients we computed the CDR sum of boxes (CDR-SoB) [47] as sum of sub-scores provided by six different domains (i.e., memory, orientation, judgment and problem solving, community affairs, home and hobbies and personal care) of CDR. The CDR-SoB compared to the total CDR score, provides different categories of dementia severity (CDR-SoB score 0= normality; 0.5-2.0=questionable impairment; 2.5-4.0= very mild dementia; 4.5-9.0= mild dementia; 9.5-15.5= moderate dementia; 16.0-18.0= severe dementia) [48]. Moreover, we assessed also the basic and the instrumental activities of daily living by using the basic Activities of Daily Living (ADL) [49] and the Instrumental Activities of Daily Living (IADL) [50], two specific questionnaires devoted to assess

self-care and the independence. All patients had to report scores ≥ 1 on the medial temporal lobe atrophy scale (MTA) [51] to fulfil the criterion of a-MCI-due to AD [42]. Patients with a Hachinski score [52] > 4 were excluded to minimize the risk of enrolling mixed forms of dementia. Major systemic, psychiatric, and other neurological illnesses (with a special attention to Parkinson disease and parkinsonisms) were carefully investigated and excluded in all patients. Finally, patients had to be right-handed as assessed by the Edinburgh Handedness Inventory [53], to reduce sample's heterogeneity on MRI data.

At baseline, patients underwent a comprehensive neuropsychological assessment and MRI scanning, separated by a maximum interval of 72 hours. Then, they were longitudinally followed-up for 24 months with clinical reassessment at six months intervals. Each follow-up visit included neuropsychological evaluation and clinical evaluation on whose basis they were reclassified in MCI Converters and non-Converters to AD, according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [54]. Twenty-four months from baseline, all patients underwent a second MRI scan using the same scanner and acquisition protocol as at baseline. This study was approved by the Ethical Committee of Santa Lucia Foundation, and written informed consent was obtained from all participants before study initiation. All procedures performed in this study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2 Neuropsychological assessment

At baseline and 24 month follow-up, all participants underwent an extensive neuropsychological battery covering all cognitive domains: a) verbal episodic long-term memory: 15-Word List (Immediate, 15-min Delayed recall and recognition) [55]; Short Story Test (Immediate and 20-min Delayed recall) [56]; b) visuo-spatial long-term memory: Complex Rey's Figure (Immediate and 20-min Delayed recall) [56]; c) short-term and working memory: Digit span

(forward and backward) and the Corsi Block Tapping task (forward and backward) [57]; d) executive functions: Phonological Word Fluency [55] and Modified Card Sorting Test [58]; e) language: Naming objects subtest of the BADA (‘‘Batteria per l’Analisi dei Deficit Afasici’’, Italian for ‘‘Battery for the analysis of aphasic deficits’’) [59]; f) Reasoning: Raven’s Coloured Progressive Matrices [55]; g) constructional praxis: copy of simple drawings with and without landmarks [55] and copy of Complex Rey’s Figure [56]; h) general cognitive efficiency: Mini Mental State Examination (MMSE) [44, 45]. Neuropsychological scores were adjusted for age and education, as reported in the corresponding references.

By using SPSS-20.0 (https://www.ibm.com/it-it/analytics/spss-statistics-software) twenty one-way ANOVAs were used to assess between-group differences (Converters vs. Non-Converters) in neuropsychological performances at baseline and 24-month follow-up. To avoid the type-I error Bonferroni’s correction was applied (p value threshold $\alpha = 0.05/20 = 0.002$).

Six one-way ANOVAs were used to compare patients in the CDR-SoB and in the activities of daily living at baseline and follow-up (Bonferroni correction p value threshold $\alpha = 0.05/6 = 0.008$) (Please see Table 1).

2.3 MRI acquisition

At both occasions, baseline and 24-month follow-up, all imaging was obtained in a single session, using a head only 3.0T MRI scanner (Siemens Magnetom Allegra, Siemens Medical Solutions, Erlangen, Germany). The MRI acquisition protocol included: (a) a dual-echo turbo spin echo (TSE) (repetition time (TR)=6190 ms, echo times (TE)=12/109 ms, echo train length (ETL)=5; matrix=256×192; FOV=230×172.5 mm2; 48 contiguous 3 mm thick slices); (b) a fast fluid attenuated inversion recovery (FLAIR) (TR=8170 ms, TE=96 ms, inversion time (TI)=2100 ms; ETL=13; with same FOV, matrix and number of slices as TSE) (c) 3D Modified Driven Equilibrium Fourier Transform (MDEFT) scan (TR=1338 ms, TE=2.4 ms, Matrix=256x224, n. slices=176, thickness=1 mm); (d) a T2*-weighted echo-planar imaging (EPI) sensitized to BOLD

contrast (TR=2080 ms, TE=30 ms, 32 axial slices parallel to AC-PC line, matrix=64×64, pixel size=3×3 mm^2, slice thickness=2.5 mm, flip angle=70°) for resting-state fMRI (total number of volumes=220). During this acquisition, subjects were instructed to keep their eyes closed, not to think of anything in particular, and not to fall asleep.

2.4 Medial temporal lobe atrophy

The Medial Temporal lobe Atrophy (MTA) scale [51] was employed to assess on volumetric images (MDEFT) the presence and severity of atrophy in each patient. This scale provides a rating score from 0 to 4, with scores≥1.5 [60] indicating the presence of significant atrophy. For each subject, we averaged the scores from the right and left hemisphere to obtain a single measure of medial-temporal lobe atrophy. As mentioned above, an MTA score equal to or higher than 1 was required to fulfil the criterion of MCI due to AD. When performing retrospective comparisons between MCI Converters and non-Converters we used a one-way ANOVA model.

2.5 Voxel-based morphometry analysis on the baseline data

T1-weighted volumes (MDEFT) obtained at baseline visit were pre-processed using the VBM protocol [61, 62] implemented in SPM8 (http://www.fil.ion.ucl.ac.uk/spm/), which consists of an interactive combination of segmentations and normalisations to produce a grey matter (GM) probability map [61, 62] in standard space (Montreal Neurological Institute, or MNI coordinates) for each subject. In order to compensate for compression or expansion that could occur during the warping of images to match the template, GM maps were “modulated” by multiplying the intensity of each voxel in the final images by the Jacobian determinant of the transformation, which corresponded to its relative volume before and after warping [61, 62]. GM, white matter (WM) and cerebrospinal fluid (CSF) volumes were computed from these probabilistic images for each subject. These images were also used to obtain participant-specific white matter and cerebrospinal fluid (CSF) masks. All GM maps were then smoothed using a 12-mm FWHM Gaussian kernel and compared voxel-wise between MCI-Converters and Non-Converters using an independent sample

T-test in SPM8. Intracranial volumes (computed as GM+WM+CSF) was used as covariate of no interest. Statistical significance was set at $p < 0.05$ FWE-corrected at cluster level ($p=0.001$ was used as threshold to cluster-forming).

2.6 Image Analysis of resting-state fMRI data

As previously described [35, 38] images were pre-processed for resting-state fMRI using Statistical Parametric Mapping version 8 (SPM8, http://www.fil.ion.ucl.ac.uk/spm/), and in-house Matlab scripts [9]. The MDEFT collected at follow-up were segmented as described in the VBM section to obtain time point-specific GM, WM and CSF masks.

The focus on VTA was driven by our previous investigation [38], in which we assessed both, VTA and locus coeruleus connectivity, but only found evidence of the former in AD. VTA is a relatively small structure whose identification was obtained following the pipeline described by Bär et al [63]. For each participant, the first four volumes of the fMRI series were discarded to allow for T1 equilibration effects. The pre-processing steps included correction for head motion, compensation for slice-dependent time shifts, and normalization to the EPI template in MNI coordinates provided with SPM8. For each data set, motion correction was checked to ensure that the maximum absolute shift did not exceed 1.5 mm and the maximum absolute rotation did not exceed 1°. In addition, we checked that the relative framewise displacement did not exceed 2.5 mm. Datasets not fulfilling these criteria were removed from the analysis. The current study design includes comparisons between patient groups defined retrospectively on the basis of clinical assessments, namely MCI-Converters and Non-Converters. To minimise the risk for our results being affected by differing degrees of motion for the 2 participant groups (MCI Converters and Non-converters), we computed and compared between them the global correlation (GCORR) [64], and the average mean displacement (root mean square or RMS of the 6 realignment parameters).

Statistical comparisons of these parameters were tested using two-way ANOVAs 2x2 (Group [MCI-Converters vs. Non-Converters] by Time [Baseline vs. Follow-up].

After fMRI data pre-processing, the signal in every voxel was regressed against the average WM and CSF signals, as well as against the 6 realignment parameters. Then, all images were filtered by a phase-insensitive band-pass filter (pass band 0.01–0.08 Hz) to reduce the effect of low-frequency drift and high-frequency physiological noise. We refer to these datasets as unsmoothed corrected data. These data were then smoothed via filtering with a 3D Gaussian kernel with 10 mm^3 full width at half maximum.

2.7 Seed-Based Analyses

As previously described [35, 38] standard space seed masks of the left and right VTA were produced using the Harvard Ascending Arousal Network Atlas in MGH152_1mm Space (https://www.martinos.org) [65].

The mean time course within each seed region was extracted (from the unsmoothed corrected data) for every participant (averaged for left and right side). This was done to minimize the partial volume contamination from neighboring nuclei. The smoothed data were then regressed voxel-wise against these time courses in a first-level SPM8 analysis [66]. The resulting beta images were taken to the second level for a random-effect group analysis. At second level, we performed a repeated measure ANOVA (implemented as a flexible factorial model in SPM8) investigating the main effect of group, the main effect of Time and their interaction. ANOVA models were used, as post-hoc analyses, to assess between-group differences (MCI-Converters vs. Non-Converters) in VTA functional connectivity, at baseline and follow-up separately. In all analyses, grey matter volumes, and the time-point of conversion, time (expressed as days) from baseline to the first clinical follow-up at which they had converted for (MCI-Converters) and the last follow-up for Non-Converters, and years of formal education were entered as covariates of no interest. As shown previously [67], education is one of the most reliable proxy measures of cognitive reserve, which modulates the

impact of AD pathology [9, 11]. To investigate the direct and indirect association between the time-point of conversion and the VTA-driven connectivity during follow-up in MCI-Converters and Non-Converters patients, we modelled an additional analysis by using the time-point of conversion (as defined above) as variable of interest.

In all comparisons, statistical significance was set at $p < 0.05$ FWE-corrected at cluster level ($p=0.001$ was used as threshold to cluster-forming).

Moreover, VTA-driven connectivity from baseline data was extracted in each subject from the peak area of difference between MCI-Converters and Non-Converters, and used for discriminant analyses (see below).

2.8 Discriminant analysis

By using SPSS-20.0 (https://www.ibm.com/it-it/analytics/spss-statistics-software) five multiple discriminant analyses (step-wise approach) were applied to the entire cohort of a-MCI patients. Individual clinical follow-up outcomes (MCI-Converters/Non-Converters) were always entered as grouping variable. In the first analysis (Model 1), VTA-driven connectivity changes in the peak area of group difference at baseline were used as predictors together with patients' MTA scores, MMSE scores, age and years of formal education. In the next steps of the discriminant analysis, the following predictors were progressively discharged: age and years of formal education (Model 2), MMSE scores (Model 3). In Model 4 and 5, the impact of the remaining 2 predictors (i.e., VTA-connectivity and MTA) were tested.

3. Results

3.1 Demographic and clinical characteristics

From the initial cohort of 45 MCI patients, 5 were excluded because their hippocampal atrophy did not support a diagnosis of MCI due to AD according with Albert et al. [42]. Two patients did not complete their MRI scan due to claustrophobia. Finally, 3 patients were excluded due to movement artifacts on at least 1 of the 2 MRI scans. Therefore, a final cohort of 35 MCI patients [mean (SD)

age: 70.5 (8.2) years; Male/Female ratio:17/18; mean (SD) years of formal education: 10.2 (4.5)] were included in the analyses.

At baseline no patient was under acetylcholinesterase inhibitors, 3 out of 35 (8.5%) patients were under benzodiazepine and 16 out of 35 (45.7 %) were under antidepressant.

At baseline, 21 patients showed an isolated deficit of long-term memory (aMCI-SD), while the remaining 14 showed a multidomain impairment characterised by additional cognitive deficits other than episodic memory (a-MCI-MD). In addition to memory impairment, a-MCI-MD showed executive dysfunctions in 78.5% (n= 11), and constructional apraxia in 21.4% of cases (n= 3).

According to their clinical follow-up patients were divided in MCI-Converters (N=16; 46%) and Non-Converters (N=19; 54%) to AD. As shown in Figure 1, the majority of patients (9 out 16) converted after 12 months, four further patients converted after 18 months; and finally, the remaining 3 patients converted after 24 months.

As shown in Table 1 there were no significant differences, at baseline, between MCI-Converters and Non-Converters in age ($F_{1,33}$=0.691, p=0.412), years of formal education ($F_{1,33}$=1.593, p=0.216), MMSE ($F_{1,33}$=1.448, p=0.237) and MTA scores ($F_{1,33}$=2.228, p=0.145). Moreover, there was no significant difference between the 2 groups (MCI-Converters and Non-Converters) in single and multiple domain distribution (Chi-square=1.23, d.f.=1, p=0.267). As expected, MMSE scores differed significantly between MCI-Converters and Non-Converters at 24-month follow-up ($F_{1,33}$=20.424, p<0.001). As shown in Table 1 at baseline visit we did not find significant differences in the functional activities between MCI-Converters and Non-Converters in each considered test. As expected, at follow-up visit MCI-Converters patients compared to Non-Converters showed significant worsening in CDR-SoB, ADL and IADL.

3.2 Neuropsychological assessment

As shown in Table 2, at baseline, there were no significant differences in cognitive performance between patients who converted to AD and those who did not during follow-up.

Table 3 summarises the cognitive performance of MCI-Converters and Non-Converters at 24-month follow-up. MCI-Converters performed significantly worse at the Copy of Complex Rey's Figure test. No other significant differences were found between groups.

3.3 Voxel-based morphometry analysis on the baseline data

We did not find significant VBM differences between MCI-Converters and Non-Converters at baseline.

3.4 VTA-driven functional connectivity in MCI Converters and Non-Converters.

There were no significant differences between groups in the considered motion parameters: GCORR (Group effect: $F_{2,32}$=0.02, p=0.891; Time effect: $F_{2,32}$=0.16, p=0.694); mean motion parameters RMS (Group effect: $F_{2,32}$=1.16, p=0.285; Time effect: $F_{2,32}$=0.72, p=0.399).

As shown in Figure 2 and Table 4 (panel 1), the ANOVA for repeated measures revealed a significant main effect of group, with MCI-Converters showing reduced VTA-driven connectivity within the right posterior cingulate cortex; no significant main effect of Time or interactions were detected.

Between group differences were further investigated at baseline and follow-up separately. As illustrated in Figure 3 panel A and summarised in Table 4 (panel 2A), MCI-Converters showed at baseline reduced functional connectivity between VTA and the posterior cingulate gyrus bilaterally (BA31) (PCC), and the precentral gyrus (BA4), when compared to Non-Converters. When comparing follow-up scans from the 2 groups, MCI-Converters revealed again reduced VTA driven connectivity in the precentral gyrus (BA4), but also in the parietal operculum and insula (BA13) in the right hemisphere (see Figure 3 panel B, Table 4 panel 2B).

Additionally, when considering the potential association between VTA-driven connectivity and the time-point of conversion we found significant direct association in the right precentral gyrus (BA4) in MCI-Converters group (see Figure 4 and Table 4, panel 3). No other significant association was found.

3.5 Discriminant analysis

The peak difference of VTA-driven functional connectivity used for the discriminant analysis corresponded to the PCC at the following MNI coordinates: x=-13; y=-9; z=35. As shown in table 5, Model 3, which included PCC patients' connectivity and MTA scores, showed the best sensitivity, specificity and accuracy (all parameters above 68%) for a correct classification of MCI patients in Converters and Non-Converters.

4. Discussion

Cholinergic dysfunction is traditionally regarded as one of the most critical processes in AD pathophysiology [17-19]. Recent evidence has shown an involvement of other neurotransmitter pathways including the dopaminergic system occurring in patients with probable AD since their early clinical stage [27].

Notably, the dysfunction of dopaminergic transmission has been hypothesized as a new player in the pathophysiology of AD [21-29, 68-70]. Post mortem studies revealed marked loss of DA receptors in the temporal and frontal lobes of AD brains [21, 23, 24], regions classically involved in cognitive decline. These dopamine D2/D3 receptors play an important role in the reciprocal activity of large groups of neurons in the high-order association cortical regions, and may promote the cognitive and behavioral impairments observed in AD [22]. D2 receptor binding was significantly reduced in the striatum of AD patients, even in the absence of overt extra-pyramidal symptoms [71]. Moreover,

neurophysiological studies showed remarkable modulatory effects of dopaminergic drugs on cortical synaptic transmission and cortical plasticity in patients with AD [25, 28, 72, 73]. Indeed, a recent randomized clinical trial confirmed the potential of dopaminergic therapy in patients with mild-to-moderate AD, highlighting the possibility to improve cognitive functions highly related to prefrontal lobe activity [74].

Degeneration of dopamine-rich neurons, which are mainly located in the VTA, has been shown to account for cognitive and behavioural symptoms in AD and MCI [36, 38]. VTA disconnection was previously observed within anatomical areas belonging to the DMN, which is typically disrupted in AD brains [38]. Part of this DMN disconnection has been consistently ascribed to deafferentation of the posterior cingulate cortex as a consequence of medial temporal lobe atrophy [75]. However, other contributions, including VTA disconnection, may play a role in determining DMN disruption, which can be regarded as a common pathophysiological mechanism accounting for the progressive accumulation of cognitive disabilities [76]. To further explore this hypothesis, in the current work, we assessed longitudinally the pattern of VTA disconnection as a surrogate marker of dopaminergic degeneration in a group of patients with a-MCI due to AD at intermediate likelihood, which is widely considered as a prodromal stage of AD [77]. To be eligible for the study, patients had to show the "cognitive core" of typical AD (i.e., long-term episodic memory deficits in isolation or in combination with additional cognitive deficits) alongside hippocampal atrophy, which was documented trough the MTA score. At 24-month follow-up, patients were re-classified as clinically stable (MCI Non-Converters) or converters to AD (MCI-Converters). At baseline, there were no between-group differences on neuropsychological testing and on tests assessing activities of daily living between MCI-Converters and Non-Converters, indicating that they belonged to a homogeneous cohort of subjects. Education (known as one of the most reliable proxy measures of cognitive reserve) was not significantly different between MCI-Converters and Non-Converters, thus making its potential role of resilience to AD pathology unlikely to account for the observed fMRI changes. Additionally, education was entered in all fMRI analyses as covariate of no interest. Interestingly, VBM analysis did not reveal

significant differences at baseline in the brain volumes between MCI-Converters and Non-Converters, reinforcing the observation that the two groups of patients were comparable both in cognitive profile and in the brain structure.

When comparing VTA driven connectivity maps we observed a significant main effect of Group, but not main effect of Time or interactions. This result indicated that MCI-Converters showed reduced functional connectivity between VTA and the right posterior cortex compared to Non-Converters both at baseline and at follow-up visit. This finding was confirmed by post-hoc analyses on group. Indeed, between MCI-Converters and Non-Converters at baseline, the former group showed a significant reduction of connectivity in the posterior cingulate cortex (BA31) bilaterally, and in the right precentral gyrus (BA4). Remarkably, within the PCC, baseline VTA-driven connectivity was able to discriminate, at a single subject level, between MCI Converters and Non-Converters to AD in short time. The posterior cingulate cortex is one of the critical nodes of the DMN, and is strictly implicated in memory functions [78]. Independently from VTA contribution, brain disconnection within the PCC was previously demonstrated in patients with MCI [15, 41, 75]. A previous study showed that functional disconnection can occur in the absence of grey matter loss in the posterior cingulate cortex of MCI patients but not in those with AD [41]. Within the limitations of a cross-sectional design, this result was interpreted as supporting the hypothesis that the local neuronal loss seen at the stage of AD may be partially due to disconnection mechanisms for which deafferentation from the medial temporal lobes is one of the major contributors. However, this is unlikely to explain the current findings, as MTA was similar between MCI-Converters and Non-Converters in our sample. This further supports the hypothesis that the difference in connectivity we observed since baseline between the two groups is genuinely due to VTA disconnection, and possibly contributes to a faster conversion to AD. This is particularly relevant for possible interventions that may be considered to delay AD progression in patients at early clinical stage. Both repetitive transcranial magnetic stimulation (TMS) applied to the posterior cingulate cortex [79] and dopaminergic therapy [74] have been shown to independently improve cognitive disabilities in patients with early AD. Future investigation are needed to challenge

the combination of pharmacological and non-pharmacological interventions, such as invasive and non-invasive brain stimulation, in modulating AD progression. VTA-driven disconnection in MCI-Converters compared to Non-Converters included also areas that do not belong to the DMN, such as the precentral gyrus (BA4), which is known to receive dopaminergic projections from VTA [80]. In rodents, these dopaminergic projections have been shown to be implicated in motor learning [81] and promoting phenomena of brain plasticity [82]. In humans, dopaminergic fibres were identified in the primary (BA4) and secondary motor cortex (BA6), as well as in somatosensory areas [83-84]. These projection fibres are carried by the mesocortical and mesolimbic pathways that originate from VTA [86]. The mesocortical and mesolimbic pathways are involved in cognition and emotion processing [86, 87], and their dysfunction might contribute to the complex cognitive and behavioural symptoms of AD [86]. From a neuropsychological perspective, the mesocortical pathway projecting to BA4 is mainly implicated in cognitive processes such as motor imagery. Motor imagery is part of the motor system but involves mainly cognitive functions, and has been recently associated with apraxia in stroke patients [89]. Patients with AD typically suffer from constructional apraxia [90, 91], which is defined as the inability to correctly compose objects or draw figures [92]. At follow-up, this cohort of MCI-Converters performed worse than Non-Converters in the domain of constructional apraxia. One might argue that disconnection between VTA and the precentral gyrus at baseline anticipates the occurrence of local atrophy that eventually results in overt apraxia at follow-up.

When considering VTA-driven connectivity at 24-month follow-up, MCI-Converters compared to Non-Converters showed a further extension of disconnection in the precentral gyrus (BA4), but also in the parietal operculum and insula (BA13). Additionally, we found among the MCI-Converters that those converted later showed higher VTA-driven connectivity in the right precentral gyrus (BA4) than patients that converted earlier. This supports the hypothesis that the involvement of the precentral gyrus might parallel the accumulation of cognitive disabilities needed for a full conversion to AD. Interestingly, we found no group-by-time significant interaction, suggesting that VTA-driven disconnection does not progress faster in the converter group.

In the discriminant analysis, the hippocampal atrophy showed the highest sensitivity but the lowest specificity in differentiating between MCI-Converters and Non-Converters. Conversely, VTA-driven disconnection within the PCC (together with the hippocampal atrophy) showed the highest ability to predict the conversion to AD 24 months in advance. These findings reinforce the idea that a combination of atrophy and disconnection is critical for AD conversion, with the latter mechanism at least partially driven by VTA disconnection. Further investigations could be carried out looking at structural connectivity, either group-wise via covariance analysis of the grey matter, or based on diffusion tractography approaches.

The main limitations of the current work are the relatively small sample size and the short follow-up interval. Further studies on larger populations are therefore needed to confirm our current findings and clarify their potential application to a single subject use in clinical settings. Additionally, strategies to improve the VTA functionality, such as TMS, could disclose whether interventions resulting in more preserved VTA connectivity might be associated with a delay in the MCI to AD conversion. Other methodological limitations include the use of a simplistic approach to correct for motion. More sophisticated approaches have been proposed [93], although they tend to work less well in clinical populations than in healthy controls [94]. In this particular study we have tried to maintain the same methodological approach described in our previous cross-sectional study on VTA connectivity [38], for consistency. In order to ensure that our results were not driven by differences in motion between groups of time points, we have compared the global correlation and the average mean displacement.

In conclusion, we demonstrated here, for the first time longitudinally, an early contribution of dopaminergic dysfunction in increasing the risk of conversion from a-MCI to AD in short time. The neurobiological substrate of this increased risk is likely related to a release of connectivity between VTA and DMN. Our findings are consistent with recent research that contributed to shed light on the strategic role of dopamine in AD pathophysiology [27, 66, 69-70].

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Figure legend

Figure 1. Time-point of conversion

The figure illustrates the distribution of MCI-Converters patients according with their time-point of conversion. X-axe reports each MCI-Converters, and Y-axe reports the different time-points. See text for further details.

Figure 3. Main effect of Group in VTA-driven connectivity

The figure showed the reduction on functional connectivity between VTA and the right posterior cingulate cortex as main effect of Group. Results are Family Wise Error (FWE) corrected at cluster level ($p < 0.05$) and overlaid onto the Ch2bet template using mricron (http://people.cas.sc.edu/rorden/mricron/).

Abbreviations: L=Left; R=Right

See text for further details.

Figure 3. Reductions in VTA connectivity between MCI Converters and Non-Converters.

The figure illustrates VTA-driven functional connectivity changes observed at baseline (panel A, in red) and 24-month follow-up (panel B, in cyan). At baseline MCI-Converters compared to non-Converters showed reduced connectivity between VTA and the posterior cingulate cortex and the right precentral gyrus. At follow-up, additional reduction of connectivity was observed in the right precentral gyrus, parietal operculum and insula. Results are Family Wise Error (FWE) corrected at cluster level ($p < 0.05$) and overlaid onto the Ch2bet template using mricron (http://people.cas.sc.edu/rorden/mricron/).

Abbreviations: L=Left; R=Right

See text for further details.

Figure 4. Association between VTA-driven connectivity changes and time-point of conversion in MCI-Converters patients

The figure shows in MCI-Converters group the direct association between VTA-driven connectivity and the time-point of conversion in the right precentral gyrus. Among the group of MCI-Converters the patients that converted later showed higher VTA-driven connectivity in the right precentral gyrus than patients that converted earlier. Results are Family Wise Error (FWE) corrected at cluster level ($p < 0.05$) and overlaid onto the Ch2bet template using mricron (http://people.cas.sc.edu/rorden/mricron/).

Abbreviations: L=Left; R=Right

See text for further details.

Table 1. Principal demographic and clinical characteristics of studied patients.

	MCI-Converters (N=16)	**Non-Converters (N=19)**	**Statistic**	**p-value**
Mean (SD) Age [years][a]	71.8 (6.8)	69.4 (9.3)	$F_{1,33}$=0.69	0.412
Sex (F/M)	12/4	6/11*		
Mean (SD) Education [years] [a]	9.1 (4.1)	11.0 (4.7)	$F_{1,33}$=1.59	0.216
a-MCI-SD/a-MCI-MD[b]	8/8	13/6	χ^2_1=1.23	0.267
Mean (SD) MMSE score (baseline) [a]	26.2 (1.6)	26.9 (1.7)	$F_{1,33}$=1.44	0.237
Mean (SD) MMSE score (24-month follow-up) [a]	23.0 (4.5)	28.2 (1.7)*	$F_{1,33}$=20.42	<0.001
Mean (SD) MTA score (baseline) [a]	2.2 (0.83)	1.8 (0.9)	$F_{1,33}$=2.22	0.145
Mean (SD) CDR-SoB (baseline)	1.4 (0.8)	1.0 (0.6)	$F_{1,33}$=2.22	0.146
Mean (SD) CDR-SoB (24-month follow-up)	4.0 (1.1)	1.4 (1.1)	$F_{1,33}$=45.1	<0.001
Mean (SD) ADL (n° of preserved activities) [range 0-6] (baseline)	5.8 (0.2)	6.0 (0.0)	$F_{1,33}$=2.55	0.119
Mean (SD) ADL (n° of preserved activities) [range 0-6] (24-month follow-up)	5.6 (0.4)	6.0 (0.0)	$F_{1,33}$=8.14	0.007
Mean (SD) IADL (n° of preserved activities) [range 0-8] (baseline)	7.1 (1.0)	7.4 (1.0)	$F_{1,33}$=1.01	0.310
Mean (SD) IADL (n° of preserved activities) [range 0-8] (24-month follow-up)	6.7 (1.2)	8.0 (0.0)	$F_{1,33}$=19.5	<0.001

* MCI Converters vs. non-Converters: p-value<0.05; [a]One-way ANOVA; [b]Chi-Square;

Abbreviations: ADL=Activities of Daily Living; a-MCI-MD= amnestic mild cognitive impairment multiple-domain; a-MCI SD= amnestic mild cognitive impairment single-domain;

CDR-SoB= Clinical Dementia Rating scale-Sum of Boxes; IADL=Instrumental Activities of Daily Living; MMSE=Mini Mental State Examination; MTA=medial temporal atrophy scale. See text for further details.

Table 2. Neuropsychological testing at baseline.

Cognitive domain **Neuropsychological test**	**a-MCI-due to AD patients**			
	MCI-Converters	**Non-Converters**		
	N=16	**N=19**	**Statistic[#]**	**p-value**
Verbal episodic long-term memory				
15-Words List				
Immediate recall (cut-off ≥ 28.5)	30.0 (6.1)	32.5 (6.9)	$F_{1,33}$=1.26	0.269
Delayed recall (cut-off ≥ 4.6)	4.1 (2.7)	5.4 (1.6)	$F_{1,33}$=3.34	0.076
Recognition: *hit rates*	10.6 (2.3)	10.3 (2.9)	$F_{1,33}$=0.09	0.763
Recognition: *false*	5.0 (4.4)	4.6 (3.9)	$F_{1,33}$=0.06	0.799
Short story test				
Immediate recall (cut-off ≥3.1)	3.8 (1.8)	4.7 (1.5)	$F_{1,33}$=2.34	0.137
Delayed recall (cut-off ≥ 2.6)	2.7 (2.7)	4.7 (1.6)	$F_{1,33}$=6.75	0.015

Visuo-spatial episodic long-term memory				
Complex Rey's Figure:				
Immediate recall (cut-off ≥ 6.4)	8.9 (6.7)	12.4 (7.4)	$F_{1,33}$=1.69	0.214
Delayed recall (cut-off ≥ 6.3)	6.7 (6.4)	11.5 (5.3)	$F_{1,33}$=4.99	0.033
Verbal short-term memory				
Digit span forward (cut-off ≥ 3.7)	4.9 (0.8)	5.0 (0.9)	$F_{1,33}$=0.039	0.846
Digit span backward	3.7 (0.9)	3.6 (1.0)	$F_{1,33}$=0.12	0.731
Visuo-spatial short-term memory				
Corsi span forward (cut-off ≥ 3.5)	4.2 (0.5)	4.3 (0.5)	$F_{1,33}$=0.17	0.680
Corsi span backward	3.6 (0.9)	3.9 (1.1)	$F_{1,33}$=0.84	0.366
Executive functions				
Phonological Word Fluency (cut-off ≥17.3)	31.4 (8.1)	33.2 (9.2)	$F_{1,33}$=0.37	0.545
Modified Card Sorting Test Criteria achieved (cut-off ≥ 4.2)	3.4 (2.1)	4.6 (1.9)	$F_{1,33}$=2.26	0.144
Modified Card Sorting Test perseverative errors (cut-off ≤7.6)	9.7 (10.2)	7.4 (1.7)	$F_{1,33}$=1.69	0.205

Language				
Naming of objects (cut-off ≥ 22)	28.2 (1.6)	28.5 (1.6)	$F_{1,33}$=0.35	0.560
Reasoning				
Raven's Coloured Progressive Matrices (cut-off ≥ 18.9)	26.1 (4.6)	28.9 (4.5)	$F_{1,33}$=3.42	0.073
Constructional praxis				
Copy of drawings (cut-off ≥ 7.1)	8.9 (1.5)	10.0 (1.3)	$F_{1,33}$=4.39	0.044
Copy of drawings with landmarks (cut-off ≥ 61.8)	65.0 (5.2)	67.6 (2.8)	$F_{1,33}$=3.55	0.069
Copy of Complex Rey's Figure (cut-off ≥ 23.7)	26.8 (8.9)	32.8 (4.4)	$F_{1,33}$=6.39	0.017

[#]One-way ANOVA

Abbreviations: a-MCI-due to AD: amnestic mild cognitive impairment due to Alzheimer's disease;

For each group of studied subjects, the table shows the mean (SD) performance scores obtained on neuropsychological testing. For each administered test appropriate adjustments for gender, age and education were applied according to the Italian normative data. Available cut-off scores of normality (≥ 95% of the lower tolerance limit of the normal population distribution) are also reported for each test. See text for further details.

Table 3. Neuropsychological testing at follow-up.

Cognitive domain **Neuropsychological test**	**a-MCI-due to AD patients**			
	MCI-Converters	**Non-Converters**		
	N=16	**N=19**	**Statistic[#]**	**p-value**
Verbal episodic long-term memory				
15-Words List				
Immediate recall (cut-off ≥ 28.5)	29.9 (9.5)	37.4 (7.4)	$F_{1,33}$=6.72	0.014
Delayed recall (cut-off ≥ 4.6)	3.9 (3.2)	5.8 (2.8)	$F_{1,33}$=3.07	0.089
Recognition: *hit rates*	11.2 (3.4)	11..9 (2.5)	$F_{1,33}$=0.50	0.484
Recognition: *false*	10.2 (6.9)	5.2 (4.4)	$F_{1,33}$=6.40	0.017
Short story test				
Immediate recall (cut-off ≥3.1)	3.5 (2.3)	5.2 (1.8)	$F_{1,33}$=3.98	0.058
Delayed recall (cut-off ≥ 2.6)	2.3 (2.6)	4.8 (2.1)	$F_{1,33}$=5.84	0.024
Visuo-spatial episodic long-term memory				
Complex Rey's Figure:				

Immediate recall (cut-off ≥ 6.4)	9.6 (8.0)	13.8 (6.7)	$F_{1,33}$=2.21	0.149
Delayed recall (cut-off ≥ 6.3)	9.6 (6.8)	12.7 (4.9)	$F_{1,33}$=2.02	0.165
Verbal short-term memory				
Digit span forward (cut-off ≥ 3.7)	5.3 (1.1)	5.1 (1.1)	$F_{1,33}$=0.23	0.637
Digit span backward	3.0 (1.5)	3.5 (1.2)	$F_{1,33}$=1.48	0.231
Visuo-spatial short-term memory				
Corsi span forward (cut-off ≥ 3.5)	4.2 (1.6)	4.1 (1.4)	$F_{1,33}$=0.01	0.892
Corsi span backward	3.1 (1.5)	3.9 (1.1)	$F_{1,33}$=2.61	0.116
Executive functions				
Phonological Word Fluency (cut-off ≥17.3)	28.4 (9.9)	29.9 (5.9)	$F_{1,33}$=0.28	0.597
Modified Card Sorting Test Criteria achieved (cut-off ≥ 4.2)	2.6 (2.1)	4.8 (1.7)	$F_{1,33}$=7.78	0.010
Modified Card Sorting Test perseverative errors (cut-off ≤7.6)	7.2 (5.0)	3.4(3.5)	$F_{1,33}$=5.05	0.034
Language				
Naming of objects (cut-off ≥ 22)	28.1 (2.1)	28.5 (2.2)	$F_{1,33}$=0.21	0.648
Reasoning				
Raven's Coloured Progressive Matrices (cut-off ≥ 18.9)	25.7 (6.5)	30.0 (4.4)	$F_{1,33}$=5.37	0.027

Constructional praxis				
Copy of drawings (cut-off ≥ 7.1)	8.4 (2.2)	9.6 (2.5)	$F_{1,33}$=1.93	0.175
Copy of drawings with landmarks (cut-off ≥ 61.8)	65.6 (5.2)	64.7 (3.8)	$F_{1,33}$=0.03	0.861
Copy of Complex Rey's Figure (cut-off ≥ 23.7)	24.2 (9.4)	32.7 (3.6)	$F_{1,33}$=12.71	0.001*

[#]One-way ANOVA; *significative p-value survived after Bonferroni's Correction

Abbreviations: a-MCI-due to AD: amnestic mild cognitive impairment due to Alzheimer's disease;

For each group of studied subjects, the table shows the mean (SD) performance scores obtained on neuropsychological testing. For each administered test appropriate adjustments for gender, age and education were applied according to the Italian normative data. Available cut-off scores of normality (≥ 95% of the lower tolerance limit of the normal population distribution) are also reported for each test. See text for further details.

Table 4. Functional connectivity changes between MCI-Converters and non-Converters at baseline and 24-month follow-up

	Side	Brain regions	Size	(Z)	MNI coordinates
1) Main effect of Group	R	Posterior Cingulate	1325	3.60	13 -17 35
2) Post-hoc analysis: MCI-Converters<Non Converters					
A) Baseline	L	Posterior Cingulate	2075	3.38	-13 -9 35
	R	Precentral gyrus		3.38	17 -21 45
Post-hoc analysis: MCI-Converters<Non Converters					
B) Follow-up	R	Precentral gyrus	2301	3.69	33 -3 19
	R	Parietal operculum		3.10	55 -3 13
	R	Insula		3.23	39 3 11
	B	Posterior cingulate			0 -19-50
3) Association between Group and Time-point of conversion					
MCI-Converters: direct association	R	Precentral gyrus	686	4.62	33 -3 61

Table 5 Discriminant analysis.

	Sensitivity	**Specificity**	**Accuracy**
Model 1	50.0%	63.2%	57.1%
Model 2	68.8%	63.2%	65.7%
Model 3	68.8%	68.4%	68.6%
Model 4	93.8%	42.1%	65.7%
Model 5	56.3%	57.9%	57.1%

Figure 1.

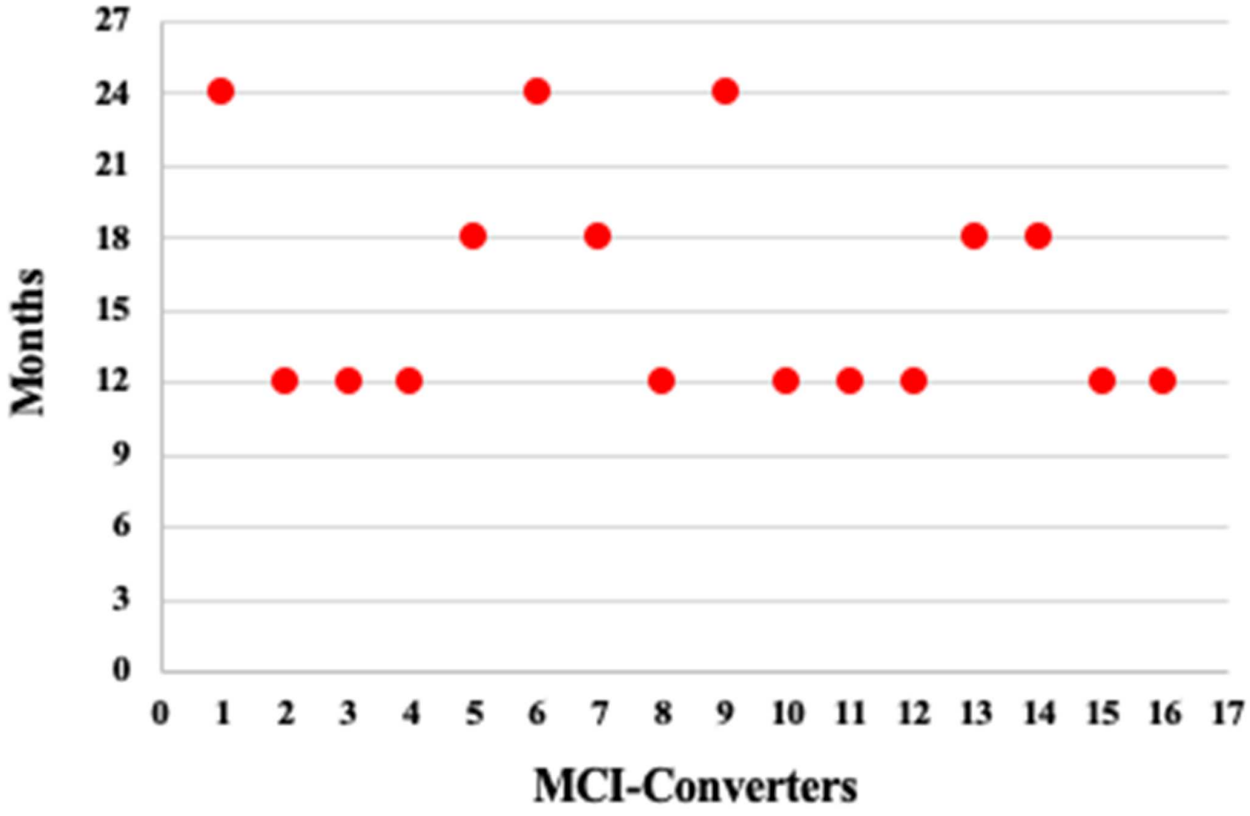

Months
27
24
21
18
15
12
9
6
3
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
MCI-Converters

Figure 2.

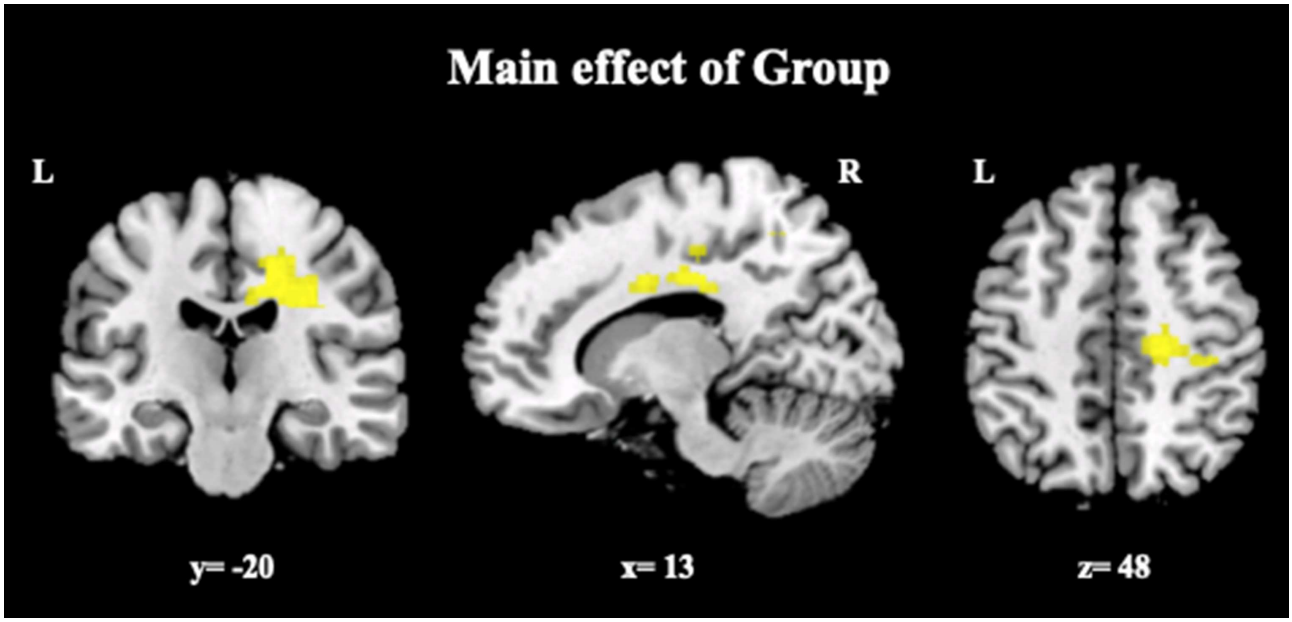


Figure 3

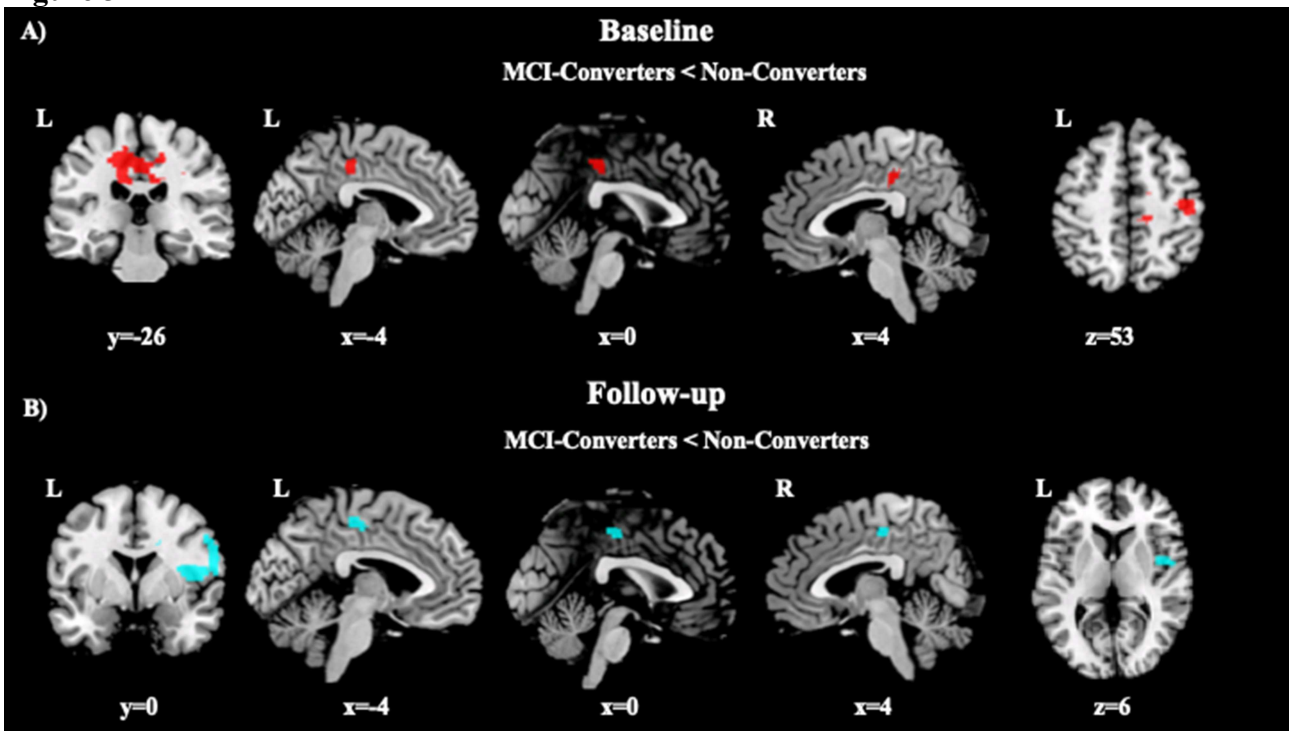
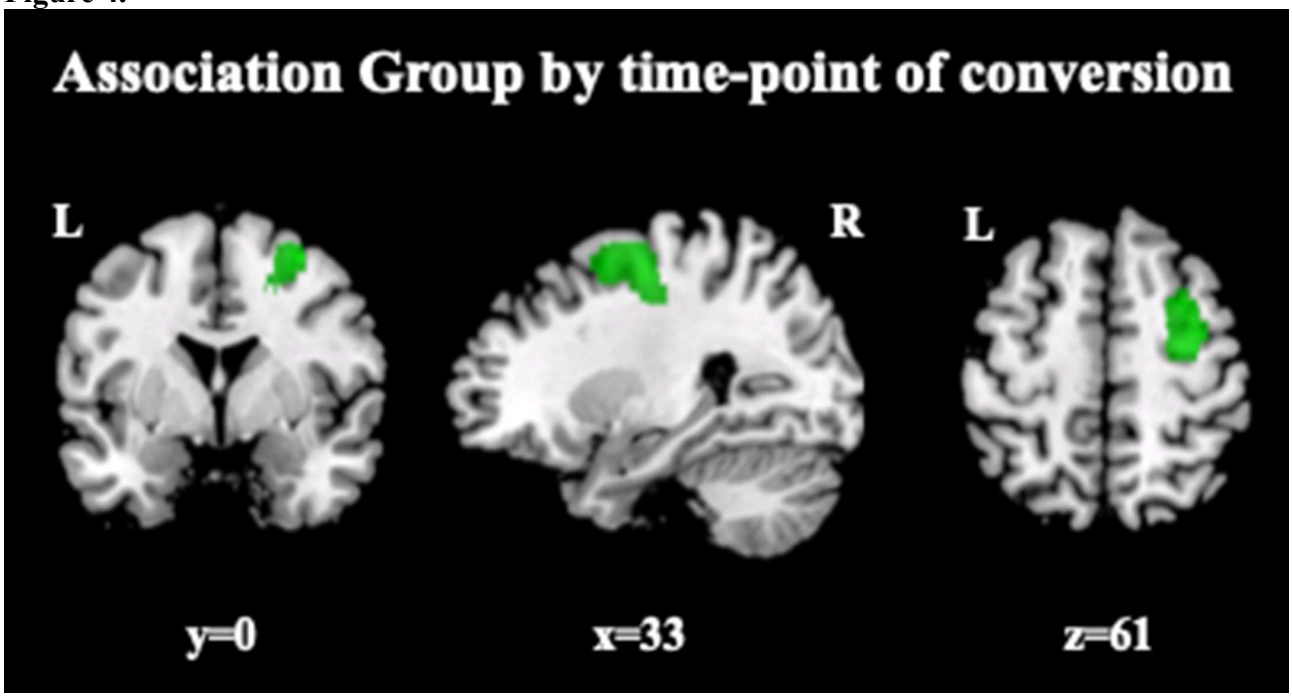

A)
Baseline
MCI-Converters < Non-Converters
L
L
R
L
y=-26
x=-4
x=0
x=4
z=53
B)
Follow-up
MCI-Converters < Non-Converters
L
L
R
L
y=0
x=-4
x=0
x=4
z=6

Figure 4.


Association Group by time-point of conversion
L
R
L
y=0
x=33
z=61