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Cholinesterase inhibitor use is associated with increased plasma levels of anti-Abeta 1–42 antibodies in Alzheimer's disease patients☆

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ABSTRACT

Acetyl-cholinesterase inhibitors (AChEI) are drugs frequently prescribed for the treatment of Alzheimer's disease (AD), exerting an effect on cognition, as well as on behavioural and psychological symptoms of dementia and activities of daily living. The efficacy of AChEI may be ascribed not only to the activation of cholinergic transmission, but also to other mechanisms, among which a putative regulation of the immune response has already been hypothesized. In the present study, we evaluated, in a cross-sectional sample of 66 AD patients and 48 healthy controls, the putative influence of AChEI on anti-Abeta 1–42 antibody plasma levels by ELISA assay. AD patients receiving AChEI therapy showed increased plasma levels of anti-Abeta 1–42 antibodies respect to untreated AD patients and antibodies levels similar to those of healthy controls, both before and after normalization by total IgG values. Our results support a potential role of AChEI in the modulation of the immune response against Abeta. We suggest that a strategy aimed at increasing the endogenous response against this peptide might represent an interesting therapeutic target to be further investigated.

It is well known that, among different mechanisms involved in Alzheimer's disease (AD) pathogenesis and progression, cholinergic dysfunction and beta-Amyloid (Abeta) peptide deposition represent key events. Interestingly, there are several indications that a relationship between the impairment of the cholinergic system and the metabolism of the amyloid precursor protein (APP) might exist [25]. Acetyl-cholinesterase inhibitors (AChEI), drugs which are frequently prescribed for the treatment of AD, may exert a positive but temporary influence on cognition [3], and might also have an effect on behavioural and psychological symptoms of dementia [30,11]; furthermore, a neuroprotective effect has been previously hypothesized [5,19,37]. This mechanism could be possibly related to the reduction of toxic Abeta fibrils amount by enhancing the trafficking and activity of the alpha-secretase ADAM10 [37]. It is also worth to notice that increasing evidences indicate that inhibition of AChE activity could regulate also the inflammatory response [24]. AChEI reduce the production of Th1 inflammatory cytokines and evoke a Th2 response, increasing IL-4 expression in peripheral blood mononuclear cells from AD patients [26]. The Th2 modulation activates B-lymphocytes and increases the antibody-mediated immune response. The efficacy of both active and passive immunization in affecting the deposition of amyloid plaques [29,1] and in reversing the memory deficits has been previously demonstrated in mouse transgenic models of AD [17], but till now clinical trials fail to show relevant clinical effects in humans [20]. Theoretically, the peripheral presence of anti-Abeta antibodies and the formation of Abeta-antibody complexes may lower Abeta blood content favouring the peptide efflux from the brain, through the blood brain barrier, resulting in "central" Abeta clearance. Moreover, published studies aimed at quantifying the amount of natural anti-Abeta antibodies in human biological fluids have so far shown conflicting results. Plasma anti-Abeta levels in AD patients, compared to controls, were found to be reduced by some authors [35,18,32,4], unchanged by others [16,2] and even increased by at least one further study [23]. In the cerebrospinal fluid, data are particularly scanty with reduced levels reported [10]. Several methodological issues, related to study design and power and also possibly related to different patient populations (treated or drug naïve), might be involved in explaining these apparently contradictory results. In the present study we measured anti-Abeta 1–42 antibody plasma levels in a cross-sectional sample of 66 AD subjects with and without AChEI treatment, in order to suggest a putative immunomodulatory effect by these drugs.

Following approval by the ethical committee of the S. Gerardo Hospital (Monza, Italy), 33 AD patients receiving AChEI (AD AChEI;

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Table 1
Demographic and clinical data by diagnostic groups.

Enrolled subjects	AD AChEI	AD w/o AChEI	CTRL
N	33	33	48
Gender M (n, %)	13 (39%)	19 (57%)	34 (71%)[#]
Age: year ± SD (range)	73.9 ± 7.4 (56–85)	75.9 ± 6.6 (60–95)	68.7 ± 4.7* (57–82)
MMSE: score ± SD (range)	17.3 ± 6.8[*] (0–26)	20.9 ± 5.3[*] (4–29)	29.0 ± 1.2 (26–30)
Disease duration: month ± SD (range)	35.7±23.4 (3–72)	26.7±12.3 (2–48)	–
ApoE ε4 allele carriers *n* (%)	19 (59%)	15 (47%)	7 (15%)[§]
Antidepressant drugs (Y/N)	9/24	6/27	None
Antipsychotic drugs (Y/N)	1/32	5/28	None

[*] $p < 0.05$ vs. the other groups.
[#] $\chi^2 = 7.93$, $p < 0.05$.
[§] $\chi^2 = 17.51$, $p < 0.0001$.

receiving donepezil 10 mg o.d. $n = 17$, or rivastigmine 4–6 mg b.i.d., $n = 12$, or galantamine 12 mg b.i.d., $n = 4$ since at least six months), 33 AD patients not receiving AChEI (AD w/o AChEI), and 48 healthy controls were recruited. Informed consent was obtained from all the subjects involved in the study by their own or, in the case of cognitive impairment, by the caregivers. AD specialists diagnosed AD according to the NINCDS-ADRDA criteria [22] and alternative diagnoses were excluded by brain imaging (MR or CAT) and an extensive neuropsychological test battery. Few patients were also taking antidepressants (SSRI or SNRI, at variable doses), or antipsychotic medications (quetiapine or promazine, variable doses) (see Table 1). Healthy controls had no personal or family history of neurological or psychiatric disorders; lack of cognitive impairment was established by a clinical interview, including a mini-mental state examination (MMSE) score $\geq$ 26. Individuals with recent infections or surgery, or under anti-inflammatory, corticosteroid or immunosuppressive drug treatments were excluded. Clinical and demographic data are shown in Table 1. Blood samples (5 ml) were collected in K_2EDTA tubes (4.08 mM final concentration), after an overnight (ON) fasting. Plasma was obtained after centrifugation ($3700 \times g$, 20 min) and stored at −80 °C until assay. The concentration of anti-Abeta 1–42 antibodies in plasma was determined by ELISA, as previously described [6]. Lyophilized human Abeta 1–42 peptide (Phoenix Pharmaceuticals) was solubilised in cold Tris–Buffer (pH 9) to obtain a 1 mg/ml solution of non-fibrillary Abeta [38]. Plates (Greiner Bio One) were coated at 4 °C ON with Abeta 1–42 diluted to 0.1 mg/ml in 50 mM carbonate buffer (pH 9.6) and sonicated 1 min twice. After washing with PBS/0.05% Tween 20 (PBST), plates were blocked with 5% FCS, 1% BSA in PBST for 90 min at room temperature (RT) and washed again with PBST. A standard curve was generated using an affinity-purified mouse (monoclonal) anti-Abeta 1–42, selective for Abeta C-terminal (The Genetics Company), at different concentrations ranging from 5 to 0.125 μg/ml (serial dilution). Blank wells with carbonate buffer alone were included to subtract out the non-specific binding of plasma antibody. Coated plates were incubated at 4 °C ON with non-diluted plasma obtained from patients and controls. After PBST washing, they were incubated for 2 h with HRP conjugated goat anti-human IgG antibodies 1:10.000 (Sigma–Aldrich) at RT. Plates were than washed again with PBST and incubated for 10 min with TMB (Sigma–Aldrich); after adding the stop solution the absorbance at 450 nm was read. The concentration of plasma antibodies was determined from the standard curve. Inter- and intra-assay variability was lower than 10%. Total IgG plasma content was evaluated by an automated immunoturbidimetric analysis on Modular P analyzer (Roche Diagnostics). To analyze ApoE genotype, total DNA was extracted from peripheral blood using a commercial DNA extraction kit (Qiagen) and DNA amplification was performed using specific primers [15].

Chi-square analysis was performed in order to detect differences for ordinal variables. One-way ANCOVA followed by Tukey *post hoc* test was used for the comparison among the three groups. Covariate analysis was used to control for possible confounders. The following variables were entered into the analysis as covariates: age, gender, MMSE score and ApoE ε4 allele status. *p* value <0.05 was considered statistically significant.

Plasma levels of anti-Abeta 1–42 antibodies were significantly increased (∼60%, $p < 0.01$) in AD patients receiving AChEI therapy (AD AChEI) with respect to AD patients not treated with AChEI (AD w/o AChEI) and similar to those measured in healthy controls (Fig. 1, Table 2). Plasma total IgG were evaluated to normalize antibody levels, considering the ratio between anti-Abeta antibodies 1–42 and total IgG the differences among the three categories were maintained (see Table 2). Noteworthy, although controls were younger with respect to AD patients (see Table 1), no age differences were detected at the comparison between AD with and w/o AChEI. A difference in MMSE score was present between the two AD groups (see Table 1), but covariate analysis failed to show any influence of this parameter on the dependent variable. Moreover, no differences in antibody levels were shown after categorizing patients according to the single AChEI molecule (e.g., either donepezil, rivastigmine, or galantamine). No effect was shown for both antidepressants and antipsychotic drugs, although only a few patients in both AD groups were taking these drugs and the study was not designed for this purpose. Finally, a difference of the ApoE ε4 carrier status was found between AD patients (regardless of the AChEI treatment status) and controls, but it did not influence anti-Abeta antibody plasma levels (Table 1).

Due to AChEI impact on the immune response and the importance of immune therapy in AD [25], we investigated the possibility that anti-Abeta 1–42 antibody plasma levels might be affected by these drugs. We found anti-Abeta 1–42 plasma levels increased in AD subjects receiving AChEI compared to untreated AD patients, and similar to control subjects. A similar result was still present

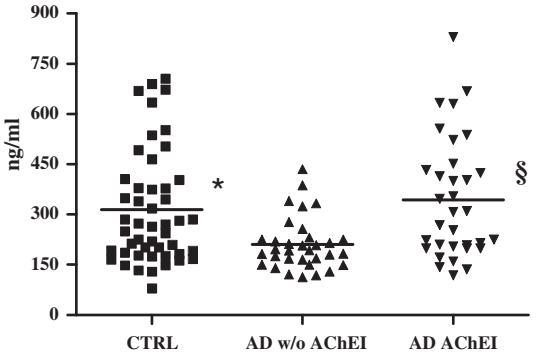

Fig. 1. Anti-Abeta 1–42 plasma levels in controls (CTRL), AD patients not receiving AChEI (AD w/o AChEI) and AD patients receiving AChEI (AD AChEI). [§]$p < 0.01$ AD AChEI vs. AD w/o AChEI, *$p < 0.01$ CTRL vs. AD w/o AChEI.

Table 2
Anti Abeta 1–42 antibodies levels.

	AD AChEI	AD w/o AChEI	CTRL
Anti-Abeta 1–42 antibodies levels (ng/ml) (mean ± SD)	344 ± 180[§]	211 ± 77	314 ± 168[*]
Ratio (anti-Abeta 1–42:total IgG) (mean ± SD)	3.76 ± 2.16[$]	2.42 ± 1.08	3.48 ± 1.96[#]

[*] $p < 0.01$ vs. AD w/o AChEI.
[#] $p < 0.05$ vs. AD w/o AChEI.
[§] $p < 0.01$ vs. AD w/o AChEI.
[$] $p < 0.05$ vs. AD w/o AChEI.

following normalization with IgG plasma content, performed to determine if the increase in antibody levels was due to a general immune response or it could be specific for the antibodies against Abeta 1–42. These results confirm a potential involvement of AChEI in modulating the immune response, and the effect appeared to be specific for the production of antibodies against Abeta 1–42. This suggests a putative restoring effect of this drug, able to maintain high anti-Abeta plasma levels, possibly mediated by the involvement of the cholinergic system expressed by human lymphocytes.

A few data about the influence of AChEI on the immune system are available in the literature. Reale and colleagues demonstrated that, following AChEI treatment, AD patients showed an increase in interleukin-4 and monocytes chemotactict protein-1 levels, two positive regulators of Th2 differentiation [26]. These data suggest that AChEI treatment may favour a Th2 mediated immune response able to induce immunoglobulins production by B lymphocytes and, among them, anti-Abeta 1–42 antibodies. Interestingly, it was shown that, in human intravenous immunoglobulin preparations (IVIg), antibodies against Abeta are naturally present [33]; hence, apart from the clinical trials, employing active and passive immunization [20], a novel approach might be represented by the administration of IVIg preparations. In fact, since 2004 Dodel demonstrated that IVIg treatment can decrease Abeta in CSF and increase the peptide in serum of AD patients [9]; more recently, Relkin confirmed a positive effect of IVIg preparations on cognitive function in AD [27]. Previous evidences showed that peripherally administered immunoglobulins against Abeta peptide can clear brain Abeta deposits in mouse models of AD, improving their cognitive performances [36]. Different mechanisms are involved in Abeta depletion by immunoglobulins [34]: (a) they can pass blood brain barrier and bind Abeta favouring its degradation by microglial cells [21]; (b) immunoglobulins can facilitate the efflux of Abeta peptide from endothelial cells [7]; (c) their binding to Abeta may lead the peptide to a reduced tendency to aggregation [31]; (d) Abeta is cleared from the brain by the "peripheral sink" effect [8]. The literature about the evaluation of plasma anti-Abeta 1–42 antibodies is still conflicting, although the amount of contrasting data may be due to the different methods and conditions used to perform the assay [10,35,2,23,32,18,16]. For example, antibodies are present in plasma free or associated to Abeta and the presence of such complexes may provide a potential explanation for the variable data reported for human studies. Another cause of variability may be represented by the conformation of the Abeta peptides used for coating the plate to perform the ELISA assay. Indeed, it is well known that Abeta exists as monomers, fibrils or oligomers and among them the last one are currently considered the most toxic [28]. We decided to use a solution of Abeta in a non-fibrillar state [38] because it may better represent the soluble forms present in plasma.

Recently, it was demonstrated that a dissociation procedure based on acidification and filtration might reveal significantly higher levels of anti-Abeta antibody in plasma from both AD patients and controls [13]. Dissociation may allow to overcome the variability of data previously obtained, revealing that antibody plasma content was significantly higher in AD subjects compared to controls and that a negative correlation with age and disease duration exists [13,14]. Our data were obtained evaluating antibodies plasma content without dissociation, but our attention was aimed to determine the potential influence of AChEI on free unbound antibodies, in order to confirm the ability of this drug to modulate the immune response, rather than indirectly assessing the amount of Abeta 1–42 produced. The ability of AChEI to increase anti-Abeta plasma content may be appealing since anti-Abeta antibodies might be able to keep under control the aggregation and/or toxic modification of Abeta peptide. Some evidences suggest, in fact, that the endogenous anti-Abeta immune response might be conformation-specific, particularly protecting against amyloidogenic toxic peptides, and that this response might decrease with advancing age and disease stage [4]. The possibility to increase the endogenous immune response against toxic Abeta might, therefore, preserve the functions of physiologic Abeta preventing possibly unexpected effects that may arise following passive immunization. Against the hypothesis that AChEI might stimulate this "immunosurveillance" towards toxic Abeta, there is the evidence that these drugs demonstrate symptomatic rather than neuroprotective effects [12]. One may think that in AD, the production of toxic Abeta species might prevail over this AChEI-mediated biological phenomenon, possibly preventing their putative immuno-mediated neuroprotective action.

Further studies, analyzing endogenous anti-Abeta antibody plasma levels before and after dissociation, will be necessary to explain their role, possibly coupled with studies aimed at clarifying more in detail the specificity of AChEI putative immunomodulatory effect. Currently, we are evaluating the production of anti-Abeta antibodies in cultured *ex vivo* B-lymphocytes following AChEI treatment in order to extend our observations. Indeed, we support the hypothesis that the study of the stimulation of the endogenous response against toxic Abeta might represent an interesting future area of therapeutic research in AD.

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