Neurovascular dysfunction in Alzheimer's disease: assessment of cerebral vasoreactivity by ultrasound techniques and evaluation of inflammatory markers and circulating progenitor cells

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Abstract: Aims: Neurovascular and endothelial dysfunction play an important role in neurodegeneration and cognitive decline of Alzheimer's disease (AD). The aim of this study was to assess the vascular dysfunction in patients with AD by investigating the frequency of circulating progenitor cells (CPCs), including hematopoietic progenitor cells (HPCs) and endothelial progenitor cells (EPCs), which have proangiogenic properties and provide a cellular reservoir for the endothelial replacement, and the blood concentration of vascular/inflammatory markers. Moreover, we want to assess the cerebral vasomotor reactivity in both patients with AD and healthy control subjects by using a Transcranial Doppler Ultrasound (TCD) study and to evaluate the possible correlation between cerebral vasoreactivity and endothelium dysfunction. Finally, we wanted to evaluate if the changes in both CPCs and cerebral vasomotor reactivity are correlated with cognitive decline. **Materials and Methods:** We recruited thirty-five AD subjects, matched for age, sex and education to seventeen healthy control subjects. All the subjects underwent brain MRI, Neuropsychological evaluation and Carotid Duplex Ultrasonography. Cerebral vasomotor reactivity was assessed using the TCD based breath-holding index test (BHI). The level of CPCs was evaluated by flow cytometry from venous blood samples and blood neurovascular/inflammatory markers were measured by enzyme-linked immunosorbent. **Results:** Both Cerebral assays blood flow velocity at the steady-state (CBFV) and BHI values were significantly lower in AD subject than in healthy control (48,55 ± 7,9 cm/s vs 55,9 ± 5,11 cm/s, $p<0,05$; 1,00 ± 0,32 vs 1,31 ± 0,29, $p<0,05$). A positive trend of correlation was found between CBFV and BHI values and MMSE scores. Then, we found that patients with AD had lower $CD45^{dim}/CD34^{+}/CD133^{+}$ HPCs counts than controls (51,43 ± 14,6 vs 62,7 ± 18,2, $p<0,05$), but such a lower HPCs number was not associated with lower MMSE. The level of circulating CD34+ cells has not been found to be correlated with CBFV and BHI in patients. Finally, a significant increased expression of MCP-1 and a trend to overexpress the chemokine RANTES was observed in AD patients as compared to healthy controls. **Conclusions:** Our results confirm that cerebral hemodynamic deterioration might be a critical marker of cognitive decline, as demonstrated by TCD study. Moreover, the results provided evidence that patients with AD have reduced circulating HPCs and a tendency to have increased inflammatory markers, but without showing a significant correlation with cognitive decline.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common type of dementia. In accordance with the vascular hypothesis of AD, first proposed by de la Torre (1), vascular risk factors might play a critical role in the development of cognitive decline. Moreover, several studies (2-4) suggest that neurovascular dysfunction might plays an important role in AD. It is now well established that the amyloid-β peptide (Aβ), a key pathogenic factor in AD, has powerful cerebrovascular effects that alter the regulation of the cerebral circulation and Aβ-induced endothelial dysfunction may be responsible for the cerebrovascular dysregulation observed in patients with AD (2). The structural and functional alterations of the brain vessels in AD markedly disrupt the homeostatic balance and cause neurovascular dysfunction and synaptic dysregulation leading to brain dysfunction (3, 4). On the other hand, in large intracranial vessels, atherosclerosis is found in more than 77 % of AD patients. Furthermore, at the microvascular level, arterioles and capillaries show reduction in density, length, and mean diameters in AD (2, 5). The consequence of these anatomical alteration is a decrease of both cerebral blood flow (CBF) and functional hyperaemia. In particular, endothelium-dependent vasodilatation is altered in peripheral vessels of AD patients (6). In addition, cerebral smooth muscle cells are converted into a hypercontractile phenotype, which leads to the stronger contractility of cerebral arterioles associated with reduced resting CBF and reactivity (7).

The vascular status of brain circulation can be assesses by neurosonological methods such as Colour Doppler flow imaging and functional transcranial Doppler sonography (TCD) (8). Cerebral vasomotor reactivity is a neurosonological parameter that may be defined as the vasodilatatory capacity of cerebral arterioles to external stimuli, providing information on cerebral hemodynamic status (9).

Peripheral blood–derived $CD34^+$ cells are circulating progenitor cells (CPCs), enriched for endothelial lineage potential. In particular, the fraction of $CD34^+/CD133^+$ cells expressing low levels of CD45 ($CD45^{dim}$) has been identified as hematopoietic progenitor cells (HPCs) able to home to ischemic or neoplastic tissues that secrete chemo-attractants and, following differentiation, to contribute to angiogenesis by secreting proangiogenic factors (10). Among those, the small subpopulation of $CD34^+/CD133^+$ cells which coexpress the VEGF-receptor 2 (KDR) is more enriched in specific endothelial progenitor cells (EPCs) (11, 12). Overall, CPCs, including HPCs and EPCs may support neurovascularization and participate in the maintenance of the endothelium by acting as a cellular reservoir for the replacement of dysfunctional endothelial cells, or by releasing angiogenic growth factors (13). Furthermore, the angiogenic process is mediated by several inflammatory cytokines, including chemokines like MCP-1 (monocyte chemotactic protein-1) and RANTES (regulated upon activation, normal T-cell expressed and secreted)/CCL5 (CC chemokine ligand 5), which in turn recruit pro-angiogenic immune cells and endothelial progenitors, promote endothelial cell migration and regulate endothelial function downstream of activation of G-protein coupled chemokine receptors (14-16). Cell adhesion molecules like sICAM-1 (soluble intercellular adhesion molecule-1), whose raised levels may be an indicator of activation of endothelial cells and VEGF (vascular endothelial growth factor) may both account for changes in angiogenesis and vascular repair responses (17, 18). Given the evidence for neurovascular dysfunction and endothelial pathology in AD, whether there are changes in the pool of CPCs and in neurovascular/inflammatory mediators needs to be determined to clarify their influence on the pathogenesis of AD.

Despite the evidence supporting the role of neurovascular and endothelial dysfunction in AD, its routine measurement in the clinical setting is challenging. The main goal of this study were to assess the cerebral vasomotor reactivity in both AD patients and health control subjects by using a

Transcranial Doppler Ultrasound (TCD) study and to assess the endothelium dysfunction by investigating the levels of HPCs and EPCs, as well as blood concentration of inflammatory vascular mediators. Furthermore, we wanted to evaluate if the changes in both circulating cells and inflammatory vascular mediators as well as in cerebral vasomotor reactivity are correlated with the cognitive decline of AD patients.

Materials and Methods

Subjects

We evaluated fifty-five AD subjects (mean age ± SD: 73,9 ± 3,5 years; 20 males; mean education ± SD: 9,8 ± 4,7 years), according to NINCDS-ADRDA criteria (19), matched for age, sex and education to twenty-five healthy control subjects (mean age ± SD 72,3 ± 4,9 years; 9 males; mean education ± SD: 10,7 ± 3,5 years), during a twelve-month period, from the Dementia Centre of Sant'Andrea Hospital (Rome, Italy). All subjects underwent TCD examination but twenty AD subjects and eight healthy control subjects did not have an adequate acoustic window to assess the vascular status of brain circulation. Therefore, we enrolled in the study thirty-five AD subjects (mean age ± SD: 72,5 ± 5,1 years) and seventeen healthy control subjects (mean age ± SD: 70,4 ± 5,6 years).

A full evaluation was made for each subject, including medical history, complete physical examination, neuropsychological assessment, cerebral imaging by Magnetic Resonance Imaging (MRI) and Carotid Duplex Ultrasonography. Cardiovascular history was established from medical records. Diagnosis of cognitive impairment was determined by a multidisciplinary staff and inclusion criteria were mild or moderate cognitive impairment defined as a score of Mini Mental State Evaluation (MMSE) >15. Subjects were excluded if they had a age major than 80 or less than 65 years, clinical history of Cardiovascular Disease (CVD), severe subcortical leukoencephalopathy or cortical infarction on MRI, carotid stenosis major than 40% as well as medical conditions and treatments interfering with the anatomic and functional properties of cerebral vessels. Subjects were also excluded from the AD group if they had evidence of other neurodegenerative disorders or cognitive impairments resulting from other diseases or a coexisting medical condition that could interfere with cognitive evaluation. The ethical committee has approved this study and informed consent was obtained by each participant.

Vascular risk factors

The presence/absence of four vascular risk factors was assessed from interviews with both subjects and informants: 1) arterial hypertension: history of blood pressure measurements greater than 160/95 mmHg or antihypertensive medication intake; 2) diabetes mellitus: plasma glucose level major than 110 mg/dl or antidiabetic drug intake; 3) hypercholesterolemia: serum cholesterol level over 220 mg/dl or statin intake; 4) smoking: more than five cigarettes per day for at least 5 years; not smoking: less than five cigarettes per day or stopped smoking for 10 years.

Neuropsychological assessment

All subjects underwent a standard neuropsychological evaluation used as a screening tool in our Dementia centre. Global cognitive functioning was assessed using the Mini-Mental State Examination (MMSE) (20), whereas other areas of cognition were investigated by means of the following tests: selective attention (Visual Search—Attentional matrices) (21), episodic long-term memory (Story Recall test) (22), non-verbal logical reasoning and problem-solving ability (Raven's Coloured Progressive Matrices) (23), word generation by phonological and semantic cues (Phonological and Semantic Verbal Fluency test) (22), auditory comprehension of complex sentences (Token test) (20), and spatial abilities and constructional praxis (Copying Drawings) (21). The raw

scores of each test were adjusted for age and education according to the distribution of Italian normative data. Functional status of subjects was assessed using Activities of Daily Living (ADL) (24) and Instrumental Activities of Daily Living (IADL) scales (25).

Brain magnetic resonance imaging

Brain MRI was obtained in all AD subjects using a 1.5-T scanner with the spin-echo technique and T1-, T2-weighted, and fluid-attenuated inversion-recovery sequences to detect possible white matter hyperintensities (WMH). These were graded according to Fakekas score, which provides an overall impression of the presence of WMH in the entire brain (26): only patients without vascular lesions (grade 0) or with small subcortical focal lesions (grade 1) were included.

Trans-Cranial Doppler ultrasound

We used the trans-temporal acoustic window to evaluate the flow velocity of the middle cerebral artery (MCAFV). Two dual 2-MHz transducers fitted on a headband and placed on the temporal bone windows were used to obtain a bilateral continuous measurement of flow velocity in the middle cerebral arteries. Depth of insonation ranged from 48 to 52 mm. Cerebral vasomotor reactivity was assessed using the TCD based breath-holding index test (BHI) (27), obtained by dividing the percent increase in flow velocity (MFV) occurring during breath-holding by the length of time (seconds) subjects hold their breath after a normal inspiration [(MFV at the end of breath-holding - rest MFV)/rest MFV x 100/s of breath-holding]. Mean flow velocity at rest was obtained by the continuous recording of a 1-minute period of normal breathing, then subjects were asked to hold their breath for 30 seconds, all subjects were normocapnic and able to hold their breath for the required period. Patients performed three evaluations, and the BHI values included in the analysis were the means of the three tests and of right and left values. TCD is a totally non-invasive and low-cost ultrasound technique, but has some limitations, indeed almost 10% of individuals have no sonic windows (28) and a similar percentage of individuals have low-quality bone windows (29), especially post-menopausal women.

Circulating progenitor cells

We analysed the level of CPCs in twenty-four AD patients (72,7 ± 5) and in nine healthy control subjects (70,8 ± 8). Twenty-six AD subjects and sixteen healthy control subjects were excluded because of laboratory logistic problems. We analysed the two CPC populations, HPCs and EPCs. The frequency of CPCs was assessed by flow cytometry analysis on fresh whole blood within 4 h after sampling. Peripheral blood was drawn into 10-ml tubes (EDTA Vacutainer, BD Biosciences, San Diego, CA, USA) and, to obtain peripheral blood leucocytes (PBL), red cell lysis was performed by addition of lysing solution (Becton Dickinson) for 30 minutes in the dark, then washed twice with PBS. PBL counts and viability were determined before and after red blood cells lysis by Trypan blue exclusion.

CPCs frequency was assessed by flow cytometry analysis as described elsewhere (12). Briefly, 5 x 10^6 PBL were incubated 20 minutes at +4°C in the dark with anti-CD34-FITC (clone AC136), anti-CD45-PerCP (clone 5B1), anti-CD133-PE (clone AC133) and anti-CD309 (VEGFR-2/KDR)-APC (clone ES8-20E6), and their isotype-matched monoclonal antibodies (all mAbs from Miltenyi Biotech). Then, stained cells were washed twice and fixed on PFA 1%. PBL were acquired with a four-color FACScalibur flow cytometer (BD) running CellQuest software (BD) with a gating strategy reported in Figure 1. In order to increase the number of EPCs analyzed, two different acquisitions were performed for each sample. In the first, 10^5 whole PBLs were acquired. In the second acquisition, 4 x 10^6 PBLs were analyzed, but only the $CD34^+$ cells were acquired. CPCs subpopulation

levels were calculated in acquired $CD34^+$ cells (gate R1) as a percentage of $CD45^{dim}/CD34^+/CD133^+$ for HPCs and as $CD45^{dim}/CD34^+/CD133^+/CD309^+$ cells for EPCs.

Inflammatory/vascular mediators

Since the angiogenic process is mediated by several potent cytokine mediators, we also evaluated neuroinflammation/endothelial indices, including MCP-1(CCL-2), RANTES (CCL-5), soluble ICAM-1 (sICAM-1) and VEGF. Briefly, plasma concentrations of MCP-1, RANTES and sICAM-1 were measured with commercially available sandwich ELISAs (Duo Set ELISA, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. In order to obtain values within the calibration curve, before the assay the plasma samples were thawed at room temperature and used undiluted for MCP-1 and diluted 1:40 for RANTES, 1:500 for ICAM-1. Regarding VEGF, because of its estimated lower concentration in plasma (30), it was optimally identified in serum and measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Values of mediators are expressed in pg/ml.

Statistics

Continuous variables are reported as mean ± standard deviations (SD), whereas categorical data were presented using counts and percentages. TCD data (MCAFV and BHI) were compared by using t–test for paired sample. Frequency of cell populations and blood concentration of inflammatory/neurovascular factors between AD and HC subjects were compared using Mann-Whitney U-test. Spearman's non-parametric rank correlation was used to correlate the TCD data and CPCs count with MMSE, ADL, IADL and other neuropsychological tests scores. Finally, the differences in the presence/absence of vascular risk factors in the two subject groups were evaluated by Chi-square and Fischer's Exact tests. Statistical significance was reached when $p<0,05$.

Results

The demographical and clinical data of the 35 AD subjects and the 17 healthy control subjects enrolled in the study are summarized in tables 1. There was no significant difference among the two groups in the demographical characteristics and the presence of vascular risk factors. Regarding the results of the TCD evaluation, both MCAFV and BHI values were significantly lower in AD subject than in healthy control subjects (45,1 ± 8,6 cm/sec vs 52 ± 9,1 cm/sec, $p<0,05$ and 1 ± 0,3 vs 1,3 ± 0,3, $p<0,05$, respectively) (Figure 2). A statistically significant correlation was found between the severity of cognitive impairment evaluated by MMSE score and both MCAFV and BHI values ($r=0,427$, $p=0,008$ and $r=0,383$, $p=0,02$; Spearman's correlation) (Figure 3). Considering the correlation between each neuropsychological test and the cerebrovascular reactivity indices we found a significant correlation between BHI and Digit Span test score ($r=0,535$, $p=0,009$; Spearman's correlation) and Rey Complex Figure Test ($r=0,550$, $p=0,006$; Spearman's correlation). We also found a significant correlation between IADL score and both MCAFV and BHI values (MCAFV: $r=0,409$, $p=0,015$; BHI: $r= 0,423$, $p=0,011$; Spearman's correlation) (Figure 4). On the contrary we did not find any significant differences between Fazekas scores and cerebrovascular reactivity indices (MCAFV: 44,2 ± 9,9 cm/sec vs 45,5 ± 8,4 cm/sec $p=0,692$; BHI: 0,97 ± 0,36 vs 1 ± 0,24, $p=0,560$).

We did not observe a significant statistical decrease in $CD45^{dim}/CD34^+/CD133^+/CD309^+$ EPCs counts or in the other endothelial biomarker (VEGF) in patients with AD, when compared to healthy controls (table 2). However, we found that subjects with AD had lower $CD45^{dim}/CD34^+/CD133^+$ HPCs counts than control subjects (51,4 ± 14,6 vs 63,9 ± 18,25 $p=0,02$) (Table 2), but the HPCs count value was not associated with the MMSE score ($r=0,183$, $p=0,32$; Spearman's correlation). The level of circulating CD34+ cells has not been found to be correlated with cerebrovascular reactivity indices

(MCAFVV and BHI) in AD patients. Furthermore, there was no correlation between both HPCs and EPCs and the clinical data of AD patients.

Finally, we reported a difference in blood inflammatory/vascular mediators between AD and healthy controls, with a significant higher chemokine CCL2 (MCP-1) level in AD patients (Figure 4), and a trend to overexpress the chemokine CCL5 (RANTES) in AD patients as compared to healthy controls (CCL2: 76 ± 51 pg/ml vs 39 ± 22 pg/ml p=0,04 and CCL5: 49,9 ± 7,9 pg/ml vs 44,5 ± 8,5 p=0,09). However, both levels of CCL2 and CCL5 did not correlate with cognitive impairment and cerebrovascular reactivity indices in AD patients.

Discussion

AD classical pathological hallmarks are intracellular neurofibrillary tangles in neurons and extracellular amyloid-β (Aβ) deposition in amyloid plaques, however cerebrovascular alterations are also present and may contribute to the pathogenesis of the disease (31, 32). Our results show that patients with AD, in comparison with healthy aged matched subjects, have a disturbance in their cerebrovascular hemodynamics, showing changes in both structure and dynamics of their cerebral blood flow circulation. That is suggested by the evidence of a reduction in CBF and the increase of the rigidity of the arterial walls, with consequent reduction of vascular compliance. In particular, MCAFV reduction is correlated with the severity of the cognitive decline, suggesting that it can influence the cognitive decline in AD subjects. Moreover, the BHI, a vasoreactivity index, was lower in AD patients and correlates with a lower MMSE score. The chronic cerebral hypoperfusion has already been proposed as a determinant factor in the accompanying cognitive deficits. It is proved that cerebral hypoperfusion can be a pathophysiologic trigger of AD. Impaired CBF in AD may be related to an engagement of microvessels as a possible consequence of various pathological processes like cerebral amyloid angiopathy, arteriolosclerosis, capillary endothelial, and basement membrane changes. Indeed, alteration of cerebral vasomotor reactivity, in the absence of neck vessels severe stenosis, may reflect increased arteriolar wall stiffness attributable to intrinsic anatomical changes (27). In the present study, we did not correlate the presence of vascular risk factors with MMSE worsening and with the changes in the cerebrovascular reactivity indices. The presence of WMH, evaluated by Fazekas score, did not correlate with altered cerebral hemodynamics. Although previous studies suggest that low cerebral blood flow and changes of cerebrovascular reactivity are related to the severity of white matter hyperintensities upon T2 weighted MRI (33), all our patients had minimal focal vascular subcortical lesions on MRI (Fazekas score 0 and 1). Furthermore, observing the results concern to the individual neuropsychological tests, we only found a correlation between BHI value and the cognitive domain of memory evaluated by the Digit Span test. We can explain it considering the high involvement of the MCA, explored by TCD study, in the supply of cerebral territory of memory domain. Our results confirm that, as demonstrated by previous studies, TCD is a valuable method to study the hemodynamic changes in patients with AD.

Endothelial dysfunction is considered to be one of the pivotal mechanisms of the structural and functional cerebral vessel alterations in AD (34, 35). Moreover, early endothelial failure and subsequent blood brain barrier (BBB) breakdown are hypothesized to be major precipitants of AD pathogenesis (34, 35).

Giving the evidence for neurovascular and endothelial dysfunction in AD, whether there are changes in the pool of CPCs needs to be determined to clarify their role in the pathogenesis of AD and to develop potential therapies for promoting endothelial regeneration. Previous studies have shown a reduction of CD34+ cells in dementia patients relative to normally aging controls (13) and then supported the notion that proangiogenic cells (CD34+CD133+ HPCs) are reduced in AD (36).

Moreover, there is evidence that CD34+ cells in general may be reduced in AD, where they correlate with amyloid-β levels in the cerebrospinal fluid (CSF) (37). A recent study suggest that the reduction of CPCs in older adults is associated with worse memory function, diagnosis of mild cognitive impairment (MCI), thinning of posterior cortical regions and hyperperfusion of the bilateral hippocampus, all of which are indicators of pathological aging linked to increased risk of AD dementia (38).

We investigated the levels of CPCs, including HPCs and EPCs in patients with AD and healthy control subjects and the association of any changes with cognitive impairment. We only found that patients with AD have reduced circulating HPCs and that results may suggest that an abnormal capacity to regenerate endothelium is associated with AD (13, 36). However, according to recent literature, the absence of a significant correlation with cognitive decline suggests that CPCs, as an endothelial biomarker is not valuable for the diagnosis and evaluation of cognitive evolution in AD, possibly because they may be too sensitive and not specific enough in older population, whatever the underlying mechanism may be (39). We also noted that CPCs levels were not associated with vascular risk factors or WML volume, but the small sample size of our subjects do not allow us to make general consideration on the association between vascular risk and progenitor cell levels in the general population.

Recently, several studies have reported an involvement of neuroinflammation in AD pathogenesis (40). Highly insoluble Aβ peptide deposits and neurofibrillary tangles could lead to microglial activation, leading to production proinflammatory cytokines and chemokines, and recruiting inflammatory cells into the brain, finally inducing neuronal dysfunction and damage (41). Cerebral hypoperfusion, up-regulates the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), markers of endothelial cell activation, and may be responsible for the increase of cytokines and chemokines production (17, 34). There is evidence suggesting that CCL2 (MCP-1) plasma level could be particularly important and possibly indicated as a biomarker to monitor the inflammatory process in AD (42, 43). Our results confirm the presence of a higher level of CCL2 in AD patients, however the absence of correlation between CCL2 and MMSE scores suggests that it may be not specific enough. A possible explanation may be given by a previous study, which revealed that CCL2 levels in the cerebro-spinal fluid (CSF), and not in plasma, are associated with an increased progression rate in prodromal AD, indicating that the inflammatory processes important for the disease progression in AD are primarily localized in the brain (44). From previous studies (43), we know that oxidative stress upregulates CCL5 (RANTES) expression in brain endothelial cells and increases CCL5 contributed to the recruitment of immune cells leading to increased neuronal death: this may explain the overexpression of the chemokine CCL5 in our AD patients; however further investigation are needed to better understand its role in neurodegeneration.

The importance of this study lies in the possibility that the assessment of cerebral vasoreactivity and endothelial dysfunction may contribute to facilitate an earlier AD's diagnosis, to identify patients at high risk of cognitive decline and to promote the development of targeted therapies to improve cerebral microcirculation. Therefore, our results indicate how the cerebral hemodynamic deterioration is a critical marker of cognitive decline and stand up once again the hypothesis of a significant pathogenetic role of vascular damage in AD (6). Further researches are needed to fully establish whether altered cerebral hemodynamics may be considered an independent factor in predicting cognitive decline or an effect of pathologic processes involved in AD (45). Moreover, our findings are consistent with the progenitor reserve hypothesis (38), which states that circulating progenitor cells are reduced in dementia due to AD, however further studies are needed to better

establish how progenitor cells may relate to disease progression and clinical manifestation. A major limitation of this study is the small sample of patients included, especially considering the prognostic value of biomarkers in patients with rapid cognitive decline.

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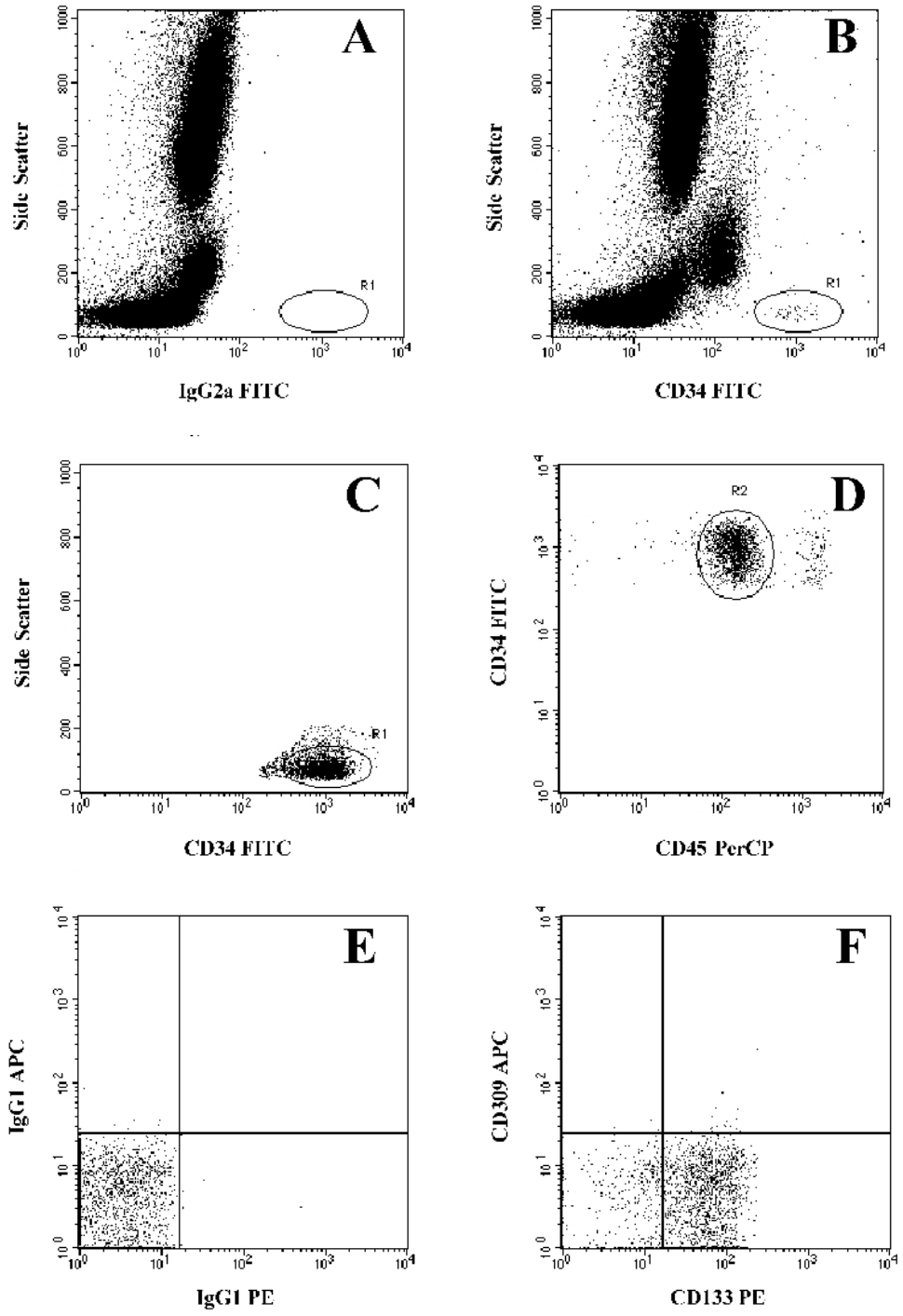
Table 1: Demographical and clinical data of AD subjects and healthy control subjects (MMSE: Mini Mental State Evaluation; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living).

	Healthy control group (n=17)	**AD group (n=35)**	**P values**
Age (years) (mean ± SD)	70,4 ± 5,6	72,5 ± 5,1	0,18
Sex (M/F)	7/10	13/22	>0,999
Education (yrs) (mean ± SD)	10,2 ± 3,3	9,6 ± 4,2	0,61
MMSE (mean ± SD)	30	22,6 ± 4,6	**<0,05**
ADL (mean ± SD)	6	5,4 ± 0,6	**<0,05**
IADL (mean ± SD)	8	6,1 ± 1,6	**<0,05**
Vascular risk factors:			
- Hypertension (n (%)):	6 (35,3%)	13 (37%)	>0,999
- Diabetes mellitus (n (%)):	1 (5,9%)	2 (5,7%)	>0,999
- Dyslipidemia (n (%)):	4 (23,5%)	8 (22,8%)	>0,999
- Current smoking (n (%)):	3 (17,6%)	5 (14,3%)	>0,999
- Carotid stenosis less than 40% (n (%))	5 (41,6%)	10 (40%)	>0,999

Table 2: Circulating Progenitor Cells levels and neuroinflammation/endothelial indices in AD subjects and healthy control subjects.

	Healthy control group (n=9) mean ± SD	**AD group (n=24) mean ± SD**	**P values**
$CD45^{dim}/CD34^{+}$ (% of PBL)	0,027 ± 0,009	0,029 ± 0,018	0,75
HPCs $CD45^{dim}$ /$CD133^{+}$ (% of $CD34^{+}$)	64,0 ± 19,4	51,9 ± 14,5	**0,02**
EPCs $CD45^{dim}/CD133^{+}/CD309^{+}$ (% of $CD34^{+}$)	2,9 ± 2,9	3,2 ± 3,0	0,79
VEGF (pg/ml)	318 ± 223	378 ± 207	0,47
CCL-2 (MCP1) (pg/ml)	39 ± 22	76 ± 51	**0,04**
CCL-5 (RANTES) (pg/ml)	44,5 ± 8,5	49,9 ± 7,9	0,09
ICAM-1 (sICAM-1) (pg/ml)	276,2 ± 81,3	216,3 ± 84,6	0,08

Figure 1. Representative dot plots showing gating strategy to identify Circulating progenitor Cells by flow cytometry.



Legend to Figure 1: Whole peripheral leukocytes were acquired (10^5 events, A and B) and $CD34^+$ cells were selected into R1 gate. In a second larger acquisition file, 4 x 10^6 PBL were analyzed by flow cytometry, but only $CD34^+$ cells were acquired (C, gate R1). In this file, HPCs were selected as $CD34^+$, $CD45^{dim}$ and $CD133^+$ cells (D and F, gates R1 and R2), whereas EPCs were identified as $CD34^+$ / $CD45^{dim}$ / $CD133^+$ / $CD309^+$ cells (F) in comparison to isotype control monoclonal antibody (E). EPC levels were calculated as percentage of positive cells in comparison to $CD34^+$ cells (gate R1).

Figure 2: MCAFV and BHI values in 35 AD subjects and in 17 healthy control subjects. (MCAFV: mean flow velocity of the middle cerebral artery; BHI: breath-holding index test).

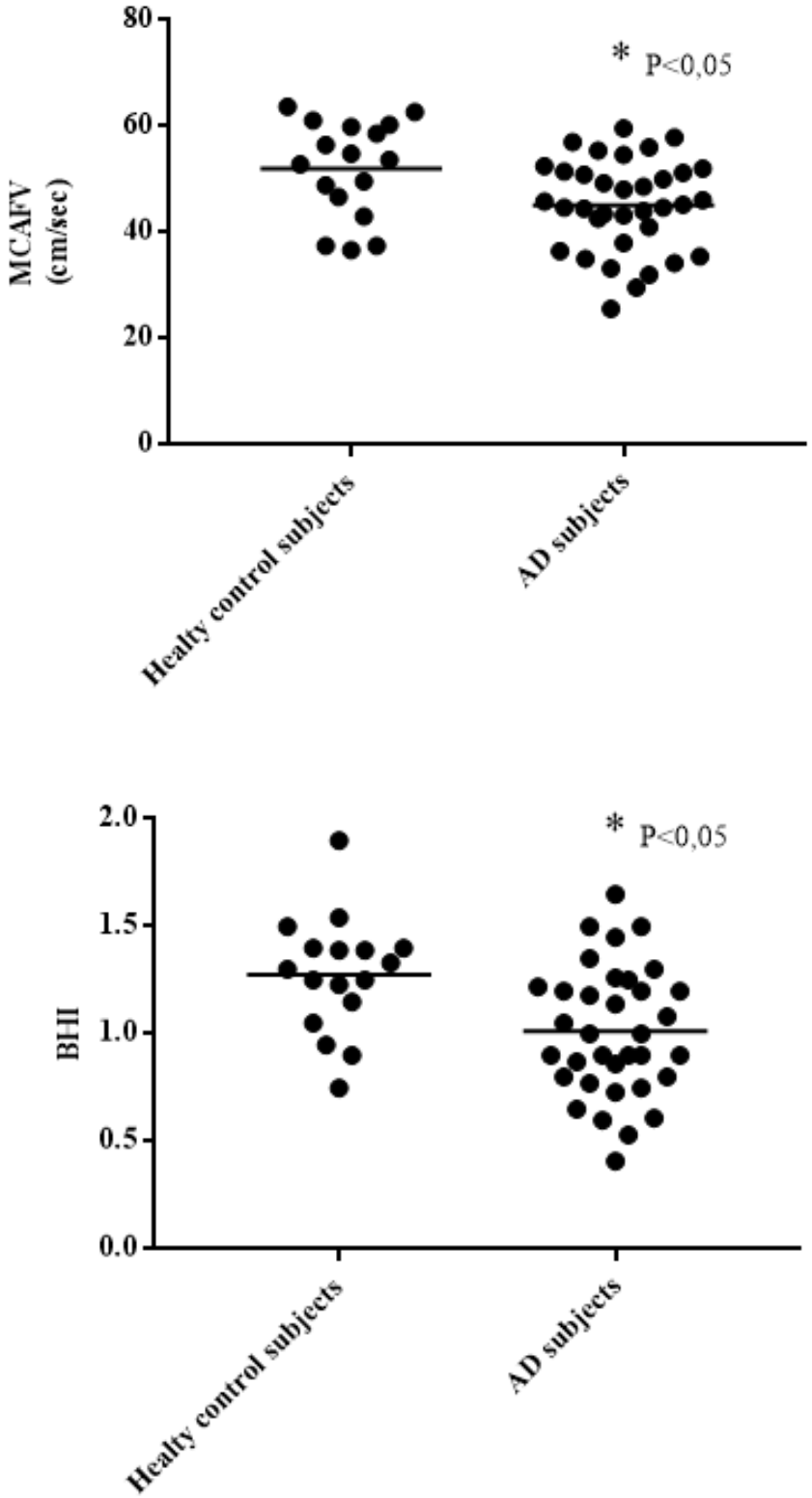


Figure 3: Correlation between MMSE score and both MCAFV and BHI in 35 AD patients. (MMSE: Mini Mental State Evaluation; MCAFV: mean flow velocity of the middle cerebral artery; BHI: breath-holding index test).

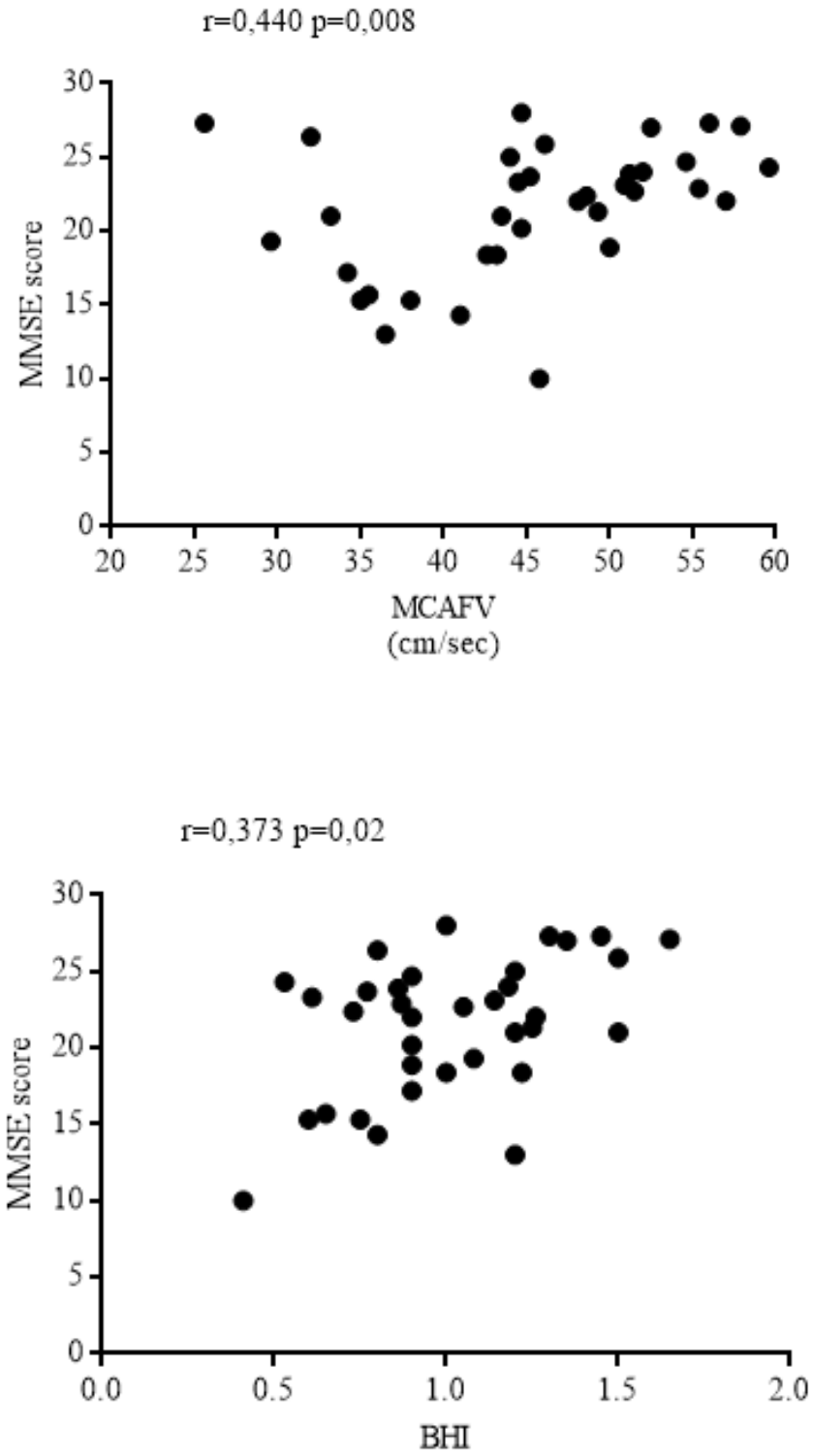


Figure 4. Correlation between IADL score and both MCAFV and BHI in 35 AD patients (IADL: Instrumental Activities of Daily Living; MCAFV: mean flow velocity of the middle cerebral artery; BHI: breath-holding index test).