Elevation of β-Amyloid 1-42 Autoantibodies in the Blood of Amnestic Patients With Mild Cognitive Impairment

Daniela Storace, PhD; Sergio Cammarata, MD; Roberta Borghi, PhD; Roberta Sanguineti, PhD; Luca Giliberto, MD; Alessandra Piccini, PhD; Valeria Pollero, BSc; Cristina Novello, BSc; Carlo Callagirone, MD; Mark A. Smith, PhD; Paola Bossù, PhD; George Perry, PhD; Patrizio Odetti, MD; Massimo Tabaton, MD

Objective: To develop a blood-based test for screening populations at risk for Alzheimer disease.

Design: Case-control study.

Subjects: A total of 180 patients with mild cognitive impairment (MCI) and 105 age-matched, cognitively normal controls.

Interventions: The titer of β-amyloid 1-42 autoantibodies in the plasma was obtained at the time of diagnosis and evaluated by enzyme-linked immunosorbent assay before and after dissociation of the antigen-antibody complexes. A total of 107 patients with MCI were followed up for 36 months; 70 of the 107 cases progressed to Alzheimer disease.

Results: The average level of β-amyloid 1-42 plasma autoantibodies in patients with MCI that progressed to Alzheimer disease, but not that of the stable cases, was significantly higher than in cognitively normal controls (P < .001).

Conclusions: The results suggest that the plasma β-amyloid 1-42 autoantibodies parallel β-amyloid 42 deposition in the brain, which is known to precede by several years the clinical onset of Alzheimer disease. The evaluation of β-amyloid 1-42 autoantibodies after dissociation of the complexes is a simple and inexpensive method that can be used to predict the occurrence of Alzheimer disease.

Arch Neurol. 2010;67(7):867-872.

The identification of a preclinical marker for Alzheimer disease (AD) is critical for the evaluation of successful disease-modifying therapy. Two approaches have been used to correlate biomarkers with the longitudinal evolution of (1) a large cohort of cognitively normal elderly people,1,2 or (2) patients with amnestic mild cognitive impairment (aMCI),3-6 a condition indicative of increased risk for the development of AD.

Potential biomarkers of AD include the main constituents of AD pathology: β-amyloid (Aβ) peptides and tau protein. Indeed, the ratio of phosphorylated tau and Aβ42 levels in the cerebrospinal fluid is so far the best indicator of the progression from aMCI to AD.1 However, a less invasive test, ie, blood-based, is necessary for screening large populations at risk of AD. To this end, the plasma levels of Aβ peptides have been analyzed in subjects at risk for AD. The average levels of Aβ42 in plasma are elevated in cognitively normal elderly subjects who progress to AD 4 to 5 years later2 as well as in patients with aMCI who progress to AD.3 However, the results of these studies greatly differ from each other,4-6 probably because the analysis of Aβ in plasma is affected by several properties of Aβ such as the tendency to aggregate, bind plasma proteins, and complex with autoantibodies. Moreover, the same variables are likely responsible for the large variability of Aβ plasma levels in the aMCI and control groups, hampering their use as reliable markers of incipient AD.3

Two of us (M.A.S. and G.P.) recently reported that serum Aβ 1-42 autoantibodies are significantly increased in patients with AD compared with age-matched controls but only after dissociation of antigen-antibody complexes.7 The blood concentration of Aβ 1-42 autoantibodies parallels the cerebral accumulation of Aβ in AD transgenic mice;8 it is, therefore, a potential peripheral marker of incipient AD. To
test this hypothesis, we measured the plasma concentration of dissociated Aβ 1-42 autoantibodies in 107 patients who were clinically followed up for 3 years.

## METHODS

### SELECTION OF CASES

The aMCI cases were selected from outpatients of 4 Italian Alzheimer disease centers. All individuals had at least 8 years of education. In all patients, results of endocrine and metabolic function tests as well as brain computed tomographic scans or magnetic resonance images were normal. Neuropsychological tests were used to evaluate multiple cognitive functions including the immediate and delayed recall of a story and word list recall, 2 tests specific for episodic memory. Patients who scored 1.5 SD below average for their age on the memory tests battery, a Clinical Dementia Rating score of 0.5, and Mini-Mental State Examination score of 26 or more of a possible 30 were diagnosed as having aMCI. All patients complained of memory disturbances for less than 1 year. Control cases were selected among cognitively normal age-matched subjects who had normal scores on the memory tests, 0 on the Clinical Dementia Rating, and more than 28 of a possible 30 on the Mini-Mental State Examination. A total of 180 patients with aMCI and 105 controls were identified. The mean ages of aMCI and control cases were not significantly different (Table).

### FOLLOW-UP EVALUATION

The patients with aMCI were evaluated every 6 months with the same battery of memory tests used at the inception of the study. Clinical Dementia Ratings and Mini-Mental State Examinations were also repeated. Diagnosis of probable AD was established according to the National Institute of Neurological and Communicative Disorders criteria. Thus, patients with deficits in multiple cognitive domains and a Clinical Dementia Rating score of 1 or more were considered to have progressed to probable AD and are referred to hereafter as having aMCI-AD. Seventy-three patients dropped out of the study. A group of 107 cases were followed up for 36 months (Table). Seventy cases evolved into AD. The rate of progression in our group was 21.8% per year, higher than previously reported.9

### DISSOCIATION OF Aβ 1-42 AUTOANTIBODES FROM Aβ PEPTIDE IN HUMAN PLASMA

The determination of human plasma Aβ 1-42 autoantibodies was performed according to the Gustaw method, with slight modifications, as described below. All human plasma samples (controls and patients with aMCI), after dilution (1:100) in dissociation buffer (phosphate-buffered saline [PBS] + 1.5% bovine serum albumin [BSA] + 0.2 mol/L glycine-acetate, pH 3.5; final volume, 500 µL), were incubated for 20 minutes at room temperature and then centrifuged at 16 000g for 25 minutes at room temperature in Centrifuge centrifugal filter device YM-50 (cutoff molecular weight, 50 000 Da; Millipore, Bedford, Massachusetts). The fraction containing the antibodies dissociated from Aβ 1-42 peptide was recovered by centrifugation at 2000g for 3 minutes at room temperature and adjusted to pH 7.0 with neutralization buffer (1 mol/L Tris buffer, 1:1 ratio). The Aβ 1-42 autoantibody recovered fraction was reconstituted to a 500 µL initial volume with dilution buffer (PBS + 1.5% BSA + 0.1% Tween-20). The plasma Aβ 1-42 autoantibody titer was measured by enzyme-linked immunosorbent assay (ELISA). In the control sample, the plasma was handled in the same way but using dissociation buffer, pH 7.0.

### Aβ 1-42 AUTOANTIBODY AFFINITY PURIFICATION

Pooled human plasma Aβ 1-42 autoantibodies that were dissociated from Aβ 1-42 peptide were dialysed against 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, at 4°C, and purified using NHS HP (N-hydroxysuccinimide–activated Sepharose high-performance) SpinTrap (GE Healthcare Bio-Science, Uppsala, Sweden). Briefly, the prepacked NHS-activated High Performance Sepharose column was equilibrated with ice-cold 1mM HCl according to the manufacturer’s instructions. The purified Aβ 1-42 peptide (AnaSpec, San Jose, California) (0.5 mg) was coupled to a 1-mL Sepharose matrix, free groups were blocked using alternate high- and low-pH buffer (0.5 mol/L ethanolicamine with 0.5 mol/L NaCl, pH 8.3, and 0.1 mol/L acetate with 0.5 mol/L NaCl, pH 4.0), and the Sepharose column was equilibrated with 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5. After extensive washes, a pool of Aβ 1-42 autoantibodies was applied to the NHS HP SpinTrap. The affinity column was washed, and the Aβ 1-42 autoantibodies fractions were eluted with 0.1 mol/L glycine-HCl, pH 2.9, and collected into tubes containing 100 mmol/L carbonate-bicarbonate buffer, pH 9.6.

### MEASUREMENTS OF HUMAN PLASMA Aβ 1-42 AUTOANTIBODY CONCENTRATION

The affinity chromatography–purified antibodies were analyzed spectrophotometrically at a wavelength of 280 nm to determine the Aβ 1-42 autoantibodies containing fractions using a Beckman spectrophotometer (Beckman Coulter Inc, Brea, Cali-
HUMAN PLASMA ANTI–Aβ 1-42 IgG REACTIVITY

The plasma Aβ 1-42 autoantibody collected fraction specificity was determined by ELISA. Briefly, 96-well microtiter plates (Costar; Corning Incorporated, Amsterdam, the Netherlands) were coated with 2.5 µg per well of Aβ42 in carbonatebicarbonate buffer, pH 9.6, overnight at 4°C. After extensive washes with PBS + 0.05% sodium dodecyl sulfate (SDS), 100 µL per well of PBS + 6% skimmed milk (wt/vol) was added for 1 hour at 37°C. After the PBS + 0.05% Tween-20 (PBS-T) washes, 50 µL per well of mouse monoclonal anti-human Aβ peptide clone 4G8 (Signet Laboratories, Covance, California) or Aβ 1-42 autoantibody fraction serial dilutions (2.5-10 µg/mL) were applied and incubated for 1 hour at 37°C. To detect clone 4G8 and Aβ 1-42 autoantibody fractions, respectively, 50 µL per well of goat antimouse and goat antihuman IgG peroxidase (Abcam, Cambridge, England) labeled at a 1:2500 dilution in PBS-T were incubated for 1 hour at 37°C. The ELISA plates were washed with PBS + 0.05% SDS followed by 100 µL per well of tetramethylbenzidine (Sigma-Aldrich) developing solution for 30 minutes. The reaction was stopped with 100 µL per well of 2mol/L H2SO4, and the color reaction was determined at 450 nm optical density on a Microplate Manager 5.0 PC (Bio-Rad).

APOLIPOPROTEIN E GENOTYPE DETERMINATION

Apolipoprotein E (APOE) genotype was determined by restriction isotyping of amplified APOE sequences, as previously described. Briefly, DNA was extracted from blood using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), and APOE sequences were amplified using the following primers: 5' TCG GCC GCA GGG CGC TGA TGG 3' forward and 5' CTC GCG GCC CCC GCC CTG GTA 3' reverse. Polymerase chain reaction products were digested using Hin6I (an isoschizomer of HhaI; Fermentas, Burlington, Canada) and then separated on a metaphor-agarose gel.

STATISTICAL ANALYSIS

Data for continuous variables are expressed as mean (standard error of mean). Nonparametric analysis was performed because the data do not follow a gaussian bell-shaped distribution; the Mann-Whitney U test was used to compare 2 groups and nonparametric analysis of variance for more than 2 (Kruskal-Wallis test with Dunnett posttest). A probability lower than 5% was considered significant.

Written informed consent was obtained from all patients (or guardians of patients) participating in the study.

RESULTS

The levels of plasma nondissociated Aβ 1-42 autoantibodies in the total group of patients with aMCI was comparable with those of age-matched controls (Figure 1A). Indeed, following dissociation of the antigen/antibody complexes, the amount of Aβ 1-42 autoantibody was significantly higher in patients with aMCI (Figure 1A). On dissociation, the average concentration of antibodies ranged from 8.1 to 9.4 µg/mL in the 3 groups of cases (Table). In aMCI, the dissociation increased by about 50% the level of antibodies. In the aMCI-AD cases, the titer of dissociated Aβ 1-42 autoantibodies trended higher than in the stable aMCI; however, the difference was not statistically significant (Figure 1B). Of note, only the aMCI-AD cases had Aβ 1-42 autoantibody concentrations that were significantly higher than those of the controls (Figure 1B). In aMCI-AD cases, the Aβ 1-42 autoantibodies increased 3-fold after dissociation (compare Figure 1 parts B and C). In accordance, before dissociation, aMCI-AD cases showed an average level of autoantibodies in the total group of patients with aMCI was comparable with those of age-matched controls (Figure 1A). As shown in Figure 2, in almost all aMCI-AD cases, the dissociation augmented the detection of autoantibodies. Instead, in about 30% of either controls or stable aMCI cases, the level of antibodies was lower after dissociation. This apparent discrepancy may depend on the loss of antibodies during the procedure (separation with Centricon filters) associated with a low amount of the complexes. The APOE ε4 allele frequencies of the aMCI-AD and stable aMCI cases were the same (Table).

COMMENT

We showed that, at the time of diagnosis, the levels of blood Aβ 1-42 autoantibodies are significantly elevated...
in patients with aMCI. Moreover, the antibodies titers are higher in patients with aMCI-AD than in the stable cases, although the difference is not statistically significant. Of note, in aMCI-AD, but not in stable aMCI, the antibodies against Aβ1-42 are significantly higher than in age-matched controls.

Previous studies have shown an increase,17 decrease,18,19 or no difference20 in Aβ autoantibody concentrations in the plasma of patients with AD. The reported variability is most likely dependent on the variation in the percentage of the antibody-antigen complexes. Indeed, dissociation of the antigen-antibody complexes yields reproducible results, showing a significant increase in Aβ1-42 autoantibodies in AD compared with age-matched controls.7

Previous studies indicate that, at the time of the clinical diagnosis of AD, Aβ42 blood levels are within the range of cognitively normal controls, mostly because Aβ shifts into the brain coincident with the accumulation of amyloid deposits, as indicated by the inversely proportional behavior of plasma and cerebral Aβ content that occurs in AD transgenic mice.21 In contrast, Aβ42 blood levels are elevated in the preclinical phase in elderly subjects who will develop AD 4 to 5 years later2 as well as in patients with aMCI that will progress to AD.3 These findings remain controversial because several discordant results have been reported.4-6 The large variability of Aβ42 plasma levels depends on the state of aggregation, the binding to plasma proteins and with autoantibodies.

In contrast, Aβ1-42 autoantibodies in plasma, following dissociation from the antigen, are not influ-
ence by such variables. The plasma anti-Aβ antibodies probably have various origins. They are, for the most part, IgG immunoglobulins that are produced in response to peripheral Aβ as well as to the Aβ deposited in the vessels of the subarachnoid space and, to a lesser extent, in the brain parenchyma that is accessible to immunocompetent cells; moreover, evidence suggests that a brain-to-blood efflux system may work across the blood brain barrier for IgG molecules, preventing the accumulation of IgG in the brain. Amyloid angiopathy occurs early in AD pathology, and its extent is proportional to the brain deposits of Aβ. In the brains of aMCI cases, abundant amyloid plaques already exist in the neocortex, with neurofibrillary pathology limited to the mesial temporal lobe. Our results in patients with AD as well as those with aMCI indicate that plasma Aβ 1-42 autoantibodies parallel the course of Aβ deposition in the brain, which is known to precede by several years the clinical onset of AD. In aMCI-AD, the concentration of autoantibodies trends higher than in the stable cases, though a statistical difference is still lacking, perhaps owing to the limited number of aMCI cases that were followed up.

This finding argues against the protective role of native Aβ autoantibodies that was suggested by the effect of active immunization in AD transgenic mice on amyloid clearance and behavior.

The analysis in blood of Aβ 1-42 autoantibodies after dissociation from the antigen is a simple, inexpensive, and reliable method that can be used to support the predictive diagnosis of AD, with potential relevance to the evaluation of incoming disease-modifying therapies.

Accepted for Publication: December 12, 2009.

Author Affiliations: Unit of Geriatric Medicine, Department of Internal Medicine and Medical Specialties, University of Genova, Genova, Italy (Drs Storace, Borghi, Sanguineti, Giliberto, Piccini, Pollero, Novello, Bossù, and Odetti); Department of Pathology, Case Western Reserve University, Cleveland, Ohio (Drs Smith and Perry); and the College of Sciences, University of Texas at San Antonio (Dr Perry).

Correspondence: Massimo Tabaton, MD, Unit of Geriatric Medicine, Department of Internal Medicine and Medical Specialties, University of Genoa, 16132 Genoa, Italy (mtabaton@neurologia.unige.it).

Author Contributions: Dr Tabaton had access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Tabaton. Acquisition of data: Storace, Cammarata, Borghi, Sanguineti, Giliberto, Piccini, Pollero, Novello, Bossù, and Odetti. Analysis and interpretation of data: Storace, Borghi, Giliberto, Caltagirone, Smith, Perry, Odetti, and Tabaton. Drafting of the manuscript: Storace, Borghi, Sanguineti, Piccini, and Odetti. Critical revision of the manuscript for important intellectual content: Cammarata, Giliberto, Pollero, Novello, Caltagirone, Smith, Bossù, and Perry. Statistical analysis: Borghi, Piccini, Perry, and Odetti. Obtained funding: Cammarata and Tabaton. Study supervision: Tabaton.

Financial Disclosure: None reported.

Funding/Support: The study was supported by grants of Italian Minister of Health (Dr Tabaton), the CARIGE Foundation (Dr Tabaton), and the CARIPLO Foundation (Dr Cammarata).

REFERENCES
