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Brain Changes in Alzheimer's Disease Patients with Implanted Encapsulated Cells Releasing Nerve Growth Factor

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Abstract. New therapies with disease-modifying effects are urgently needed for treating Alzheimer's disease (AD). Nerve growth factor (NGF) protein has demonstrated regenerative and neuroprotective effects on basal forebrain cholinergic neurons in animal studies. In addition, AD patients treated with NGF have previously shown improved cognition, EEG activity, nicotinic binding, and glucose metabolism. However, no study to date has analyzed brain atrophy in patients treated with NGF producing cells. In this study we present MRI results of the first clinical trial in patients with AD using encapsulated NGF biodelivery to the basal forebrain. Six AD patients received the treatment during twelve months. Patients were grouped as *responders* and *non-responders* according to their twelve-months change in MMSE. Normative values were created from 131 AD patients from ADNI, selecting 36 age- and MMSE-matched patients for interpreting the longitudinal changes in MMSE and brain atrophy.

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[1]Some data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu/). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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Results at baseline indicated that *responders* showed better clinical status and less pathological levels of cerebrospinal fluid (CSF) $A\beta_{1-42}$. However, they showed more brain atrophy, and neuronal degeneration as evidenced by higher CSF levels of T-tau and neurofilaments. At follow-up, *responders* showed less brain shrinkage and better progression in the clinical variables and CSF biomarkers. Noteworthy, two *responders* showed less brain shrinkage than the normative ADNI group. These results together with previous evidence supports the idea that encapsulated biodelivery of NGF might have the potential to become a new treatment strategy for AD with both symptomatic and disease-modifying effects.

Keywords: Alzheimer's disease, cerebrospinal fluid biomarkers, clinical progression, clinical trial, encapsulated cell biodelivery, nerve growth factor, neurofilaments, structural MRI, ADNI, brain changes

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, affecting more than 27 million people around the world [1, 2]. At present, the available treatment is symptomatic, e.g., cholinesterase inhibitors [3] and memantine [4]. Given the dramatic consequences that the disease imposes on patients, their families, and society, new therapies with disease-modifying effects are urgently needed. In this regard, nerve growth factor (NGF) has emerged as a potential candidate. The NGF protein has demonstrated regenerative and neuroprotective effects on basal forebrain cholinergic neurons in animal experiments [5–7]. Basal forebrain cholinergic neurons have wide projections to the cerebral cortex and the hippocampus, and have been hypothesized to degenerate in AD due to the loss of neurotrophic support from their target sites [8]. Nonetheless, these degenerating neurons are still viable for an extended period of time and may thus be amenable to rescuing pharmacotherapeutic interventions, especially in early and intermediate stages of the disease [9].

Since NGF does not pass the blood-brain barrier, local delivery of NGF is needed, but has been achieved only in few previous studies. Normalization of EEG pattern, upregulation of nicotinic receptors, and increased glucose metabolism was observed in three AD patients treated with intracerebroventricular infusion of NGF [10, 11]. However, patients developed side effects, making this route of administration nontolerable and unsafe. On the contrary, no pain-related side-effects were observed when NGF was directly infused into the brain parenchyma of rats [12] and AD patients [13]. Tuszynski and co-workers reported less cognitive decline and increased cortical glucose metabolism as evaluated by PET in six AD patients treated with genetically modified, autologous fibroblasts implanted in the basal forebrain. However, this procedure did not enable to control the injected cells. This limitation was overcome with the development of encapsulated cell biodelivery. The first clinical trial in AD patients using encapsulated NGF biodelivery to the basal forebrain demonstrated that stereotactic surgical implantation and removal of the encapsulated NGF cells was safe, well tolerated, and feasible [14, 15]. Results showed that some of the patients had an improvement in cognitive performance, EEG activity, and *in vivo* nicotinic binding-sites assessed by PET. However, no study to date has analyzed brain atrophy in AD patients treated with NGF.

Brain atrophy in AD is at least in part the result of a sequence of neuropathological changes starting in the hippocampus and entorhinal cortex, spreading later to other limbic structures, and eventually affecting other parts of the cerebral cortex [16]. These macroscopic brain changes can be detected with structural MRI. Two frequently used MRI markers for AD diagnosis and disease progression are whole brain atrophy and hippocampal atrophy. Both markers correlate with cognitive decline and show promise for assessing drug efficacy [17, 18]. In this study we report the MRI results of the first clinical trial in AD patients using encapsulated NGF biodelivery to the basal forebrain [14, 15]. The first aim was to describe structural brain characteristics at baseline as well as longitudinal changes at twelve months follow-up. The second aim was to calculate normative values from the Alzheimer's Disease Neuroimaging Initiative (ADNI) dataset in order to interpret these baseline results and longitudinal changes. The third aim was to study the relationship between brain atrophy and several outcome variables. We hypothesized that improved cognition would be related to less brain shrinkage. Moreover, patients with less brain shrinkage would show better clinical progression, less cognitive decline, and better outcome on cerebrospinal fluid (CSF) biomarker levels.

MATERIALS AND METHODS

Study design and NGF treatment

The study design is described in detail elsewhere [14, 15]. Briefly, six mild to moderate AD patients were enrolled and received the NGF treatment during

12 months (see *NGF cohort* below). Prior to device implantation, all patients underwent baseline evaluations including medical examination, assessment of somatic, neurological and psychiatric status, cognitive tests, MRI, EEG, and PET studies, as well as blood and CSF sampling. Following baseline assessments, the patients were implanted stereotactically under general anesthesia. The first three patients received single bilateral encapsulated NGF implants targeting the nucleus basalis of Meynert (Ch4 region). The other three received double bilateral implants targeting the vertical limb of the diagonal band (Ch2) in addition to the Ch4 region. Stereotactic coordinates are detailed elsewhere [15]. All patients were carefully monitored during the clinical trial. Additional clinical, cognitive, MRI, EEG, PET, and CSF evaluations were performed at 3 and 12 months after implantation. The study was approved by the Swedish Medical Products Agency and ethical approval was obtained from the regional human ethics committee of Stockholm. Both patient and caregiver gave written informed consent prior to study entry.

Participants

NGF cohort: Six patients were recruited from the memory clinic at the Karolinska University Hospital (Huddinge, Sweden) using the following inclusion criteria: 1) NINCDS/ADRDA criteria for probable AD; 2) Clinical Dementia Rating scale (CDR) total score of 0.5 or 1; 3) Mini-Mental State Exam (MMSE) scores between 15 and 24; 4) age between 50–80 years; 5) patient community dwelling and living with a caregiver; and 6) stable cholinesterase inhibitors (ChEI) treatment for at least 3 months before enrollment and remain on stable ChEI for the study period. Exclusion criteria were ongoing medical and/or psychiatric conditions treated with antipsychotic drugs. Stable doses of other baseline medications were maintained. Noteworthy, the diagnosis of AD was histopathologically confirmed in five of the patients from cortical brain biopsies obtained during the implantation procedure. In one patient, biopsy only provided fibrotic tissue but not neurons. Diagnosis on this patient was based on core clinical criteria and supported by an AD profile of pathological CSF and neuroimaging biomarkers.

ADNI cohort: Apart from the six patients described above, we also included a group of AD patients from the ADNI cohort. The ADNI was launched in 2003 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, the Food and Drug Administration, private pharmaceutical companies and non-profit organizations. It is a longitudinal multisite study including over 50 universities and medical centers across the United States and Canada [19]. The project was established to develop standardized imaging techniques and biomarker procedures in normal subjects and Mild Cognitive Impairment and AD patients. For this study we selected the group of AD patients scanned on a 1.5T MRI system and with 12 months follow-up MRI available, according to the recommendations made by Wyman et al. [20]. Data were obtained from the ADNI database (http://adni.loni.usc.edu/, PI Michael M. Weiner). A detailed description of the inclusion criteria can be found on the ADNI webpage (http://www.adni-info.org). Diagnostic criteria for the AD patients were largely the same as for the NGF cohort: 1) NINCDS/ADRDA criteria for probable AD; 2) CDR of 0.5 or 1; 3) MMSE scores between 20 and 26; and 4) age between 55–90 years. Subjects were excluded if they had any significant neurologic disease other than AD. Specific psychoactive medications were excluded. Stable doses of other baseline medications, ChEI among them, were permitted if listed in the ADNI procedures manual (http://www.adni-info.org/Scientists/ADNIStudyProcedures.aspx). The ADNI was approved by the institutional review board at each site. Informed consent was obtained from all subjects.

Magnetic resonance imaging

Data acquisition: Protocols for MRI acquisition were very similar for the NGF and ADNI patients. The NGF patients were scanned on the same 1.5T Magnetom Avanto MR scanner (Siemens, Erlangen, Germany) at the Karolinska University Hospital (Huddinge, Sweden). A three-dimensional T1-weighted MPRAGE sequence was acquired in coronal plane (TR/TE/TI = 2400/2.56/1000 ms; flip angle = 8°; slice thickness = 1.3 mm; FOV = 250 × 250 mm; matrix size = 192 × 192). The ADNI patients were also scanned with a 3D T1-weighted MPRAGE sequence, acquired in the sagittal plane (TR/TE/TI = 2400/3/1000 ms; flip angle = 8°; slice thickness = 1.2 mm; FOV = 240 × 240 mm; matrix size = 192 × 192). Full brain and skull coverage was required and detailed quality control was carried out on all MR images according to previously published criteria [21, 22].

Data analysis: Cortical reconstruction and volumetric segmentation were performed using the FreeSurfer 5.1.0 image analysis suite (http://surfer.nmr.mgh.harv-ard.edu/). To extract reliable volume estimates,

images were automatically processed with the longitudinal stream [23]. An unbiased within-subject template space and image was created for each participant using robust, inverse consistent registration, including time point one (Baseline) and time point two (12 months follow-up) [24]. Image processing was then initialized with common information from the within-subject template, significantly increasing reliability and statistical power. Briefly, this processing includes: (1) motion correction; (2) removal of nonbrain tissue [25]; (3) automated Talairach transformation; (4) segmentation of the subcortical structures [26]; (5) intensity normalization [27]; (6) tessellation of the gray matter white matter boundary; (7) automated topology correction [28]; (8) surface deformation following intensity gradients to optimally place the gray and/or white and gray and/or CSF borders at the location where the greatest shift in intensity defines the transition to the other tissue class [29, 30]; (9) registration to a spherical atlas [31]; (10) parcellation of the cerebral cortex into units based on gyral and sulcal structure [32]; and (11) creation of a variety of surface based data. This segmentation approach has previously been used for multivariate AD classification [33], imaging-neuropsychological analysis [34], imaging-genetic analysis [35], and biomarker discovery [36]. Data management and image processing was done through TheHiveDB [37].

Visual quality control was performed on all output data. All steps involving brain extraction, automated Talairach transformation, tessellation, surfaces reconstruction, and subcortical segmentation were carefully checked. After image processing, volumetric measures were taken from the segmentation routine and an index of brain atrophy was calculated using the following formula:

BV/CSF index = (total GM volume + total WM volume) / total CSF volume

BV stands for brain volume, which is the total volume of the gray matter (total GM volume) plus the total volume of the white matter (total WM volume) in the brain. CSF is the volume of the brain cerebrospinal fluid (lateral ventricles + third ventricle + fourth ventricle + sulcal cerebrospinal fluid). This index represents brain volume in relation to total CSF volume at a given time point (e.g., baseline) or longitudinally (e.g., difference between 12 months follow-up and baseline). Both in normal aging and pathological conditions, the volume of the brain tends to decline with time while the CSF volume increases. Therefore, lower values of the BV/CSF index represent greater brain atrophy (cross-sectionally) or shrinkage (longitudinally). The hippocampal volume for each patient was also analyzed, normalized to each subject's intracranial volume [38].

Outcome measures

The CDR [39] and Instrumental Activities of Daily Living (IADL) scales [40] were applied for assessing clinical and functional status, respectively. Cognition was evaluated using the MMSE [41] and the Alzheimer's disease Assessment Scale-cognitive subscale (ADAS-Cog) [42]. Higher scores on the CDR, IADL, and ADAS-Cog, and lower scores on the MMSE represent worse performance.

Several biomarkers were measured in the CSF. $A\beta_{1\text{-}42}$ was included as a marker of brain amyloid-β protein deposition. Total tau (T-tau) and neurofilaments light (NFL) were studied as markers of neurodegeneration. The CSF samples were aliquoted in polypropylene tubes and stored at -80°C until analysis. The Luminex xMAP technology using the Inno-Bia AlzBio 3 kit (Innogenetics, Gent, Belgium) was performed for the analysis of $A\beta_{1\text{-}42}$ and T-tau as described previously [43]. NFL was analyzed using ELISA as described elsewhere [44].

Statistical analyses

Spearman's rank correlations were conducted to study relationships among variables. Wilcoxon signed-rank test (Z) was used for the analysis of repeated measures. The effect size (ES) was calculated by dividing the Wilcoxon test statistic (i.e., Z) by the $\sqrt{}$sample size [45]. ES values above 0.50 were considered large according to Cohen's benchmark. Means (and Standard Deviations) are presented in the results section. Categorical variables were analyzed with the Chi-square test. In all the analyses, p-values of $p \leq 0.05$ were adjusted with the Bonferroni correction for multiple comparisons. All analyses were performed using SPSS 20.0 for Mac.

RESULTS

Demographic and clinical characteristics at baseline

The NGF cohort consisted of four women and two men with a mean age of 62.2 (6.46) (Table 1). The mean MMSE total score was 22.2 (1.83), with scores between 19 and 24. CDR total scores were either 0.5 or 1. All patients were *APOE* ε4/ε4 homozygotes,

Table 1
Demographic and clinical characteristics at baseline

	NGF	ADNI-Total	NGF versus ADNI-total (*p*-value)	ADNI-matched	NGF versus ADNI-matched (*p*-value)
Sample size, n	6	131		36	
Age, mean (Sd)	62.2 (6.46)	74.8 (7.60)	**<0.001**	67.4 (5.20)	0.084
range	55–73	55–89		55–72	
Gender, n female	4	63	0.373	19	0.527
MMSE, mean (Sd)	22.2 (1.83)	23.4 (1.89)	0.139	22.4 (1.27)	0.838
range	19–24	20–26		20–24	
CDR	0.5 or 1	0.5 or 1	0.985	0.5 or 1	0.999
APOE ε4/ε4 carriers, %	83	23	**0.003**	36	0.076
Duration of ChEI at inclusion in months, mean (SD)	15 (6.93)	na		na	

Bonferroni correction for five comparisons: $p \leq 0.010$. ChEI, cholinesterase inhibitors; ADNI, Alzheimer's Disease Neuroimaging Initiative; APOE, Apolipoprotein E; CDR, Clinical Dementia Rating scale; NGF, nerve growth factor; MMSE, Mini-Mental State Examination; na, non-available information.

except patient 6 who had the *APOE* ε3/ε3 genotype. All patients had been treated with ChEI as concomitant medication for a mean duration of 15 (6.93) months at study start, and continued on stable treatment throughout the study. Patient two was taking donepezil and the other five patients were taking galantamine. Out of the 133 AD patients available from the ADNI dataset, two were excluded due to errors when processing their MRI data. The remaining 131 ADNI AD patients were significantly older than the NGF patients ($t_{(135)} = -3.998$; $p < 0.001$) (Table 1). In order to make the groups comparable in age, ADNI patients beyond the NGF age range of 55–73 years were excluded. Further, ADNI patients with a MMSE total score higher than 24 were also excluded to match the NGF group. The final ADNI-matched subgroup included 36 AD patients. This group was statistically comparable with the NGF group in age, gender, MMSE, CDR total score, and APOE genotype distribution (Table 1). It was not possible to compare the duration of ChEI at inclusion because that information was not available for the ADNI dataset. Nonetheless, 33 out of the 36 ADNI patients had stability in permitted baseline medications, ChEI among them.

Definition of Responders and Non-responders based on the MMSE

The NGF patients were grouped as *responders* and *non-responders* to further evaluate the possible clinical effect of the NGF treatment. This categorization was made based on their 12-months change in the MMSE total score. As standard cut-off, we used the mean value of the 12-months change in MMSE from the ADNI-matched subgroup, which was −2,72 (in practice, a cut-off of −2 points was used, also coinciding with the 12-months typical decline previously described in AD ChEI-treated patients [46, 47]). NGF patients one, four, and six showed decline equal or less than 2 points and were thus considered *responders*. The other three patients (two, three, and five) declined more than −2 points and were considered *non-responders* (Table 2). Following the same procedure, the ADNI-matched subgroup was divided in two groups in order to have specific control groups for *responders* and *non-responders* (see *responders* ADNI control group and *non-responders* ADNI control group in Supplementary Table 1).

Additional MMSE assessments were also performed at 12 months or more prior to the NGF treatment, as well as 15, 19, and 27 months after device implantation. Figure 1A shows the MMSE trajectories for *responders* and *non-responders*. Pre-treatment MMSE trajectories were comparable in both groups. However, *responders* showed a clear improvement in MMSE during the NGF treatment. This pattern was observed for ADAS-Cog as well (Fig. 1B). After treatment, *responders* showed slower rate of decline in MMSE and certain stabilization at 19–27 months follow-up, as compared to *non-responders.*

We also evaluated the possible effect of the number of implants (two implants in Ch4 versus four implants in Ch2 and Ch4). The relationship between response to treatment and number of implants was not significant ($\chi^2 = 0.667$; $p = 0.414$). Further, the number of implants did not correlate with baseline values or longitudinal changes of any of the demographic, neuroimaging, and outcome variables. Figure 1C shows the MMSE trajectories for patients with two and four implants and Fig. 1D and 1E show the percentage of change in the BV/CSF index and hippocampal volume, respectively. As both groups had mostly comparable values, results in the next sections are presented regardless of the number of implants.

Table 2
MMSE total score and BV/CSF index at baseline and change at 12-months follow-up

		MMSE			BV/CSF index	
Cohort		Baseline	Absolute 12-months change	Percentage of change	Baseline	Percentage of change
NGF patients	1 (R)	23	+4	+17%	15.5	−13%
	2	19	−3	−16%	14.8	−19%
	3	24	−9	−38%	20.7	−16%
	4 (R)	21	−1	−5%	15.7	−21%
	5	23	−9	−39%	31.0	−22%
	6 (R)	23	−2	−9%	15.7	−11%
	Mean (SD)	22.2 (1.83)	−3.33	−15%	18.9 (6.30)	−17%
ADNI-matched ($n = 36$)		22.4 (1.27)	−2.72	−12%	22.1 (9.77)	−14%
ADNI-total* ($n = 131$)		23.4 (1.89)	−2.48	−11%	20.0 (9.13)	−10%

Responders are marked with (R), according to their 12 months longitudinal change in the MMSE total score (equal or less than the −2.72 normative cut-off from the ADNI-matched cohort: patient 1 = +4; patient 4 = −1; patient 6 = −2). *Results for the ADNI-total sample are not used in this study but are reported given their normative value; Absolute 12-months change = variation in MMSE points at 12 months follow-up; Percentage of change = percentage of change at 12 months follow-up. ADNI, Alzheimer's Disease Neuroimaging Initiative; BV/CSF index, Brain volume/cerebrospinal fluid volume index; MMSE, Mini-Mental State Examination; NGF, nerve growth factor.

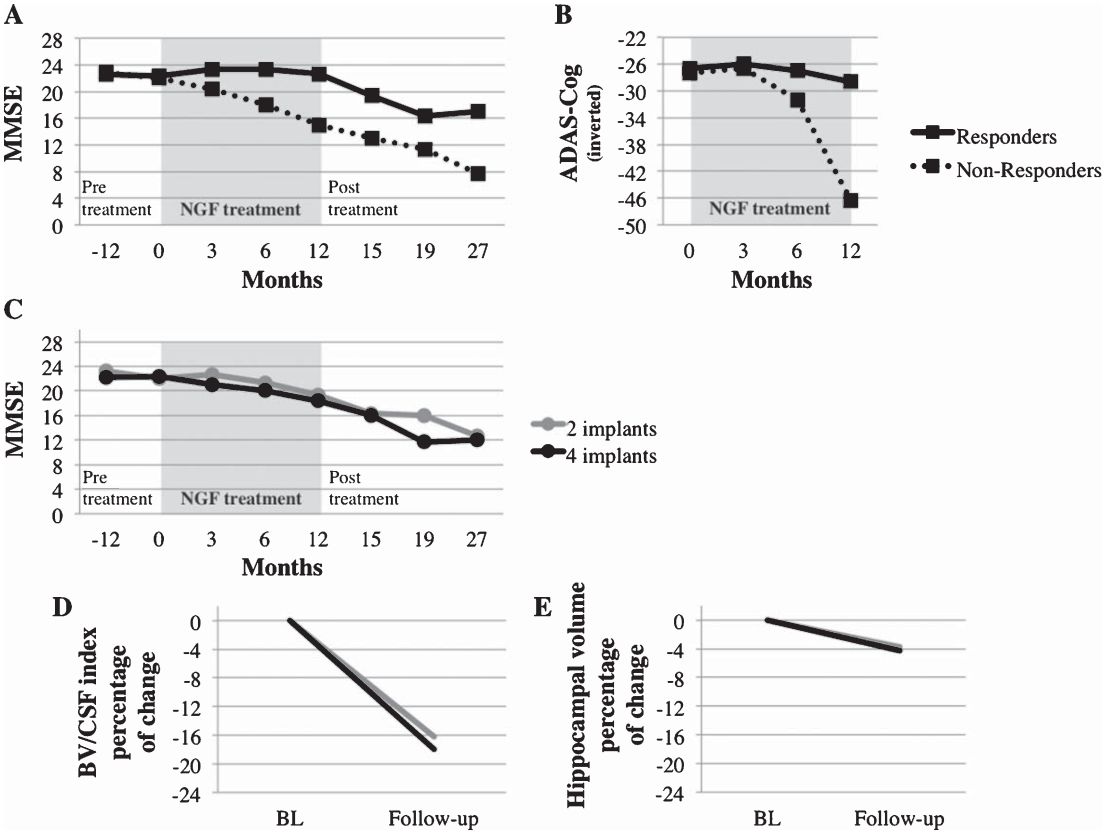

Fig. 1. Trajectories in *Responders* and *Non-responders* (A, B), and in patients with two implants and four implants (C-E). ADAS-Cog scores are inverted in (B) in order to facilitate comparability with MMSE in (A) (lower score means worse performance). Hippocampal volume was corrected by the total intracranial volume before calculating the percentage of change to account for between-individuals differences.

Brain atrophy in Responders and Non-responders

At baseline, the BV/CSF index was lower in *responders* (15.6 ± 0.12), compared to *non-responders* (22.2 ± 8.20) (Table 3 and Fig. 2A). *Responders* were thus initially more severely affected as evaluated by degree of MRI atrophy, although variability was greater among the *non-responders*. This pattern was inversed for the ADNI control groups: at baseline, the BV/CSF index was lower in the *non-responders*

Table 3
Demographic, clinical, cognitive, and neuroimaging variables, and CSF biomarkers in *Responders* and *Non-responders*

	Responders		Non-responders	
	Baseline	Percentage of change	Baseline	Percentage of change
Group size, n	3	–	3	–
Age	60.0 (5.00)	–	64.3 (8.08)	–
Gender, n female	2	–	1	–
MMSE	22.3 (1.15)	+2%	22.0 (2.65)	−32%
AD diagnosis, y	1.7 (1.15)	–	1.7 (0.58)	–
ChEI, months	14.7 (5.51)	–	15.3 (9.45)	–
CDR, 0.5/1	2/1	–	1/2	–
CDR-SOB	3.5 (0.87)	+24%	4.2 (1.44)	+128%
IADL	11.0 (2.00)	+42%	17.0 (3.61)	+49%
ADAS-Cog	26.7 (6.43)	+7%	27.3 (0.58)	+70%
BV/CSF index	15.6 (0.12)	−15%	22.2 (8.20)	−19%
Hippocampus	0.0034 (0.0004)	−4%	0.0034 (0.0004)	−4%
$A\beta_{1\text{-}42}$ (pg/mL)	162.3 (43.98)	+2%	139.7 (24.70)	−11%
T-tau (pg/mL)	161.0 (15.72)	+5%	115.0 (56.79)	−41%
NFL (pg/mL)	286.7 (87.37)	+16%	125.0 (0)	+45%

Mean (Sd) is presented for all the variables except for gender and CDR, where number of females and CDR0.5/CDR1 is reported instead. Hippocampus volume was corrected by the total intracranial volume to account for between-individual differences; Percentage of change = percentage of change at 12 months follow-up. $A\beta_{1\text{-}42}$, amyloid-β-peptide 1-42; ChEI, cholinesterase inhibitors; AD, Alzheimer's disease; ADAS-Cog, Alzheimer's disease Assessment Scale-cognitive subscale; BV/CSF index, Brain volume/cerebrospinal fluid volume index; CDR, Clinical Dementia Rating scale; CDR-SOB, Clinical Dementia Rating scale–sum of boxes; IADL, Instrumental Activities of Daily Living; MMSE, Mini-Mental State Examination; T-tau, Total level of tau protein; pg/mL, picograms per milliliter.

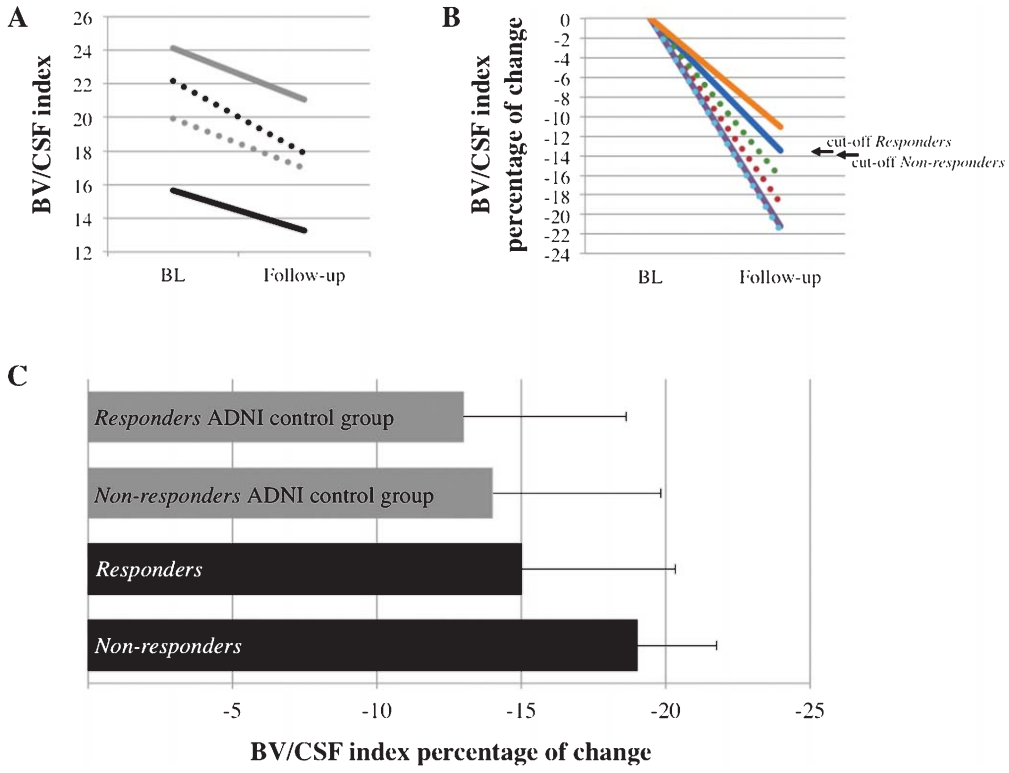

Fig. 2. BV/CSF index in *Responders* and *Non-responders*. A) NGF cohort in black; ADNI cohort in gray; *Responders* and *Responders* ADNI control group in solid lines; *Non-responders* and *Non-responders* ADNI control group in pointed lines. B) *Responders* in solid lines; *Non-responders* in pointed lines; Patient one in solid black, patient two in pointed light gray, patient three in pointed dark gray, patient four in solid dark gray, patient five in pointed black, and patient six in solid light gray; The arrows shows the normative cut-off calculated from the *responders* ADNI control group and the *non-responders* ADNI control group.

ADNI control group (19.9 ± 7.47) compared to the *responders* ADNI control group (24.12 ± 11.27) (Supplementary Table 1 and Fig. 2A). On average, the six NGF patients had a mean BV/CSF index of 18.9 (6.30) at baseline. At follow-up this value declined to 15.6 (4.67), reaching statistical significance ($Z = -2.201$; $p = 0.028$; ES $= -0.90$). Interestingly, *responders* showed slower decline than *non-responders* in the BV/CSF index (Table 3, Fig. 2A, C), while the ADNI control groups showed similar decline (Supplementary Table 1, Fig. 2A, C). Moreover, decline in *responder* patients one and six was above the standard cut-off calculated from the ADNI control group (Fig. 2B). On the contrary, all *non-responders* showed a percentage of change below this standard cut-off (Fig. 2B). Regarding hippocampal volume, both groups had the same mean volume at baseline and percentage of change at follow-up (Table 3).

Normative values of the BV/CSF index for the ADNI-total sample ($n = 131$) are presented in Table 2. At baseline, the BV/CSF index had a mean value of 20.0 (9.13). At follow-up, this value declined to 18.0 (8.30), corresponding to a percentage of change of 10%, and reaching statistical significance ($Z = -9.772$; $p < 0.001$; ES $= -0.85$).

Clinical variables, cognition, and CSF biomarkers in Responders and Non-responders, and correlation with brain atrophy

Table 3 shows the results for the CDR sum-of-boxes, IADL, ADAS-Cog, $A\beta_{1\text{-}42}$, T-tau, and NFL. At baseline, *responders* showed qualitatively less clinical severity and biomarker pathology. In particular, *responders* exhibited less clinical and functional impairment according to CDR-SOB and IADL, and showed higher level of $A\beta_{1\text{-}42}$. On the contrary, they also showed higher levels of T-tau and NFL.

At follow-up, *responders* declined less on the CDR sum-of-boxes, IADL, ADAS-Cog, and $A\beta_{1\text{-}42}$, and showed less increase in NFL compared to *non-responders*. In contrast, they showed more increase in T-tau. When analyzing the longitudinal changes in the six NGF patients as a group, no significant changes were observed once the Bonferroni correction was applied ($p \leq 0.008$ for six comparisons). Nonetheless, some trends were observed in the CDR sum-of-boxes and IADL (CDR-SOB: $Z = -2.214$; $p = 0.027$; ES $= -0.90$; AIDL: $Z = -2.207$; $p = 0.027$; ES $= -0.90$).

Baseline BV/CSF index did not correlate with any other baseline measure. On the other hand, it did significantly correlate with longitudinal changes in $A\beta_{1\text{-}42}$ ($r = -0.943$; $p = 0.005$) (Fig. 3A). Patients with greater brain atrophy at baseline (mostly *responders*) showed less worsening in $A\beta_{1\text{-}42}$. Furthermore, longitudinal changes in the BV/CSF index significantly correlated with longitudinal changes in T-tau ($r = 0.943$; $p = 0.005$) (Fig. 3B), and showed a trend for the IADL at baseline ($r = -0.816$, $p = 0.019$), and longitudinal changes in the CDR sum-of-boxes ($r = -0.912$; $p = 0.011$) ($p \leq 0.008$ after Bonferroni correction) (Fig. 3C, D). The pattern of correlations indicates that patients with less longitudinal brain shrinkage (mostly *responders*) were more functionally preserved (IADL) at baseline and experienced less worsening in disease severity at the follow-up (CDR-SOB). Moreover, they showed stability in T-tau levels.

DISCUSSION

In this study, MRI results are presented from the first clinical trial performed in AD patients using encapsulated NGF biodelivery to the basal forebrain. Patients were grouped as *responders* and *non-responders* according to their twelve-months change on the MMSE total score. At baseline, *responders* showed better clinical status according to the CDR-SOB and IADL, and less pathological levels of $A\beta_{1\text{-}42}$. However, they showed more brain atrophy and neuronal degeneration as evidenced by lower BV/CSF index and increased T-tau and NFL levels. Despite this unfavorable initial profile, *responders* showed considerably better clinical progression as demonstrated by the CDR-SOB, IADL, and ADAS-Cog. According to our definition of response to treatment, *responders* also showed noticeably better progression in the MMSE total score. Moreover, they showed less brain shrinkage at follow-up, stability in both $A\beta_{1\text{-}42}$ and T-tau, and less increase in NFL levels.

The fact that *responders* had more brain atrophy and neuronal degeneration at baseline, but at the same time better clinical status, poses an interesting contradiction. This demarcation between pathophysiological severity and the clinical manifestation of the disease has previously been attributable to brain resiliency or reserve (e.g., cognitive reserve, brain reserve) [48]. The concept of reserve can be understood as the ability to tolerate higher levels of brain injury without exhibiting or exhibiting less clinical symptoms. Therefore, in this study *responders* might have more reserve. The fact that *responders* showed less brain shrinkage, less clinical worsening, and less

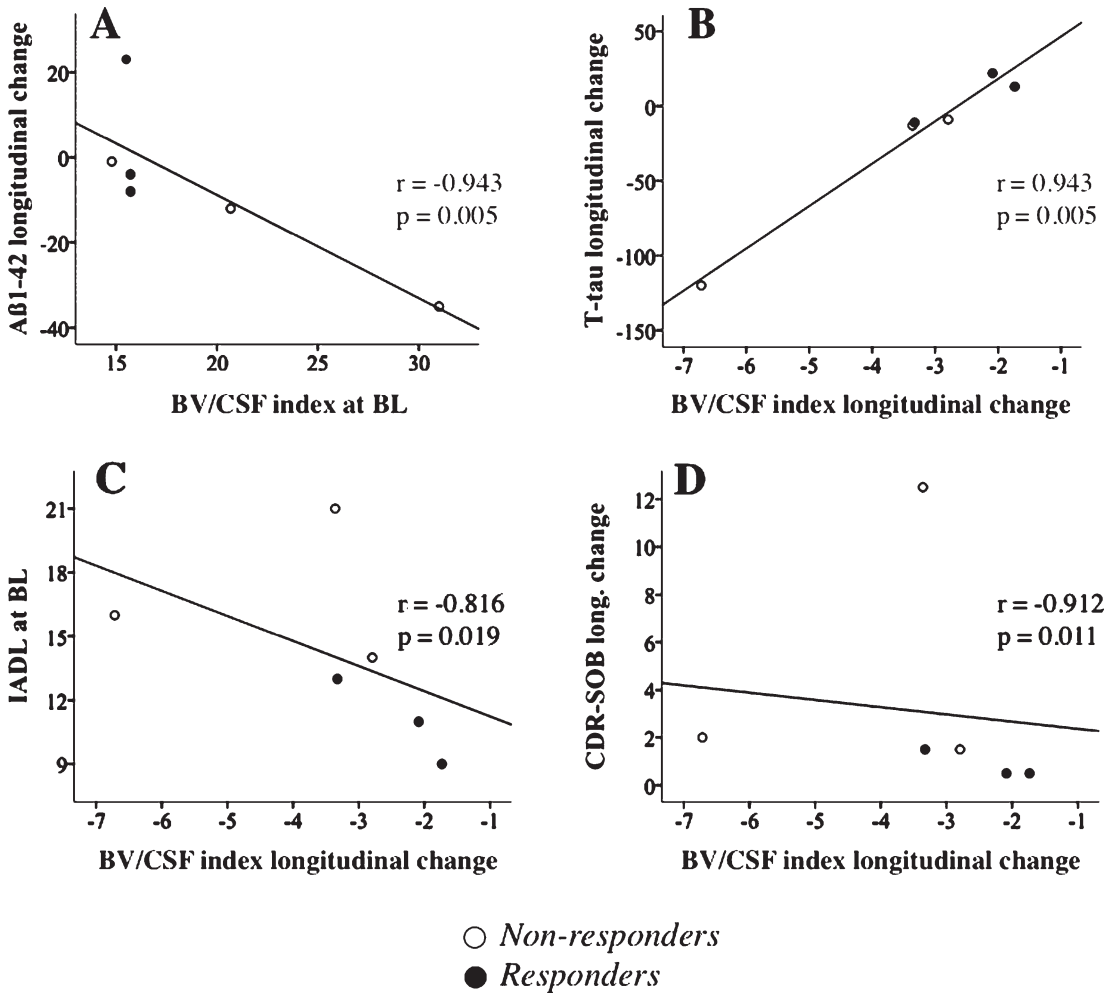

Fig. 3. Correlation between the BV/CSF index and clinical variables, cognition and CSF biomarkers. Y-axis in Figure A, B, and D represents differences (follow-up minus baseline); Y-axis in Figure C represents raw scores in IADL; X-axis represents values in the BV/CSF index at baseline in Figure A, and differences (follow-up minus baseline) in BV/CSF index values in Figure B, C, and D; BL, baseline.

marked CSF biomarker changes at the follow-up supports this hypothesis. Likewise, ADNI patients with better progression in MMSE had less brain atrophy at baseline. It may be argued that *responders* showed less brain shrinkage because they were more atrophied at baseline and thus had less potential range for deterioration. However, their rate of shrinkage was comparable to the one exhibited by the respective ADNI control group, which had less brain atrophy from the beginning (see Fig. 2A). One may also argue that higher reserve per se and not response to treatment might be the main explanation for the trajectories obtained on brain atrophy and the outcome variables. However, it is not likely that the improvement in MMSE obtained in this study is exclusively explained by higher reserve in the *responders*. For example, years of education, a proxy of cognitive reserve [48], was not correlated with either baseline or longitudinal changes in MMSE, both in the NGF patients and the ADNI-matched subgroup (Supplementary Table 1). Given these results, it is tempting to speculate that the biological mechanisms involved in brain resiliency, together with environmental influences and other protective factors such as younger age in *responders*, may have had a positive role facilitating some treatment effect in *responders*. For instance, *responder* patient four had high education and intelligence quotient, was relatively young (57 years old), and was in good health with no comorbidity and no other medication than the anti-dementia drug.

Our definition of *responders* and *non-responders* was based on twelve-month change in the MMSE total score. As standard cut-off, we used the same value from

a subgroup of ADNI AD patients comparable in age, gender, MMSE, disease severity (CDR), and APOE $\varepsilon 4/\varepsilon 4$ distribution. The standard cut-off was set at -2 points. This cut-off is also supported by other studies including AD patients treated with ChEI. For instance, declines of -2.6 and -4 points have been reported after 2 years of donepezil treatment [46, 47], and declines of -6.4 points after 3 years of rivastigmine treatment [49]. Moreover, these figures are confounded by the typical initial improvement in MMSE performance after treatment beginning. Thereafter, treated patients decline at similar rates than a placebo group (minimum 2 points per year) [46]. In this regard, the inclusion criteria in our study required patients to have stable treatment before enrollment. Figure 1 shows the MMSE trajectories for *responders* and *non-responders*. As shown, after comparable pre-treatment trajectories *responders* had a clear improvement in the MMSE during the NGF treatment, with slower rate of decline and certain stabilization at 19–27 month follow-ups, as compared with *non-responders.* Two aspects incline us to speculate that these trajectories in MMSE might be more related to the NGF treatment than to ChEI use. First, our patients should already have reached the stable stage of ChEI treatment benefit, and moreover, changes in MMSE perfectly coincide with implantation. This is supported by the *responders*' pre-NGF slope and the inflection point shown in Fig. 1. Second, the main clinical trials on ChEI demonstrate that treatment with ChEI only manage to moderately modify the decline in MMSE [46, 47]. To our knowledge, the MMSE trajectory showed by the *responders* in this study has not been previously described in AD patients treated with ChEI. Alternatively, our findings could be due to chance, placebo effect, or rater bias. However, the MMSE changes in this cohort are associated with a variety of biological markers. Previous results from these six patients show an association between improved cognition and increased EEG activity and nicotine binding assessed by PET [14], with these markers less prone to chance, placebo effect, or rater bias. Tuszynski and co-workers [13] also demonstrated an improvement in MMSE score and cerebral glucose metabolism after NGF delivery in AD patients. MRI results from automatic segmentation tools (such as the one applied in this study) are also free of rater bias, placebo effect, or chance. Due to this reason, MRI has become a common outcome measure in clinical trials [50].

To our knowledge, this is the first study reporting longitudinal brain changes in AD patients treated with encapsulated NGF producing cells. We also propose the BV/CSF index as a useful marker of global brain atrophy and longitudinal shrinkage. Noteworthy, we report normative values for this index from the ADNI study, worldwide reference for studying biomarkers of AD. Brain atrophy in the ADNI-total AD sample ($n = 131$) is 19.95 (9.13) at baseline and the twelve-months percentage of shrinkage is -10%. These values are presented in Table 2 for their normative use. Using the cut-off calculated from the respective ADNI control group, we found that the rate of shrinkage was slower in two of the three *responders.* The other *responder* patient had the lowest percentage of change in brain volume (also below the ADNI control cut-off), but had the highest CSF volume increase (Supplementary Fig. 1). This patient was the one with more years of education and higher intelligence quotient, two proxies of cognitive reserve [48]. However, this patient had presented disease symptoms during only one year. This is consistent with recent studies showing that high reserve may primarily influence the ability to tolerate AD pathophysiology for a longer period of time, but may also be associated with rapid decline after a "tipping point" is reached and compensatory mechanisms begin to fail [51, 52].

The cholinergic neurons located at the basal forebrain have wide projections to the cerebral cortex and the hippocampus [53, 54]. Given that neurons located in the vertical limb of the diagonal band (Ch2) directly project to the hippocampus, we determined whether the three patients with implants in Ch2 and Ch4 had less hippocampal decline than those with implants only in Ch4. However, both groups showed the same rate of hippocampal atrophy. In addition, as commented above, *responders* exhibited less global brain shrinkage at follow-up compared to *non-responders*, but hippocampal atrophy was similar in both groups. Therefore, the results of this study suggest a more widespread response in the brain, consistent with wide cerebral projections, rather than a specific reaction limited to the hippocampus. The fact that the Ch2 region is usually less affected in AD than the Ch4 region could explain this result [55].

The main limitation of this study is the small number of patients in the NGF cohort. The exceptional characteristics of the encapsulated NGF therapy make this method subject to strict regulatory and ethical conditions at this phase of the research. Acknowledging this, we carefully designed the statistical approach and performed a rigorous analysis of the data. We also included the ADNI-matched subgroup in order to have a widely accepted external reference to support the evaluation of longitudinal changes in MMSE and the

BV/CSF index. A control group from the same center was not included in the clinical trial because the primary objective was to explore procedures' safety and tolerability [14, 15]. Although using the ADNI group may decrease the direct comparability of results, it has other advantages such as counting on a substantially bigger sample for the calculation of normative values. Noteworthy, the diagnosis of AD was histopathologically confirmed in five of the six NGF patients and inclusion criteria allowed us to recruit a quite homogeneous group of patients in terms of demographic, clinical, and biomarker characteristics. These aspects make it possible to reduce sample size in clinical trials [56, 57]. In addition, the pattern of findings presented here is coherent itself and also with previous reports from the same cohort [14, 15]. Nonetheless, these results must be interpreted with caution and confirmed in larger studies. A second drawback in this study is the non-expected T-tau concentration at the follow-up. *Non-responders* showed a decrease of −31% in T-tau, while *responders* remained basically stable. Nevertheless, this percentage is confounded by *non-responder* patient five. This patient showed decreased T-tau (−67%) but increased NFL (+108%), which is contradictory because both measures should change in the same direction (see Supplementary Fig. 2). Given that the other two *non-responders* showed stability in T-tau levels, and that longitudinal changes in T-tau were not significant either in *non-responders* ($Z = -1.604$; $p = 0.109$), or in the six NGF patients as a group ($Z = -0.420$; $p = 0.674$), stability in T-tau levels in *non-responders* seems to be a more appropriate interpretation. Other limitation is that, although our results might be explained by factors related to brain resiliency (e.g., cognitive reserve), a bigger sample as well as other proxies of cognitive reserve (e.g., engagement in social and intellectual activities, etc.) and brain reserve (e.g., status of functional brain networks) are needed to profoundly test this hypothesis. Finally, possible response to treatment was based on the MMSE given that clinical progression and cognitive performance is one of the main outcome measures in current clinical trials. Since MMSE is a crude measure of cognition, the use of other outcome measures could have been more robust. Of note is that the ADAS-Cog results were in line with the MMSE changes in each individual patient. In this study we propose MRI as a more robust outcome measure. Results on MRI were associated with clinical progression and MRI is less prone to bias than assessment of cognitive performance. Besides the anticipated structural brain response, the inclusion of the functional MRI-resting state modality in future studies of this kind may be of great interest to analyze a possible response in brain connectivity. In addition, we are currently working on alternative outcomes based on cholinergic CSF biomarkers.

In conclusion, we have previously demonstrated that deep brain implantation of encapsulated NGF-producing cells is feasible, safe, and well tolerated by AD patients [14, 15]. Moreover, improved cognition was accompanied with improvement in nicotine binding and EEG activity in a subset of the six AD patients also included in the current study [14]. In this study, improved cognition was also associated with less brain atrophy. Moreover, this slower rate of brain atrophy was positively related with clinical progression, cognitive decline, and CSF biomarker progression. These results support the idea that encapsulated biodelivery of NGF might have the potential to become a new treatment strategy for AD with both symptomatic and disease-modifying effects. Although this strategy is probably not suitable for all AD patients, it may become an option for those AD patients that are younger, have mild clinical and cognitive impairment, and high cognitive or brain reserve. We also tested the usefulness of the BV/CSF index for monitoring brain longitudinal changes. We believe that this BV/CSF index may be a useful outcome measure not only for future clinical trials, but also for any research where a marker of brain atrophy and shrinkage is needed. It can be used in other neurological conditions and, interestingly, given its simplicity, it could be easily applied in clinical routine for monitoring dementia progression, stratifying patients according to their degree of brain atrophy, or staging the disease based on a MRI marker.

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SUPPLEMENTARY MATERIAL

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