Progression markers of Parkinson’s disease are crucial for successful therapeutic development. Recently, a diffusion magnetic resonance imaging analysis technique using a bitensor model was introduced allowing the estimation of the fractional volume of free water within a voxel, which is expected to increase in neurodegenerative disorders such as Parkinson’s disease. Prior work demonstrated that free water in the posterior substantia nigra was elevated in Parkinson’s disease compared to controls across single- and multi-site cohorts, and increased over 1 year in Parkinson’s disease but not in controls at a single site. Here, the goal was to validate free water in the posterior substantia nigra as a progression marker in Parkinson’s disease, and describe the pattern of progression of free water in patients with a 4-year follow-up tested in a multicentre international longitudinal study of de novo Parkinson’s disease (http://www.ppmi-info.org/). The analyses examined: (i) 1-year changes in free water in 103 de novo patients with Parkinson’s disease and 49 controls; (ii) 2- and 4-year changes in free water in a subset of 46 patients with Parkinson’s disease imaged at baseline, 12, 24, and 48 months; (iii) whether 1- and 2-year changes in free water predict 4-year changes in the Hoehn and Yahr scale; and (iv) the relationship between 4-year changes in free water and striatal binding ratio in a subgroup of Parkinson’s disease who had undergone both diffusion and dopamine transporter imaging.

Results demonstrated that: (i) free water level in the posterior substantia nigra increased over 1 year in de novo Parkinson’s disease but not in controls; (ii) free water kept increasing over 4 years in Parkinson’s disease; (iii) sex and baseline free water predicted 4-year changes in free water; (iv) free water increases over 1 and 2 years were related to worsening on the Hoehn and Yahr scale over 4 years; and (v) the 4-year increase in free water was associated with the 4-year decrease in striatal binding ratio in the putamen. Importantly, all longitudinal results were consistent across sites. In summary, this study demonstrates an increase over 1 year in free water in the posterior substantia nigra in a large cohort of de novo patients with Parkinson’s disease from a multi-site cohort study and no change in healthy controls, and further demonstrates an increase of free water in Parkinson’s disease over the course of 4 years. A key finding was that results are consistent across sites and the 1-year and 2-year increase in free water in the posterior substantia nigra predicts subsequent long-term progression on the Hoehn and Yahr staging system. Collectively, these findings demonstrate that free water in the posterior substantia nigra is a valid, progression imaging marker of Parkinson’s disease, which may be used in clinical trials of disease-modifying therapies.

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Abbreviations: A/PSN = anterior/posterior substantia nigra; PPMI = Parkinson’s Progressive Marker Initiative; SBR = striatal binding ratio; STN = subthalamic nucleus

Introduction

The shortage of dopaminergic input to the striatum along the nigrostriatal pathway in Parkinson’s disease results in a wide range of motor impairments, which are typically managed with symptomatic therapy (Jankovic and Aguilar, 2008; Fox et al., 2011; Rascol et al., 2011; Kordower et al., 2013). Notably, there is no approved treatment that stops or slows down disease progression in individuals with Parkinson’s disease. It is believed that any progress toward a breakthrough in neuroprotective or disease-modifying therapeutics has been slowed, in part, by the lack of a biomarker that can objectively detect brain changes related to Parkinson’s disease and monitor these changes as the disease progresses (Olanow et al., 2009a; Athauda and Foltynie, 2015). That is, the identification of a robust biomarker for monitoring disease progression represents a critical yet unmet need in the field of Parkinson’s disease (Henchcliffe and Severt, 2011).

Imaging studies in Parkinson’s disease provide a rich avenue for biomarker development (Seppi and Schocke, 2005; Du et al., 2012; Lehericy et al., 2012; Bowman et al., 2016). Over the years, diffusion MRI has improved the understanding of the pathophysiology of Parkinson’s disease by demonstrating microstructural alterations within the substantia nigra (SN), such as changes in the direction of diffusing water molecules (Vaillancourt et al., 2009; Cochrane and Ebmeier, 2013; Schwarz et al., 2013; Langley et al., 2016). However, the results of a recent meta-analysis of nigral fractional anisotropy changes question the stability and validity of this measure as a Parkinson’s disease biomarker (Schwarz et al., 2013). More recently a novel method for processing and analysis of diffusion MRI data has proved useful in assessing brain changes associated with Parkinson’s disease and its progression (Ofori et al., 2015a, b). This new computational approach fits a bitensor model instead of a single-tensor model to the imaging data, separating the diffusion properties of water in brain tissue from those of water in the extracellular space (Pasternak et al., 2009; Albi et al., 2017). The procedure allows the calculation of the fractional volume of free water within a voxel, which is expected to increase in neurodegenerative disorders (Wang et al., 2011) and can otherwise bias diffusion indices and lead to reduced fractional anisotropy and increased mean diffusivity values (Metzler-Baddeley et al., 2012). In a recent multi-site study of healthy adults it was demonstrated that free water measures had a higher sensitivity than conventional diffusion MRI measures to detect within- and between-group effects (Albi et al., 2017).

Previously, it was shown that free water levels in the posterior substantia nigra (PSN) were elevated in Parkinson’s disease relative to healthy controls across single- and multi-site cohorts (Ofori et al., 2015a), and this region is consistent with the ventrolateral tier of the SN where dopaminergic cell loss is greatest in Parkinson’s disease (Jellinger, 2012). Furthermore, free water in PSN was also elevated in atypical parkinsonian syndromes, consistent with more severe pathology in these disorders as compared to Parkinson’s disease (Planetta et al., 2016). Most importantly, the results of a prospective, single-site longitudinal study revealed that free water in PSN increased over the course of 1 year in Parkinson’s disease but not in controls, and baseline free water predicted changes in bradykinesia and cognitive scores (Ofori et al., 2015b). Of note, how free water in PSN changes over an extended follow-up period, beyond 1 year of disease progression, has not yet been described.

In the context of imaging approaches to Parkinson’s disease, a multi-site validation of a neuroimaging progression marker would be a critical step toward using an imaging endpoint in future disease-modifying clinical trials. Such an effort is also consistent with the goal of the Parkinson’s Progressive Marker Initiative (PPMI), a multicentre international study set up to identify and validate progression markers in newly diagnosed Parkinson’s disease. Data used in the preparation of this article were obtained from the PPMI database and consisted of serial diffusion MRI scans collected across 1 and 4 years. Briefly, the specific goals of the current study were to: (i) contrast 1-year changes in free water in a multi-site study of de novo Parkinson’s disease to 1-year changes in healthy controls; and (ii) further examine the pattern of change in free water in patients with Parkinson’s disease with a 4-year follow-up. As free water imaging provides an indirect measure of dopaminergic degeneration within the SN and predicts subsequent clinical changes (Ofori et al., 2015b), we hypothesized that free water levels in the PSN would increase in Parkinson’s disease compared to controls, and continue to increase with disease progression over the 4-year period. We also tested the hypothesis that the increase in free water in PSN over a short period of time (1–2 years) will be associated with long-term (4-year) changes in Hoehn and Yahr status. Finally, we hypothesized that long-term (4-year) changes in free water in PSN would relate to changes in dopamine transporter imaging (DaTscan) measures in the putamen.

Materials and methods

Participants

Data were downloaded between January and August 2016 through a standard application process from the PPMI website...
Table 1. Demographic and clinical characteristics of study cohorts

| Demographics | Clinical data | Controls (dMRI) | 1-Year PD Cohort 1 (dMRI) | 4-Year PD Cohort 2 (dMRI) | 4-Year PD Cohort 3 (dMRI and DaTscan) |
|--------------|---------------|----------------|--------------------------|--------------------------|--------------------------------------|
| n            | 49            | 103            | 46                       | 30                       |                                      |
| Age at baseline, years | 60.29 (10.44) | 60.04 (9.49)   | 59.07 (9.67)             | 58.66 (10.33)             |                                      |
| Sex: M / F | 29 / 20       | 69 / 34        | 34 / 12                  | 22 / 8                   |                                      |
| Disease duration, years | –          | 0.58 (0.58)    | 0.63 (0.58)              | 0.63 (0.59)               |                                      |
| MDS-UPDRS-III at baseline | 0.61 (1.41) | 20.05 (8.83)   | 21.30 (8.66)             | 22.00 (7.37)              |                                      |
| Hoehn and Yahr stage at baseline: 1 / 2 | –          | 44 / 59        | 17 / 29                  | 11 / 19                   |                                      |
| Hoehn and Yahr stage at Year 4: 1 / 2 / 3 | –          | –              | 9 / 21 / 1              | –                        |                                      |
| MoCA | 28.27 (1.09) | 27.50 (1.99)   | 27.35 (2.31)             | 27.57 (1.88)              |                                      |

Data represent mean (±SD) or count. Motor severity at baseline (MDS-UPDRS-III, Hoehn and Yahr stage) was assessed OFF medication. At Year 4, only 31 of 46 patients with Parkinson’s disease in Cohort 2 had an evaluation of the Hoehn and Yahr stage OFF medication. DaT = dopamine transporter imaging; dMRI = diffusion MRI; F = female; M = male; MDS-UPDRS-III = motor section of the Movement Disorders Society Unified Parkinson’s Disease Rating Scale; MoCA = Montreal Cognitive Assessment; PD = Parkinson’s disease.

Clinical scores obtained OFF medication including the Hoehn and Yahr stage and the ratings for the motor section of the MDS-UPDRS (Goetz et al., 2007) and the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005) are provided in Table 1.

Imaging data acquisition

Diffusion MRI

Data were collected at various sites on 3 T TIM Trio Siemens scanners (software version VB15 or higher) equipped with a 12-channel matrix head coil. Prior to data collection, participating sites were supplied with the software version specific electronic protocol to be imported into the scanner. The PPMI diffusion sequence had the following parameters: b = 1000 s/mm², 64 diffusion gradient directions with one b0 image, image matrix = 116 × 116 × 72, flip angle = 90°, and a voxel resolution of 1.98 × 1.98 × 2.0 mm.

123I-FP-CIT SPECT

The 30 patients with Parkinson’s disease in Cohort 3 underwent DaTscan imaging at recruitment, 12 months, 24 months, and 48 months according to the PPMI imaging protocol. Single photon emission computerized tomography (SPECT) imaging was performed 4 ± 0.5 h after injection with a dose range of 111–185 MBq or 5.0 mCi of DaTscan™. Data were acquired into a 128 × 128 matrix stepping each 3° for a total of 120 (or 4° for a total of 90) projections into 20° symmetric photopeak windows centred on 159 KeV and 122 KeV.

Diffusion MRI analysis

Data preprocessing

FMRIB Software Library (FSL, http://www.fmrib.ox.ac.uk/fsl/) and custom UNIX shell scripts were used to preprocess the data (Ofori et al., 2015a, b; Burciu et al., 2016; Planetta et al., 2016). Each scan was corrected for signal distortions due to eddy currents and head motion. Next, gradient directions were rotated in response to the eddy current corrections, and non-brain tissue was removed. Free water maps and free water-corrected diffusion tensor maps were calculated using MATLAB R2013a (The Mathworks, Natick, MA) (Pasternak et al., 2009). The bitensor model calculates the signal attenuation as the sum of attenuations arising from two compartments: one that models free water and a tissue compartment. The free water-corrected tensor maps were also used to calculate free water-corrected diffusion MRI analysis.
fractional anisotropy. Finally, the b0 image at each time point was registered to a standardized T2-weighted image in MNI space (2 × 2 × 2 mm) by affine transformation with 12 degrees of freedom and tri-linear interpolation using FLIRT (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FLIRT). The resulting transformation matrix was then applied to the free water and free water-corrected fractional anisotropy maps.

**Regions of interest**
Consistent with previous work (Ofori et al., 2015a, b; Burciu et al., 2016; Planetta et al., 2016), regions of interest were hand-drawn by an experienced rater on the b0 image of each subject in MNI space at each time point, and then used to extract values from the corresponding free water and free water-corrected fractional anisotropy maps. Importantly, regions of interest were drawn blinded to the free water map, group status, and visit. The size of the regions of interest was chosen to fit within the brain structure across all subjects, and its location was consistent with that reported in previous studies (Ofori et al., 2015a, b; Burciu et al., 2016; Planetta et al., 2016). In each hemisphere, the region of interest covered two consecutive slices, and consisted of eight voxels (one voxel = 2 × 2 × 2 mm). Figure 1 illustrates the procedure used to draw regions of interest in the anterior substantia nigra (ASN) and PSN. Positioning of the regions of interest was guided by anatomical landmarks and not by the MNI coordinates corresponding to the substantia nigra. Consistent with previous work in parkinsonian disorders and healthy individuals (Vaillancourt et al., 2012; Ofori et al., 2015a, b; Planetta et al., 2016), we assessed both inter-rater and intra-rater reliability of the region of interest placement. To calculate an index of inter-rater reliability we had a second rater draw the SN regions of interest on 25 b0 images randomly selected. The second rater was also blind to the free water map and clinical information pertaining to group status or visit. For the intra-rater reliability calculation, we had the main rater draw the subset of 25 subjects twice. Finally, we also included a control region of interest in the subthalamic nucleus (STN), a region where structural changes in Parkinson’s disease are not expected. Consistent with previous studies (Ofori et al., 2015a, b; Burciu et al., 2016; Planetta et al., 2016), for each region of interest the mean free water and mean free water-corrected fractional anisotropy were calculated bilaterally.

**DaTscan SPECT analysis**
DaTscan SPECT data were processed per PPMI protocol, and outcome measures included striatal binding ratios (SBR) for the caudate and putamen. The SBR values for Parkinson’s disease in Cohort 3 were downloaded from the PPMI website. For the purpose of our analysis, the SBR was averaged across hemispheres.

**Statistical analysis**
All statistical analyses were performed with IBM SPSS Statistics 24 (SPSS Inc., Chicago, Illinois), and results were
corrected for multiple comparisons using the Benjamini-Hochberg (1995) false discovery rate (FDR) method at $P_{\text{FDR}} < 0.05$. An intraclass correlation coefficient (ICC) was calculated for the free-water measures in the SN extracted from the hand-drawn regions of interest. A two-way mixed model with absolute agreement was used. Differences in age at baseline between Parkinson’s disease in Cohort 1 and controls were assessed with an independent samples $t$-test, whereas group differences in sex distribution were examined with Pearson’s chi-square test. Imaging data were tested for normality before being subjected to further statistical analysis. Whenever the assumption of normality was not met, non-parametric statistics were applied to the data.

**Cohort 1**

First, we compared 1-year changes in free water and free water-corrected fractional anisotropy in anterior SN (ASN), PSN and STN between Parkinson’s disease in Cohort 1 and healthy controls using a univariate model with the difference between time points as the dependent variable, group and PPMI site as fixed factors, and the imaging measure at baseline as covariate. Significant group effects were followed-up by a supplementary analysis that assessed differences between Parkinson’s disease and controls on the baseline measure.

**Cohort 2**

In Cohort 2 longitudinal changes in free water and free water corrected fractional anisotropy in ASN, PSN, and STN were analysed using a two-way mixed ANOVA model with time (Baseline, Year 1, Year 2, Year 4) as a within-group factor, and PPMI site as a between-group factor. The data met the variance-covariance, and sphericity assumptions. Where appropriate, significant effects were followed up with pairwise comparisons. In case of a time effect on an imaging measure, an additional two-way mixed ANOVA analysis was performed evaluating shorter-term time effects (Baseline, Year 1, Year 2) and whether these changes over time were consistent across data collection sites. We were also interested in whether the progression of free water in PSN over 4 years followed a different pattern in the more affected patients versus the less affected patients. For this, we used the median value of the total MDS-UPDRS-III at baseline (20 points) to group the 46 Parkinson’s disease in Cohort 2 into ‘mild Parkinson’s disease’ and ‘moderate Parkinson’s disease’. Severity of symptoms at baseline (mild versus moderate) was used as a between-group factor in a two-way mixed ANOVA model. In Cohort 2 we also examined the baseline predictors of long-term changes in free water using a multiple regression analysis and the backward elimination method. The dependent variable was the relative per cent change over 4 years in free water in PSN, while the predictors were the following measures at baseline: age, sex, total MDS-UPDRS-III, disease duration, MoCA, free water in PSN. Of note, we also assessed how free water related to the clinical progression of Parkinson’s disease. A Spearman rank correlation was run in 31 Parkinson’s disease patients from Cohort 2 who had the Hoehn and Yahr stage scored OFF antiparkinsonian medication at Year 4 in order to determine the relation between changes over 4 years in the Hoehn and Yahr stage and (i) free water at baseline; (ii) 1-year relative per cent change in free water in PSN; and (iii) 2-year relative per cent change in free water in PSN.

**Cohort 3**

For the 30 Parkinson’s disease patients in Cohort 3 who underwent both diffusion MRI and DaTscan four times over the 4-year period, the Pearson’s product-moment correlation coefficient was calculated between the relative per cent change over 4 years in free water in the PSN and the relative per cent change over 4 years in SBR in the putamen and caudate. Finally, we also report required sample size at 80% and 90% power for future clinical trials that would use relative changes in free water in the PSN and/or SBR in the putamen to test the effect of a potential disease-modifying drug. For the control group, the mean changes over 2 years and 4 years were estimated from 30 patients who had undergone both diffusion MRI and DaTscan. For the treatment group, we assumed 50% or 70% effect of a drug. Power analysis was conducted using two ways of estimating within group standard deviation: with and without adjusting covariates sex and baseline measure.

**Results**

No significant differences were found between Parkinson’s disease and controls in age at baseline ($P = 0.887$), or sex distribution ($P = 0.369$). The imaging metrics corresponding to the three Parkinson’s disease cohorts and control group are listed in Table 2. In the assessment of inter-rater reliability between Raters 1 and 2, there was a strong agreement for both ASN (ICC = 0.86, $P < 0.001$) and PSN (ICC = 0.90, $P < 0.001$). The ICC analysis also revealed a high intra-rater reliability coefficient (ASN = 0.91, PSN = 0.92, $P$-values < 0.001).

**Cohort 1: 1-year changes in diffusion MRI**

A significant group effect was found on the 1-year difference in free water in PSN, with patients with Parkinson’s disease having an increase in free water compared to controls [$F(1,152) = 4.618$, $P = 0.033$; Fig. 2A]. The analysis revealed no site effect ($P = 0.276$) or Group × Site interaction ($P = 0.936$). Furthermore, a secondary analysis revealed that free water in PSN at baseline differed between Parkinson’s disease and controls, with Parkinson’s disease having more free water than controls ($U = 2011$, $P = 0.043$). No significant group, site, or Group × Site interaction effects were found for free water in ASN and STN, and free water corrected fractional anisotropy in ASN, PSN, and STN ($P$-values > 0.05).

**Cohort 2: 2- and 4-year changes in diffusion MRI**

A significant time effect for free water in PSN was found in the cohort of 46 Parkinson’s disease patients [$F(3,114) = 9.165$, $P < 0.001$; Fig. 2B], but not for free water in ASN or STN, or free water corrected fractional anisotropy in ASN, PSN, and STN ($P$-values > 0.05). In PSN, post hoc comparisons revealed significant increases...
| Measure     | Study cohorts | ROI | Baseline | Year 1 | Year 2 | Year 4 |
|-------------|---------------|-----|----------|--------|--------|--------|
| dMRI FW     | Controls (n = 49) | ASN | 0.127 (0.03) | 0.130 (0.03) | – | – |
|             |               | PSN | 0.138 (0.03) | 0.145 (0.03) | – | – |
|             |               | STN | 0.092 (0.02) | 0.096 (0.02) | – | – |
| PD Cohort 1 (n = 103) | ASN | 0.140 (0.04) | 0.138 (0.05) | – | – |
|             |               | PSN | 0.155 (0.05) | 0.173 (0.06) | – | – |
|             |               | STN | 0.092 (0.02) | 0.095 (0.02) | – | – |
| PD Cohort 2 (n = 46) | ASN | 0.134 (0.03) | 0.136 (0.04) | 0.143 (0.04) | 0.141 (0.04) |
|             |               | PSN | 0.155 (0.04) | 0.174 (0.05) | 0.185 (0.06) | 0.198 (0.04) |
|             |               | STN | 0.096 (0.02) | 0.094 (0.02) | 0.092 (0.02) | 0.096 (0.23) |
| PD Cohort 3 (n = 30) | ASN | 0.129 (0.03) | 0.136 (0.05) | 0.147 (0.04) | 0.138 (0.03) |
|             |               | PSN | 0.148 (0.04) | 0.172 (0.04) | 0.194 (0.06) | 0.189 (0.04) |
|             |               | STN | 0.923 (0.02) | 0.957 (0.02) | 0.948 (0.02) | 0.939 (0.01) |
| dMRI FAf   | Controls (n = 49) | ASN | 0.568 (0.06) | 0.568 (0.07) | – | – |
|             |               | PSN | 0.621 (0.05) | 0.618 (0.05) | – | – |
|             |               | STN | 0.574 (0.05) | 0.569 (0.05) | – | – |
| PD Cohort 1 (n = 103) | ASN | 0.550 (0.06) | 0.558 (0.06) | – | – |
|             |               | PSN | 0.613 (0.06) | 0.626 (0.05) | – | – |
|             |               | STN | 0.558 (0.04) | 0.565 (0.04) | – | – |
| PD Cohort 2 (n = 46) | ASN | 0.555 (0.05) | 0.553 (0.06) | 0.565 (0.06) | 0.582 (0.06) |
|             |               | PSN | 0.626 (0.06) | 0.627 (0.05) | 0.631 (0.06) | 0.637 (0.05) |
|             |               | STN | 0.568 (0.05) | 0.563 (0.05) | 0.562 (0.04) | 0.562 (0.04) |
| PD Cohort 3 (n = 30) | ASN | 0.555 (0.06) | 0.542 (0.05) | 0.558 (0.05) | 0.581 (0.07) |
|             |               | PSN | 0.628 (0.06) | 0.625 (0.05) | 0.630 (0.06) | 0.633 (0.05) |
|             |               | STN | 0.566 (0.05) | 0.555 (0.05) | 0.557 (0.04) | 0.555 (0.05) |
| DaT SBR    | PD Cohort 3 (n = 30) | PUT | 0.785 (0.26) | 0.700 (0.25) | 0.670 (0.29) | 0.581 (0.26) |
|             |               | CAU | 2.044 (0.60) | 1.933 (0.51) | 1.761 (0.55) | 1.598 (0.60) |

Data represent mean (±SD). CAU = caudate; DaT = dopamine transporter imaging; dMRI = diffusion MRI; FAf = free-water corrected fractional anisotropy; FW = free water; MoCA = Montreal Cognitive Assessment; PD = Parkinson’s disease; PUT = putamen; ROI = region of interest.

Figure 2. Longitudinal changes in free water in PSN. Panels show differences (adjusted for free water at baseline) between patients with Parkinson’s disease from Cohort 1 and healthy controls (A), and free water in PSN over the course of 4 years in the patients with Parkinson’s disease from Cohort 2 (B). (C) An example of a free water map at each time point of a single Parkinson’s disease patient. Error bars represent the standard error. CON = controls; FW = free water; PD = Parkinson’s disease.
in free water from baseline to Year 1 (P = 0.008), baseline to Year 2 (P = 0.003), baseline to Year 4 (P = 0.003), Year 1 to Year 4 (P = 0.006), but no significant change in free water from Year 1 to Year 2 (P = 0.154), or Year 2 to Year 4 (P = 0.154). Similar to the 1-year analysis, there was no site effect (P = 0.733) and also no Time × Site interaction in free water in PSN (P = 0.244). Figure 2C depicts an example of a free water map at each time point of a single Parkinson’s disease patient, showing an increase over time in free water in Parkinson’s disease. Time, Site and Time × Site effects were also explored in an analysis evaluating 2-year changes. A significant time effect was found [F(2,76) = 5.884, P = 0.004], but no Site (P = 0.723) or Time × Site interaction (P = 0.194).

When comparing mild and moderate Parkinson’s disease, we found no significant Disease severity × Time interaction (P = 0.996). In the free water corrected fractional anisotropy data, there was no significant time effect in any of the regions of interest (P-values > 0.05). A significant regression model [F(2,46) = 18.86, R = 0.684, P < 0.001] indicated that baseline free-water values (β1 = −495.58, P < 0.001) and sex (β2 = −24.73, P < 0.01) predicted the change over 4 years in free water in the PSN. That is, the 4-year change in free water in PSN was greater for males than females, and greater for those patients with Parkinson’s disease who had a lower baseline free water than patients with Parkinson’s disease who started off with a higher baseline free water. Finally, results showed that the progression over 4 years on the Hoehn and Yahr scale was significantly related to the relative per cent change in free water in PSN over 1 year (ρo = 0.363, P < 0.05, Fig. 3A) and 2 years (ρo = 0.379, P < 0.05, Fig. 3B), but not to the free water levels at baseline (P = 0.806). That is, disease progression of 1 point on the Hoehn and Yahr scale was associated with a greater increase in free water in PSN than the increase observed in those patients who did not progress on the scale.

**Cohort 3: Relation between 4-year diffusion MRI and 4-year DaTscan changes**

An inverse association was found between relative changes over 4 years in free water in PSN and relative changes over 4 years in SBR in the putamen (r = −0.431, P < 0.05; Fig. 4), but not between changes in free water and changes in SBR in the caudate (P = 0.332).

The results of the power analyses based on free water in PSN and SBR in the putamen are shown in Table 3. Briefly, the power analysis for a two-arm study (treatment versus placebo) using relative change in free water in PSN over 2 years as an outcome measure indicated that to detect a significant effect of a neuroprotective agent with 90% power and 50% predicted change, 176 subjects are required across groups. When assuming a 70% effect of a drug, the sample estimate for 2-year changes in free water in PSN at 90% power is 90 subjects. Table 3 provides additional sample size estimations for 4-year changes in free water, and 2- and 4-year changes in SBR in the putamen.

**Discussion**

Using serial diffusion MRI data from the PPMI study we confirmed an increase over 1 year in free water in PSN in a large cohort of Patients with Parkinson’s disease, and characterized for the first time the pattern of progression of free water over an extended period of time. Specifically, we showed a progressive increase of free water over 4 years, with a steeper change in free water in the early stages of the follow-up compared to the later stages of the follow-up. A key finding was that 1-year and 2-year changes in free water were associated with 4-year changes on the Hoehn and Yahr scale. This indicated that the short-term increase in free water is related to the long-term progression of motor symptoms. Moreover, sex and baseline free water levels significantly predicted the rate of change in free water in PSN over 4 years, and the overall increase in free water was related to a decrease in DaTscan SBR in the putamen. Together, these results demonstrate the potential of free water as an imaging biomarker for Parkinson’s disease progression.

The results of the current study confirmed previous single site, and longitudinal results that free water levels in the PSN increase over a 1-year period of progression (Ofori et al., 2015b). Further, our findings provide the first validation that this increase in free water levels with 1-year progression is evident also in newly diagnosed de novo Parkinson’s disease, and when using a multi-site setup. To our knowledge, this is the first study to measure non-invasively disease-related changes in SN over an extended period of time. Importantly, the analysis of free water in the 46 Parkinson’s disease patients from Cohort 2 further revealed that free water in PSN continues to increase over 4 years of disease. In early unmedicated Parkinson’s disease, this increase appears to be greater in the first years following diagnosis compared to the later years of the follow-up.

A key aspect in this study is the fact that results over 1, 2 and 4 years of disease progression were consistent across sites/scanners. The consistency of longitudinal results across sites/scanners (Philips 3 T in Ofori et al., 2015b, Siemens 3 T in PPMI study) is of utmost importance in evaluating surrogate markers, and validates the feasibility and reliability of free water imaging as a progression marker of Parkinson’s disease. As suggested earlier, free water in PSN could be a valid outcome measure in future clinical trials of disease-modifying therapies. Additional evidence in support of the use of free water in clinical longitudinal intervention designs comes from the inverse relation between 4-year free water changes in PSN and DaTscan measures showing that the greater the increase in free water, the greater the progression-related decline in dopamine transporter integrity in
the putamen. This finding consolidates previous results from cross-sectional data relating diffusion MRI and dopaminergic terminal dysfunction (Schlerf et al., 2013; Ofori et al. 2015a), and suggests that changes in free water in the SN parallel disease-related changes in the functional status of dopaminergic nerve terminals of the basal ganglia, a hallmark of Parkinson’s disease.

The results of the free water analysis in those Parkinson’s disease from Cohort 3 who underwent both diffusion and dopamine transporter imaging at each time point also warrant some attention (Table 2). Although in both Cohorts 2 and 3 the free water changes from Year 2 to Year 4 were not significant, patients in Cohort 3 appeared to have rather modest changes in free water during this time period compared to Cohort 2. This difference may be related to variability in this group of Parkinson’s disease, which is a subset of Cohort 2, but also to possible effects of antiparkinsonian medication on the progression of free water. While naïve at enrolment, many Parkinson’s disease patients may have been placed ON medication by Year 2 of the follow-up. It was previously shown in a retrospective study that patients with Parkinson’s disease who had taken the MAO-B inhibitor rasagiline as part of their treatment regimen had lower free water in PSN than Parkinson’s disease patients who had not taken this medication chronically (Burciu et al., 2016). This suggests that therapeutic interventions may alter the progression of free water in SN, an interesting hypothesis that should be further tested in placebo-controlled, randomized clinical drug studies.

Most importantly, current results show a strong link between free water changes and changes in an important clinical endpoint. Specifically, free water in PSN at baseline does not predict the progression on the Hoehn and Yahr staging system, but 1-year and 2-year longitudinal changes do. The per cent change in free water from baseline to 1-year and baseline to 2-years is significantly greater in those Parkinson’s disease patients who progressed on the Hoehn and Yahr scale over the course of 4 years than in those Parkinson’s disease patients with no changes in the clinical stage. These results advance the hypothesis that slowing down the rate of increase of free water in PSN may slow down the clinical progression of the disease. Also, the fact that free water changes in the first 1–2 years following diagnosis were more pronounced than free water changes later in the follow-up suggest that this may be an optimal time window for an intervention that would try to alter the course of these changes earlier on in order to prevent worsening of symptoms.

Figure 3 Relative per cent change in free water in PSN over 1 year (A) and 2 years (B) across subgroups determined based on the 4-year changes in the Hoehn and Yahr stage. In both plots, the per cent change from baseline is greater in patients with Parkinson’s disease who had a 1-point increase on the Hoehn and Yahr scale (n = 8) than in patients with Parkinson’s disease who did not progress on the scale (n = 19). Parkinson’s disease patients who had a decrease of 1 point on the scale had almost no change in free water (n = 4) compared with the other two groups. Error bars represent the standard error. FW = free water; PD = Parkinson’s disease.

Figure 4 Correlation plot illustrating the relation between 4-year relative per cent change in free water in PSN and 4-year relative per cent change in SBR in the putamen. FW = free water; PUT = putamen.
Interestingly, sex and baseline free water levels were significantly related to 4-year changes such that male patients had a greater increase in free water levels than female patients, and patients with a lower baseline free water had a steeper increase of free water compared to patients who started off with a higher baseline free-water value. Over the years, data from death rates and prevalence studies in Parkinson’s disease patients from around the world suggested the relative risk of Parkinson’s disease was ~1.5 times greater in males than in females (Schrag et al., 2000; Van Den Eeden et al., 2003; de Lau et al., 2004; Wooten et al., 2004). The current findings suggest sex as a factor to consider in future clinical trials aiming to assess the efficacy of treatments in slowing the progression of nigral pathology in Parkinson’s disease.

Finally, using power analyses of the imaging progression data, sample size estimates were provided for detecting a slowing of free water progression in a potential future clinical trial of a possible disease-modifying therapy. Specifically, for a 50% reduction in the rate of change of free water in PSN over 2 years in the group receiving an experimental treatment versus a placebo group, ~150–200 subjects across groups would be required. These estimates are consistent with the sizes estimated using functional imaging markers such as DaTscan (Table 3). Of note however, when designing future clinical trials of neuroprotective agents, one important aspect to be taken into account is the dropout rate, which would require increasing the sample size. Overall, these imaging-based sample sizes are smaller than the sample sizes of current trials of disease-modifying therapies for Parkinson’s disease, which have not been able to utilize such a marker. Samples from recent Parkinson’s disease studies ranged from sample sizes such as 1176 subjects for the ADAGIO study (Olanow et al., 2009b) and 336 subjects in the ongoing STEADY-PD trial (NCT02168842).

Taken together, the current study validated free water in the PSN as a Parkinson’s disease progression marker at 1- and 4-year follow-up, in an international multi-site cohort of early initially medication-naive Parkinson’s disease patients, and showed a strong association between free water changes and progression of a crucial clinical endpoint. This study takes another step towards validating a novel imaging biomarker for Parkinson’s disease, capable of measuring disease progression, and paving the way for potential future use in testing disease-modifying therapies for Parkinson’s disease.

**Table 3 Sample size estimation for future clinical trials based on relative changes in neuroimaging endpoints**

| Cohort (n) | Outcome | Covariates | Control group mean SD | Within group SD | Sample size estimate assuming 50% effect of a drug | Sample size estimate assuming 70% effect of a drug |
|-----------|---------|------------|------------------------|----------------|-----------------------------------------------|-----------------------------------------------|
| 30 FW PSN (dMRI) | 4-Year change | No | 0.3374 | 0.3225 | 0.5231 | 116 | 154 | 0.7323 | 60 | 80 |
| | | Yes | 0.3374 | 0.2338 | 0.7216 | 62 | 82 | 1.0102 | 32 | 42 |
| 2-Year change | No | 0.3509 | 0.3885 | 0.4515 | 156 | 208 | 0.6321 | 80 | 106 |
| | Yes | 0.3509 | 0.3577 | 0.4904 | 132 | 176 | 0.6866 | 68 | 90 |
| 30 SBR Putamen (DaTscan) | 4-Year change | No | -0.2563 | 0.2068 | 0.6197 | 82 | 110 | 0.8675 | 42 | 56 |
| | Yes | -0.2563 | 0.2115 | 0.6059 | 86 | 116 | 0.8483 | 44 | 60 |
| 2-Year change | No | -0.1434 | 0.1920 | 0.3733 | 226 | 302 | 0.5236 | 116 | 154 |
| | Yes | -0.1434 | 0.1960 | 0.3657 | 236 | 316 | 0.5120 | 120 | 162 |

Relative change is defined as (Year 4 values / baseline value − 1). Covariates include sex and baseline free water for the diffusion tensor imaging power analysis, and sex and baseline SBR for the DaTscan power analysis. Sample estimates are across the two groups (treatment/placebo). DaT = dopamine transporter imaging; dMRI = diffusion MRI; FW = free water; PD = Parkinson’s disease.

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