Original Article

Brain Atrophy, Anti-Smooth Muscle Antibody and Cognitive Impairment: An Association Study

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ABSTRACT: Cortical atrophy, neuronal loss, beta-amyloid deposition, neuritic plaques, and neurofibrillary tangles are neuropathological key features in the Alzheimer's disease (AD). Antibodies against beta-amyloid, neurotransmitters, microvascular endothelium components and microglial cells have been detected in AD serum suggesting that AD could be another autoimmune disease and provides a link between vascular pathology, endothelium dysfunction and neuronal cells death. Aim of the present study was to evaluate the association between autoantibody profile and cognitive impairment in geriatric patients, accounting for ApoE genotype as a potential confounding factor. Three hundred and forty-four geriatric patients, attending the clinic for the cognitive decline, underwent a biochemical and immunological profile, chest X-ray, cerebral computed tomography scan and complete cognitive evaluation. All patients were also screened for the ApoE genotype. A significantly higher prevalence of Anti-Smooth Muscle Antibody (ASMA) positivity was found in 89/204 (43.63%) patients with diagnosed neuroradiological signs of cerebral atrophy compared with 15/140 (10.71%) patients without the condition (p<0.001). Multivariable logistic model evidenced that such association was independent of patient's age, gender and Mini-Mental State Examination (OR=8.25, 95%CI: 4.26-15.99) and achieved a good discriminatory power (c-statistic=0.783). Results were also independent of ApoE genotype, which resulted not associated both with the presence of brain atrophy and with the presence of ASMA positivity. Our results shows a strong association between brain atrophy and ASMA positivity and are consistent with several studies that focused attention on the mechanisms of endothelial immune response in the development of dementia.

Key words: brain atrophy, anti-smooth muscle antibody, cognitive impairment

Interaction between the immune and the nervous systems is bidirectional and occur under both physiological and pathological conditions. The effects of the aging process on the constituents of both these systems result in important functional changes in their interactions; such effects, in turn, alter susceptibility to autoimmune, neoplastic and degenerative disease of the nervous system.

Cortical atrophy, neuronal loss, beta-amyloid deposition, neuritic plaques, and neurofibrillary tangles are neuropathological key features in the Alzheimer's disease [1]. Amyloid deposits have been observed in microvessels and are often associated with degenerating endothelium, damaged smooth muscle cells and pericytes, and are associated with various abnormal basement membrane alterations which are all components of blood-brain-barrier (BBB) damage. Then the major pathological role of beta-amyloid in AD may be to induce vascular and BBB function damage [2-4]. Impairment of BBB function can occur in conditions that are commonly associated with aging, such as atherosclerosis, hypertension, cerebrovascular ischemia and stroke; all of which have

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been found to be risk factors for AD. In addition, altered blood brain barrier has been associated with mutations of the apolipoprotein E (ApoE) gene [5,6]. Current concepts regarding the pathogenesis of AD include the participation of mechanism of endothelial dysfunction associated to inflammatory and autoimmune components [7,8]. Autoimmunity can be involved in many human diseases, and even though the causes of the autoimmune disorders are unknown, the specific targets of the autoantibodies characterize each autoimmune disease. Autoantibodies can also affect the central nervous system (CNS) reacting with neurons in neurological diseases including Huntington's chorea [9], Sydenham's chorea [10], cerebral lupus [11,12], multiple sclerosis [13,14], experimental allergic encephalomyelitis [15], Rasmussen's encephalitis [16,17] and recently in Alzheimer's disease [18,19]. Anti-neuronal autoantibodies have been detected in AD serum, but their significance still remains obscure and their use as diagnostic tools has partially been dismissed due to the presence of similar amounts of these antibodies in healthy donors [20]. Is already known that antibodies against beta-amyloid are present in AD patients' serum and in non-demented individuals [21]. There is a growing evidence that lipid metabolism and oxidative stress may also participate in the pathogenesis of AD. Oxidized low-density lipoproteins (OxLDL) are also known to be highly immunogenic and induce systemic antibodies with important functional properties in health and disease [22]. Ganglioside GM1 tightly binds to beta-amyloid and may inhibit its conformational changes (from alpha-helix to beta-sheet). Several studies reported an increase in anti-GM1 titers in the AD patients' serum as well as from other age–related dementias [23-24]. It is well known that during the progression of AD there is a global loss of neurotransmitters but there is a lack of significant data linking autoimmunity to loss of neurotransmitters in AD [25]. Autoantibodies to glutamate were detected in blood plasma from patients with AD. Importantly, plasma concentration of immune complexes and antibodies to serotonin and dopamine were higher in patients with AD compared to mentally healthy volunteers of the same age [26]. A great body of evidence exists about the fact that antibodies to microvascular endothelium might have a role in the BBB damage, inducing activation and/or apoptosis in endothelial cells; Delunardo et al. identified rabaptin 5 (RABPT5) as a new endothelial autoantigen in AD, recognized by serum IgG in 65% of patients with AD [27]. Mruthinti et al. reported that anti-RAGE IgG titers were higher in AD-diabetic patients [28]. Serum anti-ATP synthase autoantibodies were detected in 38% of patients with AD. Antibodies directed against microglial cells were found in serum and CSF of AD patients [29]. Serum antibodies to spectrin, peroxidise, thyroglobulin and myelin basic protein are significantly increased in patients with AD. In summary, these data in the context of the underlying mechanisms of many autoimmune diseases,indicated that AD could be another autoimmune disease and provides a link between vascular pathology, endothelium dysfunction and neuronal cells death [30-32]. Aim of the present study was to evaluate the association between autoantibody profile and cognitive impairment according to age.

MATERIALS AND METHODS

Patient recruitment

This was a monocentric cross sectional study fulfilling the Declaration of Helsinki (availabe at: www.wma.net/en), the guidelines for Good Clinical Practice (available at: www.ema.europa.eu) the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (available at: www.strobe-statement.org) and the National Institute for Health and Clinical Excellence (NICE) requirements (available at: www.nice.org.uk). The approval of the study for experiments using human subjects was obtained from the local Ethics Committees on human experimentation. Written informed consent for research was obtained from each patient or from relatives/legal guardian in the case of critically disabled demented patients prior to participation in the study. All patients included in this study were Caucasians, with most individuals living in Central and Southern Italy from at least two generations. All subjects included in this study were selected among patients consecutively attending the clinic for cognitive decline of the Geriatrics Unit of the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) "Casa Sollievo della Sofferenza" (Italy) from July 2012 to July 2014.

Inclusion/exclusion criteria

Inclusion criteria were: 1) age $\geq$ 65 years as we are evaluating a geriatric population; 2) all kind of cognitive status (i.e. no cognitive impairment (CTRL), Alzheimer Disease (AD), Depression, mild cognitive impairment (MCI), Parkinson's Disease (PD), diagnosis of vascular dementia (VaD), 3) given informed consent for research. Exclusion criteria were: 1) diagnosis of mixed dementia (MxD); 2) presence of immuno-rheumatological diseases; 3) presence of serious comorbidity, tumors, other diseases or physiological status (ascertained blood infections, vitamin B12 deficiency, anemia, disorders of the thyroid, kidneys), that could be causally affect to cognitive function; 4) history of alcohol or drug abuse; 5) head trauma; 6) clinical, biochemical and radiological findings, consistent with liver and/or lung disease).

Clinical and instrumental assessment

All patients attending the clinic for the cognitive decline, underwent a standard diagnostic tool. Briefly, a physical examination, electrocardiogram, venous blood sampling for biochemical profile, chest X-ray, cerebral computed tomography scan and complete cognitive evaluation were assessed.

Blood tests include complete blood count, electrolytes, renal and liver function, lipid profile, glycaemia, homocysteine, vitamin B12, folate, thyroid function, total protein electrophoresis, serum calcium, ESR, CRP and autoantibody profile (i.e. Anti-nuclear antibody – ANA, Anti-mitochondrial antibody – AMA, Anti-smooth muscle antibody – ASMA, Anti–citrullinated protein antibody – APCA, assessed by semi-quantitative determination (Immunofluorece BIO RAD Kallestad).

Cognitive-functional evaluation and diagnosis

Baseline demographic and clinical characteristics were collected by a structured interview, a clinical evaluation and a review of records from patient's general practitioners.

In all patients, cognitive status was screened by means of the Mini-Mental State Examination (MMSE) [33] the Alzheimer's Disease Assessment Scale - Cognitive Section (ADAS-Cog) [34], and the Clinical Dementia Rating scale (CDR) [35]. Dementia (CDR 1+) was confirmed and diagnosed by the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DMS-IV), whereas diagnosis of questionable dementia (QD) was made according to a CDR value of 0.5+ [36]. Diagnosis of mild cognitive impairment (MCI) was made, according to the Petersen criteria [37], in subjects with CDR 0.5+ and an MMSE value from 24 to 27. Diagnosis of possible/probable AD was made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) [38]. Diagnosis of Parkinson's disease was made according to the NICE guideline for diagnosis and treatment of Parkinson's disease. Diagnosis of vascular dementia (VaD) was made according to the criteria of the National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences Work Group (NINDS-AIREN) [39]. Differential diagnosis between AD and VaD was also based on the Hachinski Ischemic Score to address unclear AD/VaD diagnoses [40]. In particular scores ≤ 4 were considered as probable AD, scores ≥ 7 were included into the VaD group. Scores between 5 and 6 were diagnosed as mixed dementia

(MxD) and were excluded from the study. Diagnosis of AD or VaD was always supported by neuroimaging evidences (computed tomography scan and/or nuclear magnetic resonance). In particular, the presence of multiple cortical/subcortical infarcts or an infarct in a strategic area such as the thalamus or temporal lobe and/or lesions of the white matter indicated probable VaD. The absence of the above mentioned cerebrovascular lesions indicated AD. Functional status was evaluated using the Activities of Daily Living (ADL) index [41] and the Instrumental Activities of Daily Living (IADL) scale [42].

Genetic analysis

Genomic DNA was purified from fresh/frozen blood samples following salting-out method [43]. In the present study, for all patients we analyzed the *APOE* genotypes as previously described [44].

Statistical Analysis

Patients' characteristics were reported as mean ± standard deviation (SD) and frequencies (column percentages) for continuous and categorical variables, respectively. Comparisons between patients with a diagnosed brain atrophy and those without a diagnosed brain atrophy were performed using two-sample t test and Pearson Chi-square test (or Fisher exact test as appropriate), for continuous and categorical variables, respectively. Normal distribution assumption was checked by means of Q-Q plot, Shapiro-Wilks and Kolmogorov-Smirnov tests. Associations between ASMA positivity and the presence of brain atrophy were assessed using both univariable and multivariable logistic regressions and results were reported as odds ratio (OR), along with their 95% confidence intervals (95% CI).

For multivariable analysis, the following adjustment covariates were considered: patients' age, gender, MMSE value, APOE genotype, neurological diagnosis (i.e. CTRL, VaD, MCI, PD, AD and depression). The stepwise variable selection criterion (significance level for entry into the model: p=0.10, significance level for staying into the model: p=0.05) was used to select, among all candidate covariates, the ones to be included into the final multivariable model. Models' discrimination, i.e. the ability to distinguish patients with diagnosed brain atrophy from patients without the diagnosed brain atrophy, was assessed by computing the concordance (or c) statistic using the models' predicted probabilities [45]. Comparison between c-statistics was carried out using DeLong's test [46]. Two-sided p-values <0.05 were considered statistically significant. All statistical analyses were performed using SAS Release 9.3 (SAS Institute, Cary, NC).

Table 1. Demographic and clinical patients' characteristics (overall and according to the presence of brain atrophy)

Variable	All subjects (N = 344)	Without diagnosed atrophy (N = 140)	With diagnosed atrophy (N = 204)	*p*-value*
Age (years)	77.81 ± 8.20	75.19 ± 8.69	79.63 ± 7.33	<0.001
Gender - N of males (%)	131 (38.08)	45 (32.14)	86 (42.16)	0.060
Positivity for Anti-Smooth Muscle Antibody - N (%)	104 (30.23)	15 (10.71)	89 (43.63)	<0.001
Mini-Mental State Examination (MMSE)	19.96 ± 6.45	21.43 ± 5.75	18.95 ± 6.72	<0.001
Number of patients with MMSE<25 - N (%)	251 (72.97)	93 (66.43)	158 (77.45)	0.024

*p-value from Pearson Chi-Square test and two-sample t-test for categorical and continuous variables, respectively.

RESULTS

A total of 344 eligible geriatric patients (131 males/213 females, with a mean age of 77.81 ± 8.20 years and a mean MMSE of 19.96 ± 6.45) were enrolled in this study. Neuroradiological signs of brain atrophy were found in 204 (59.3%) patients. Demographic and clinical patients' characteristics (overall and according to the presence of brain atrophy) are summarized in Table 1.

A significantly higher prevalence of Anti-Smooth Muscle Antibody (ASMA) positivity was found in patients with brain atrophy compared to those without the condition (43.63% *vs*. 10.71%, p<0.001). The diagnostic accuracy of the ASMA positivity to predict a brain atrophy was characterized by a sensitivity of 46.3%, a specificity of 89.3%, a positive predictive value of 85.6% and a negative predictive value of 52.1%. Moreover, patients with brain atrophy were significantly older than those without the condition (79.63 ± 7.33 vs. 75.19 ± 8.69 years, *p*<0.001) and had a significantly lower MMSE score (18.95 ± 6.72 vs. 21.43 ± 5.75, *p*<0.001).

In Table 2 patients are stratified on the basis of clinical diagnosis and genotype profile overall and according to the presence of brain atrophy. As expected, significantly higher prevalence of AD (34.31% vs. 19.29%, *p*=0.002) was found in patients with cerebral atrophy. No significant differences in ApoE genotype distribution were found.

Table 2. Patients' clinical diagnosis and genotype profile (overall and according to the presence of brain atrophy)

Variable	Category	All subjects (N = 344)	Without diagnosed atrophy (N = 140)	With diagnosed atrophy (N = 204)	*p*-value*
Diagnosis – N (%)	No cognitive impairment	22 (6.40)	8 (5.71)	14 (6.86)	0.669
	Alzheimer disease	97 (28.20)	27 (19.29)	70 (34.31)	0.002
	Depression	92 (26.74)	53 (37.86)	39 (19.12)	0.001
	Mild Cognitive Impairment	90 (26.16)	37 (26.43)	53 (25.98)	0.926
	Parkinson's disease	12 (3.49)	5 (3.57)	7 (3.43)	0.945
	Vascular dementia	31 (9.01)	10 (7.14)	21 (10.29)	0.316
	Missing values (n)	3	1	2	
APOE – N (%)	e2/e2	2 (0.59)	0 (0.00)	2 (0.99)	0.516[#]
	e2/e3	22 (6.45)	6 (4.32)	16 (7.92)	0.183
	e2/e4	3 (0.88)	1 (0.72)	2 (0.99)	1.000[#]
	e3/e3	208 (61.00)	88 (63.31)	120 (59.41)	0.468
	e3/e4	91 (26.69)	36 (25.90)	55 (27.23)	0.785
	e4/e4	15 (4.40)	8 (5.76)	7 (3.47)	0.311

*p-value from Pearson Chi-Square test and two-sample t-test for categorical and continuous variables, respectively.
p-value from Fisher exact test.

Table 3 Results from univariable and multivariable logistic models, in the evaluation of the association between positivity to Anti-Smooth Muscle Antibody and the presence of brain atrophy

Model	OR (95% CI)*	*p*-value	c-statistic (95% CI)^
Univariable	6.447 (3.529 - 11.780)	<0.001	0.665 (0.622 - 0.707)
Multivariable#	8.250 (4.257 - 15.986)	<0.001	0.783 (0.734 - 0.831)

*OR: Odds ratio along with their 95% confidence interval (95% CI); ^C-statistic along with 95%CI;
#Multivariable model adjusted for: age, gender and MMSE (stepwise selection criterion with significance level for entry into the model: p=0.10 and significance level for staying into the model: p=0.05)

Results from univariable and multivariable logistic regressions are reported in Table 3. As mentioned above, the presence of ASMA positivity was significantly associated to the presence of brain atrophy in the univariable analysis (OR=6.45, 95%CI: 3.53-11.78, *p*<0.001), achieving a fair overall discriminatory power (c=0.665). This association was also confirmed by the multivariable analysis (OR=8.25, 95%CI: 4.26-15.99, p<0.001), where the following adjustment covariates were selected and included into the logistic regression: age, gender and MMSE. Such multivariable model achieved a good discriminatory power (c=0.783) which was significantly higher than c=0.665, from univariable model (*p*<0.001). Furthermore, such results were independent by the ApoE genotype, which resulted not associated both with the presence of brain atrophy and with the presence of ASMA positivity.

DISCUSSION

An endothelial dysfunction, in many diseases, including chronic renal failure, heart failure, hypertension, diabetes, and immunologic diseases has been demonstrated [47-50] but its role in developing cognitive impairment is unknown and of increasing interest [51]. In addition, recent data have shown that endothelial function is impaired in AD patients, suggesting that such dysfunction might represent a link between vascular and beta-amyloid hypothesis of Alzheimer's disease [52-54]. The endothelium is a monolayer of endothelial cells lining the lumen of the vascular beds and is mechanically and metabolically located, separating the vascular wall from the circulation and the blood components [55]. Brain capillaries are normally dynamic, cylindrical tubes that are lined by endothelial cells with tight junctions, a basement membrane and, at irregular intervals, by pericytes [56,57]. Evidences suggest that pericytes may play a role in the regulation of blood flow by their contractile response and supporting this notion is the presence of myosin, actin and tropomyosin within pericytes [58,59]. Anti Smooth Muscle Antibodies (ASMA) react with actomyosin and different antigens of smooth muscles that constitute the cell cytoskeleton. ASMA, not species specific, are almost exclusively IgG, more rarely IgM. These antibodies can be detected with techniques of indirect immunofluorescence or, more recently, with fluorimetric immunoassay techniques [60,61]. According to current knowledge, the anti-G actin are specific to chronic active hepatitis. These antibodies are associated with pathological conditions with a strong inflammatory and immunological component (chronic hepatitis, viral diseases and lung disease). Also are present in approximately 20-30% of apparently healthy subjects, a situation in which it is not yet known the significance of their presence.

The autoantibody profile observed in patients enrolled, showed that positivity for ASMA was greater in people with diagnosed atrophy as compared to those who did not present this abnormality. This difference was present regardless the level of cognitive impairment a shown in Table 1. Table 2 showed that cognitive status distribution was substantially related to the presence/absence of diagnosed brain atrophy and as expected brain atrophy was greater in AD. Moreover, ApoE polymorphism (a well-known risk factor for AD) didn't show any difference according to presence/absence of brain atrophy. Table 3 strongly evidenced the association between asma profile and brain atrophy both in univariate and multivariate approach.

A recent study indicates that Alzheimer's disease is a vascular disorder with neurodegenerative consequences [62]. Critically Attained Threshold of Cerebral Hypoperfusion (CATCH) is a haemodinamic microcirculatory insufficiency that destabilize neurons, synapses, neurotransmission with consequent neurodegenerative state characterized by the formation of senile plaques, neurofibrillary tangles and amyloid angiopathy [63]. Clinical evidence suggests that are fundamental in the development of CATCH duration and severity of hypoperfusion and the age of subjects. Pathological studies showed that brain capillaries in AD develop basement membrane thickening, pericyte degeneration, endothelial cell shape changes, and luminal buckling. These pathological changes cause altered

microvascular blood flow and lower concentrations of glucose and oxygen in neuronal targets, creating an energy crisis at the mitochondrial level that causes unbalanced energy system in brain [64]. At present the major question is: how these vascular changes leading to chronic hypoperfusion cause neurodegeneration? Our results are in agreement with the growing evidences that there is a link between inflammation, endothelial dysfunction, brain immune system and pathogenesis of dementia. Moreover, accumulation and infiltration of activated immune cells have been well documented in the AD brain in addition to classical neuropathological signs i.e. amyloid plaques and NFT [65,66]. Clustering of activated microglia [67] and astrocytes is observed in the AD brain, associated with upregulated expression of a variety of inflammatory cytokines, including interleukin-1 (IL-1) [68], interleukin-6 (IL-6) [69], tumor necrosis factor-α (TNF-α) [70], and transforming growth factor-β (TGF-β) [71]. Moreover, innate immune receptors, such as toll-like receptor and lipopolysaccharide (LPS) receptor (CD 14), are upregulated in the human AD brain [65]. Than, it has been shown that some markers of glial activation are elevated even before the development of amyloid deposition, implying that inflammation precedes typical AD neuropathological changes and has some causative effect in AD pathogenesis [72]. Our results lead us to assert that ASMA positivity explain only brain atrophy maybe as a consequence of endothelium damage. The usefulness of our results is to identify ASMA positivity as a potential risk factor of brain atrophy. As it is well known, this clinical condition can be a prelude to cognitive impairment. For this reason, it seems reasonable to propose ASMA profile as a tool to screen population at risk of developing cognitive impairment during aging process in a large cohort study.

Acknowledgements

Conflicts of Interest

All authors agree with the presented findings, have contributed to the work and declare no conflict of interest.

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