LOOKING AHEAD

AMYLOID-TARGETED THERAPEUTICS IN ALZHEIMER'S DISEASE: USE OF HUMAN ALBUMIN IN PLASMA EXCHANGE AS A NOVEL APPROACH FOR Aβ MOBILIZATION

The fact that 90% of circulating Aβ is bound to albumin led to the hypothesis that if endogenous albumin were replaced through a plasma exchange schedule, the existing dynamic equilibrium set between the CSF and plasma Aβ may be altered.

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CURRENT CONCEPTS ABOUT ALZHEIMER'S DISEASE

Symptoms, epidemiology and diagnosis

Alzheimer's disease (AD) is a chronic, progressive and ultimately fatal neurodegenerative disorder in which normal thinking and memory appear to be disrupted, probably due to impaired or blocked transmission of complex messages between brain cells. Symptoms that characterize AD can be grouped by cognitive dysfunction symptoms (memory loss, language difficulties, impaired intellectual and coordination skills), psychiatric symptoms (depression, hallucinations, delusions, agitation) and a series of symptoms associated with difficulties in performing daily life activities such as shopping, driving and, in severe cases, dressing and eating unaided.

AD primarily affects the elderly. About 6% of people aged over 65 are affected[1] and AD is the most common cause of dementia in this population (50–70%), followed by vascular dementia (30–40%), and mixed dementia (15–20%). It is estimated that 24.3 million people have dementia today worldwide, with 4.6 million new cases of dementia every year.[1] The prevalence of dementia increases exponentially from approximately 1% at 60–65 years of age to more than 30–35% in people older than 80 years. The direct and indirect costs of AD and other dementias are enormous, with the worldwide average annual cost per person with dementia estimated to be USD 10,700 in 2005.[2]

Late-onset AD is considered a complex disorder in which multiple genetic and non-genetic factors must work together to produce the clinical phenotype. APOEε4 allele is the only well-established major genetic risk factor involved in late-onset AD.[3] Carriers of one APOEε4 copy have a 2- to 4-fold risk of developing AD as compared with noncarriers, and APOEε4 homozygotes multiply their AD risk by 154. In addition to *APOE* gene, there may be other loci that could be associated with an increased or decreased risk; however, the true effects of these loci remain controversial as many reported associations are not subsequently confirmed in other studies. Reduced sample sizes, different recruitment sources and strategies or diverse inclusion or exclusion criteria are methodological problems inher-

SUMMARY

A clinical investigation program was carried out to replace endogenous albumin of patients with mild to moderate Alzheimer's disease (AD) with 5% *Human Albumin Grifols®* through a plasma exchange (PE) schedule, in order to alter the dynamic equilibrium between albumin-bound Aβ in plasma and Aβ in cerebrospinal fluid. In a pilot proof-of-concept study, 7 patients underwent 6 PE in 3 weeks and 1 year of follow-up. Plasma Aβ determinations demonstrated a variation pattern in levels in relation with the PEs. Cognitive status scores (MMSE and ADAS-Cog) were more stable than expected. In a phase II clinical trial, 29 patients were randomized into PE-treated and control groups with 1 year follow-up. Interim results point toward the occurrence of $A\beta_{40}$ mobilization in the PE-treated patients, who scored better in cognitive tests (differences at 9 months: 2.5 in MMSE and 5.5 in ADAS-cog). These results suggest that a PE program with 5% *Human Albumin Grifols* may have a promising role in the treatment of mild to moderate AD.

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ent to case-control genetic analyses that may explain part of the controversies.[4]

Early diagnosis of AD has become increasingly important as disease-modifying approaches to treatment are being developed. Although the only definitive diagnosis of AD can be made via brain biopsy or autopsy, there currently are diagnostic criteria allowing standardization of the diagnostic process for physicians. Such criteria include clinical observation of symptoms, neurologic examination, and results from diagnostic tests such as memory screening and psychometric tests, neuroimaging, and cerebrospinal and other fluid markers assessments.[5-7]

Pathophysiology and molecular mechanisms

Neuropathological characteristics of AD include the presence of extracellular neuritic plaques and intraneuronal neurofibrillary tangles in areas of the brain parenchyma involved in memory and/or in brain vessels, predominantly in the amygdala, hippocampus and neocortex.[8] β-Amyloid peptide (Aβ) is the proteinaceous component of the amyloid fibrillar deposits that are usually present in neuritic plaques. Neurofibrillary tangles are composed of paired helical filaments of which hyperphosphorylated tau proteins form the primary component. These lesions are found in nerve cell bodies and in apical dendrites provoking cytoskeletal changes in AD-affected neurons. Although there is an inter-relation and a synergetic effect between Aβ aggregation and the propagation of tau pathology,[9] observations from autopsied AD brains indicate that plaques precede tangles. It is currently accepted that Aβ production and deposition are central to the pathogenesis of AD,[10] although whether Aβ is the ultimate cause is still under debate.

The presence of amyloid plaques is associated with neurotoxic events, oxidative stress and neuroinflammatory reactions.[11,12] However, it is still unclear whether Aβ neurotoxicity is a preliminary cause or rather a late event in the pathophysiology of AD. Affected neurons become dysfunctional, show synaptic and dendritic desarborization, have reduced levels of neurotransmitters, and finally undergo neuronal apoptosis.[13,14]

The amyloid cascade: Aβ aggregation and neuritic plaques

The amyloid cascade hypothesis is one of the several hypotheses that nowadays try to explain the pathogenesis observed in AD.[10] According to this hypothesis, pathologic metabolism of β-amyloid precursor protein (Aβ PP or APP), the originator of the Aβ peptide,[15] is the initiating event, subsequently leading to the aggregation of Aβ to form neuritic plaques, which would favor the formation of neurofibrillary tangles, loss of synaptic connections, death of tangle-bearing neurons and dementia.

APP is a type-I integral membrane glycoprotein containing the Aβ region (4 kD) that is synthesized in the neuronal rough endoplasmic reticulum.[16] Secretory vesicles containing full-length APP are transferred through the Golgi apparatus to the trans-Golgi network and are then axonally transported to the presynaptic outer plasma membrane. APP is processed by several different proteases called secretases, following either a nonamyloidogenic pathway or a pathogenic amyloidogenic pathway (Fig. 1).

In the nonamyloidogenic pathway, membrane-bound APP is constitutively cleaved by α-secretase within the Aβ sequence, therefore preventing the formation of amyloidogenic peptides. APP cleavage by α-secretase gives rise to the release of a large soluble *N*-terminal fragment named sAPP-α and a 10-kD membrane-bound 83-residue COOH-terminal fragment (C83) into the extracellular space. Subsequently, γ-secretase cleaves C83 to produce a nonpathogenic soluble peptide (p3) and a residual APP intracellular domain (AICD). The sAPP-α fragment has been described as possessing neurotrophic and neuroprotective properties.[17]

In the amyloidogenic pathway, APP is cleaved by β-secretase (BACE1) releasing a soluble *N*-terminal fragment (sAPP-β) and a membrane-bound 12-kD *C*-terminal fragment (C99), which is cleaved by γ-secretase

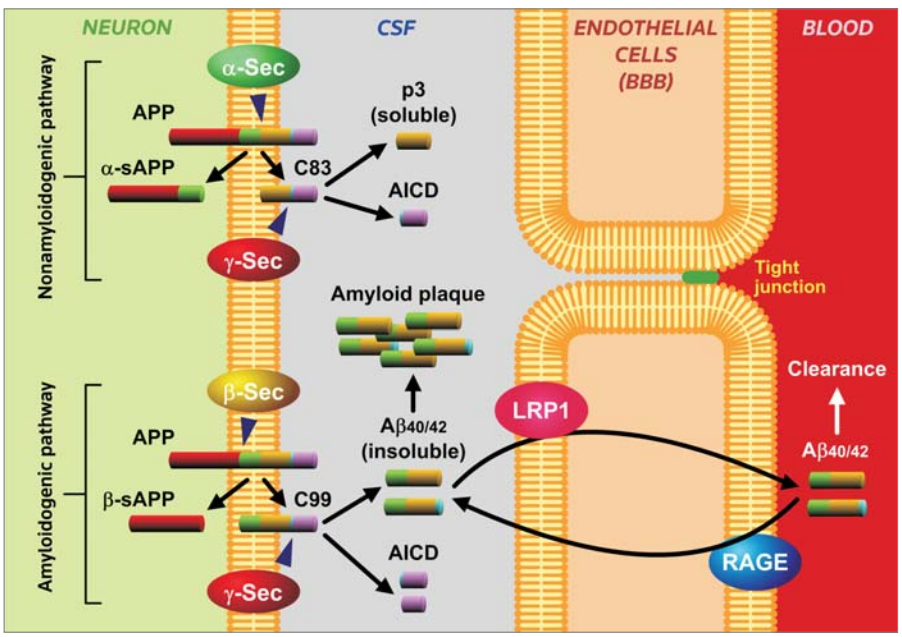

Figure 1. The amyloid cascade hypothesis and Aβ clearance. In the nonamyloidogenic pathway, membrane-bound β-amyloid precursor protein (APP) is constitutively cleaved by α-secretase (α-Sec), giving rise to the release of a soluble *N*-terminal fragment (α-sAPP) and a membrane-bound fragment (C83), which is subsequently cleaved by γ-secretase (γ-Sec) to produce a nonpathogenic soluble peptide (p3) and a residual APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β-secretase releasing a soluble *N*-terminal fragment (β-sAPP) and a membrane-bound fragment (C99), which is cleaved by γ-secretase to produce AICD and a heterogeneous generation of $A\beta_{40}$ and $A\beta_{42}$, whose hydrophobic properties facilitate the formation of amyloid plaque in the cerebrospinal fluid (CSF). Aβ can be cleared from the CSF by transcytosis through the blood–brain barrier (BBB) mediated by low-density lipoprotein receptor-related protein-1 (LRP1), while the receptor for glycation end products (RAGE) mediates Aβ influx into the brain across the BBB.

to produce AICD and a heterogeneous generation of $A\beta_{40}$ and $A\beta_{42}$, whose hydrophobic properties facilitate the formation of amyloid plaque in the brain cerebrospinal fluid (CSF).[18,19] Aβ accumulates not only in the core of neuritic plaques but also on the vessel walls (amyloid angiopathy). The spread of AD pathology can be mediated by soluble extracellular Aβ that induces neurotoxicity and tau hyperphosphorylation in surrounding cells.

Aβ can be cleared off the CSF by transcytosis through the blood–brain barrier (BBB) mediated by low-density lipoprotein receptor-related protein-1 (LRP1), while the receptor for glycation end products (RAGE) mediates Aβ influx into the brain across the BBB (Fig. 1).[20]

Tau hyperphosphorylation and neurofibrillar tangles

Tau proteins are found in all cell types and are major components of neurons where they are predominantly associated with axon microtubules. The main function of the tau protein is to modulate microtubule formation dynamics by site-specific phosphorylation. Normal microtubule assembly occurs in two separate phases: nucleation and elongation. Nucleation occurs when tubulin dimers polymerize to form protofilaments which posteriorly arrange in groups through lateral contacts to form a hollow cylinder; subsequently these microtubules elongate by the continuation of this process. Tau proteins dynamically stabilize microtubules by binding to several tubulin molecules simultaneously. When decreased axonal transport is required, certain motifs within the microtubule binding repeats of tau are phosphorylated by affinity-regulating kinases, thus reducing the binding of tau to microtubules and favoring disassembly.[21,22] In pathological conditions, tau can be hyperphosphorylated at additional sites, thus increasing the propensity of tau to oligomerize and accumulate as intracellular paired helical filaments that can eventually form insoluble aggregates as neurofibrillary tangles. Disruption of normal phosphorylation events results in the deregulation of neurite outgrowth and impaired axonal transport.[23]

Apoptosis and neuronal loss

The concept that the accumulation of large amounts of Aβ in brain amyloid plaques inducing neuronal death is the hallmark of AD still remains controversial. Neuronal loss is particularly difficult to assess, and opposite views have been expressed concerning its course as well as its relation with Aβ and severity in AD. Albeit some studies suggest Aβ is neurotoxic to cells,[13,24] some authors have identified a weak correlation between dementia and neuritic plaques.[25,26] Increasing evidence indicates that neuronal death in AD is the result of apoptotic mechanisms, with Aβ playing a key role.[27,28] Aβ may exert its neurotoxic effects in a variety of ways, including disruption of mitochondrial function,[29] induction of apoptotic genes,[30,31] formation of ion channels[32] triggering loss of calcium homeostasis,[24] stimulation of the JNK pathway,[33] or activation of microglia cells leading to the expression of proinflammatory genes[12,34] and an increase in reactive oxygen species.[11,35] For some authors, caspases might play a dual role in AD influencing the proteolytic processing of APP and increasing Aβ formation, ultimately regulating the apoptotic death of neurons.[36]

THERAPEUTICS

Current management: preventing decline of cognitive mechanisms

Despite years of intensive research, a safe and effective treatment has not yet been encountered. The currently approved therapies are only available for the symptomatic treatment of AD, show no long-term efficacy, and do not prevent disease progression. Current therapeutic agents include cholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) receptor antagonists.

There is evidence that biological dysfunction or imbalance in neurotransmission, particularly cholinergic and glutamatergic, is involved in the etiology of AD. The neurotransmitter acetylcholine is essential for processing memory and learning. Deficits in both concentration and function of acetylcholine have been found in patients with AD, caused by either a loss of cholinergic neurons or decreased acetylcholinesterase activity.[37] Cholinesterase inhibitors have a moderate but worthwhile effect in stabilizing symptoms. The current drugs (donezepil, rivastigmine and galantamine) are adequate for mild and moderate AD.[38-41] On the other hand, overactivation of NMDA receptors, which are pivotal in learning and memory, by the neurotransmitter glutamate has been linked to neuronal damage that may result in cognitive decline in patients with AD.[37] Memantine is a partial NMDA receptor antagonist that appears to be effective in slowing down cognition decline in moderate to severe AD patients.[42]

However, the search for effective treatment strategies for AD continues and special attention is being paid to potential targets for drug and therapeutics development, such as the enzymes and molecules involved in the mechanisms that can lead to the development of the disease. A number of products targeting not only Aβ formation and aggregation but also tau pathology, oxidative stress, inflammation, excitotoxicity and neurodegeneration are currently under active investigation.[43-45]

New perspectives: targeting neuritic plaque

Among the many novel therapeutic approaches under investigation for AD, strategies oriented towards reducing the production of cytotoxic Aβ in order to prevent the accumulation of amyloid deposits or to reduce the existing neuritic plaque seem particularly appealing.[46] Pharmacologic targets as detailed in the following sections and points of action of putative therapeutic agents can be seen in Figure 1.

Reduction of APP

Modulation of APP production is the top upstream targeting of Aβ. Intracellular trafficking of APP may be regulated by multiple factors such as signal transduction enzymes or hormone stimulation. Interfering with these factors may affect intracellular levels of APP and thus the proteolytic processing of APP, thereby reducing the overall levels of Aβ. Compounds such as phenserine, an acetylcholinesterase inhibitor, and deferoxamine, a Fe^{3+} chelator, have been also described as possessing the capacity to lower the rate of APP messenger RNA synthesis, resulting in a substantial reduction of Aβ levels.[47,48] In a phase III clinical study, however, phenserine failed to demonstrate efficacy compared to placebo in cognition tests.

Activation of α-secretase

Favoring APP processing through the neuroprotective, nonamyloidogenic pathway

seems to be a logical alternative strategy to reduce the burden of cytotoxic Aβ. This process should involve the pharmacological activation or upregulation of α-secretase. Multiple enzymes have been identified as possessing α-secretase-like activity. Four members of the ADAM (a disintegrin and metalloproteinase) family, ADAM 9, ADAM 10, ADAM 17 (TACE) and more recently ADAM 19, have been proposed as α-secretases.[49,50] In particular, ADAM 10 has been postulated to exert a predominant role in vivo as the physiologically relevant constitutive α-secretase.[51] Competition between α-secretase and β-secretase for the substrate APP has been demonstrated in vivo, and evidence suggested that overexpression of ADAM 10 inhibited the production of Aβ, prevented plaque formation and alleviated the associated neurological effects.[52] Several mechanisms for α-secretase upregulation have been described, including *ADAM10* gene expression enhancement and stimulation of molecular signaling.[51] Moreover, low cholesterol levels have been associated with higher levels of α-secretase ADAM 10 activity.[53] Statins (e.g., batimastat, marimastat, simvastatin, atorvastatin) are well-known cholesterol-lowering drugs that have been suggested to regulate α-secretase resulting in anti-AD efficacy.[54,55]

Inhibition of β-secretase

As one of the major players involved in the neurotoxic Aβ-generating amyloidogenic pathway, β-secretase may be a key therapeutic target against AD. β-Secretase is an integral membrane aspartyl protease primarily expressed in the brain and often termed BACE1 for β-site APP-cleaving enzyme 1.[56] While recent reports indicate that BACE1 expression is tightly regulated, proposed physiological roles include participation in a wide range of processes such as axonal growth, brain development and myelination, although many of these functions within the central nervous system are not completely understood.[57] Overexpression of BACE1 is associated with neurodegeneration and BACE1 is upregulated in at least some AD brains.[58] Development of effective BACE1 inhibitors has proven challenging, mainly due to difficulties found in successful BBB crossing and delivery to the brain.[59] Current BACE1 inhibition agents under investigation include OM-99-1, OM-99-2, ATG-Z1 and CTS-21166.[55,60]

Inhibition/modulation of γ-secretase

γ-Secretase is a high-molecular-weight complex composed of four major membrane proteins: presenilin 1 (PS1), nicastrin (NTC), presenilin enhancer 2 (PEN-2) and anterior pharynx defective 1 (Aph-1). γ-Secretase is ubiquitously expressed and can cleave a number of different membrane proteins besides C99. Notch 1 receptor is a particularly relevant substrate of γ-secretase.[61] Notch signaling regulates the capacity of neurons to extend and elaborate neurites but it is also involved in embryogenesis as well as cell differentiation and maturation events in adulthood. For these reasons, γ-secretase inhibitors (e.g., begacestat, MK-0752 and flurizan, although the latter failed in a phase III clinical study) can interfere with vital physiological processes causing toxicity.[62] Research for alternatives to γ-secretase inhibitors is focused on the development of selective C99 proteolysis blockers (e.g., imatinib, LDDN-9918) and γ-secretase modulators capable of reducing the formation of pathogenic $A\beta_{40}$ and $A\beta_{42}$ (e.g., ibuprofen, indomethacin), allowing γ-secretase to generate shorter, less fibrillogenic Aβ peptides.[63]

Interfering with Aβ aggregation

AD neurotoxicity is thought to result from the aggregation of Aβ into growing amyloid fibrils that form neuritic plaques.[64] As a consequence, downstream strategies targeting Aβ with the intention to inhibit this aggregation or to disrupt the already formed amyloid plaque in brain tissue are currently under investigation (e.g., immunotherapy, small-molecule pharmacotherapy, metal chelation).[65]

Immunotherapy with Aβ-specific antibodies, which includes active (vaccination) or passive immunization, is thought to act through several mechanisms of action. Antibodies against Aβ can prevent the formation of plaques in some animal models and in humans,[66] although these treatments are associated with deleterious immune reactions.[67,68] Antibodies can bind Aβ in fibrils and plaque, thus favoring disaggregation, producing soluble forms of Aβ that can be eliminated from the body.[69] However, plaque-directed antibodies are required to cross the BBB. It is thought that antibodies can enter the brain by passive diffusion at sites deficient in BBB.[70] Moreover, Aβ-antibody complexes may be cleared by FcRn receptor-mediated transcytosis across the BBB.[71]

Small-molecule inhibitors of Aβ aggregation under active development include Colostrinin, AZC-103, SEN-606, and even natural products derived from *Gingko biloba*, curcumin and nicotine. The Aβ aggregation inhibitor tramiprosate (*Alzhemed®*) failed to show significant differences versus placebo in AD patients. Amyloid plaque degradation enhancers include small molecules such as aleplasinin (PAZ-417) as well as short synthetic peptides that could be active in disrupting the stability of the β sheet.

There is evidence that certain metal ions (Cu^{2+}, Fe^{3+} and Zn^{2+}) play a role in the precipitation of cytotoxic Aβ. In this sense, the possible capacity of metal chelators such as iodochlorohydroxyquin and PBT-2 (the product that replaced the withdrawn clioquinol) to reverse amyloid-β plaque deposition is under investigation.[72,73]

Aβ clearance

In addition to antibody- or drug-mediated Aβ degradation in brain, extracellular monomeric Aβ can be cleared from the brain to the periphery, where it can then be degraded or removed. The concentration of Aβ in brain interstitial fluid is tightly regulated through transport across the BBB (Fig. 1). LRP1 is the major cell surface transporter protein involved in Aβ clearance through transcytosis from the brain to the blood,[74] while the RAGE mediates soluble Aβ influx into the brain across the BBB.[75] RAGE is a potential target for therapies aimed at lowering the Aβ load in brain. Inhibitors of RAGE–Aβ binding currently in the pipeline for mild and moderate AD include PF-04494700 (phase II development).

There is growing evidence that Aβ levels in AD are increased in plasma and decreased in CSF.[76] This observation has led to the design of novel therapeutic strategies proposed to clear Aβ from the brain through the induction of an unbalance of Aβ transport dynamics across the BBB. Thus, the sequestration of Aβ in plasma may both increase the transport of free Aβ from CSF to plasma and reduce Aβ transport into the brain in order to restore the intrinsic equilibrium between brain and blood Aβ levels.[77] Immunotherapy with antibodies binding to and clearing plasma Aβ has the advantage

of not having to undergo BBB crossing and is proven capable of reducing brain amyloid burden in mouse models.[78]

A novel approach: plasma exchange with albumin replacement

Plasma exchange is a process used to eliminate patient's plasma and replacing it with another solution in order to maintain normal volemia and osmotic balance. To achieve this effect, albumin or other colloids have been used, as well as fresh frozen plasma and crystalloids. The purpose of this procedure is to eliminate toxic substances from patient plasma, such as autoantibodies, alloantibodies, immune complexes, proteins or toxins. Plasma exchange is widely used in the treatment of different pathologies. Specifically, this procedure has been applied to the following disorders: Guillain-Barré syndrome,[79] multiple sclerosis,[80] inflammatory demyelinating polyradiculoneuropathy,[81] acute inflammatory demyelinating disease of the CNS[82] and other peripheral neurological alterations.[83]

Here, plasma exchange is presented as a novel approach for the treatment of AD with a focus on plasma Aβ clearance, taking into account the fact that 90% of circulating Aβ may be bound to albumin.[84] Hence, the potential mobilization of plasma Aβ bound to therapeutic albumin through plasma exchange could in turn translate into a mobilization of brain Aβ and, as a consequence, lead to an improvement of the patient's cognitive functions.

With this in mind and taking into account that preliminary studies have demonstrated that *Human Albumin Grifols®* is able to bind Aβ peptide,[85] a clinical investigation program using *Human Albumin Grifols* through a plasma exchange regimen in patients with mild to moderate AD was carried out.

CLINICAL INVESTIGATION PROGRAM OF Aβ MOBILIZATION THROUGH ALBUMIN BINDING AND PLASMA EXCHANGE IN MILD TO MODERATE AD

Pilot study (proof-of-concept)

The first clinical study carried out was a pilot study aimed to assess whether *Human Albumin Grifols* was able to mobilize plasma Aβ peptide when used in a therapeutic plasma exchange program at a rate of two plasma exchanges per week during 3 weeks, that is, 6 plasma exchanges in total. Furthermore, a possible change in the cognitive status was also assessed through neuropsychological evaluations.

During each plasma exchange procedure, a complete plasma volume was removed from the patient and was simultaneously replaced by a similar volume of 5% *Human Albumin Grifols*, which is a concentration of albumin similar to that found naturally in plasma. Preferably, plasma exchanges were performed through a double-lumen central line, although peripheral access was also permitted. After each procedure, blood count, calcium, activated partial thromboplastin time, prothrombin time and fibrinogen were monitored before patients were discharged.

Plasma $A\beta_{40}$ and $A\beta_{42}$ levels were determined at baseline, before and after each plasma exchange and once a month during 6 months of follow-up. On the other hand, CSF $A\beta_{40}$ and $A\beta_{42}$ were determined through a regular spinal tap at baseline, at the end of the plasma exchange period and at 3 and 6 months after the plasma exchange period. Determinations of plasma $A\beta_{40}$ and $A\beta_{42}$ were carried out with a sandwich-type ELISA test (β-amyloid [1-40] ELISA kit, Zymed, U.S.A. and Innotest β-amyloid [1-42] CE, Innogenetics, Belgium) originally commercialized for CSF determinations, following a protocol variation recommended by the manufacturer so that it could be more suitable for plasma determinations. It is important to note that at that moment a validated ELISA test for plasma $A\beta_{40}$ and $A\beta_{42}$ was not commercially available.

In addition to biochemical determinations, cognitive status was evaluated at baseline and at 3 and 6 months after the plasma exchange period through the Mini-Mental Status Examination (MMSE)[86] and the Alzheimer's Disease Assessment Scale, cognitive subscale (ADAS-Cog) examination.[87]

Finally, neuroimaging studies were also performed. Morphological assessments consisted of a magnetic resonance imaging (MRI) performed at baseline and at 3 and 6 months after the plasma exchange period to assess changes in the volume of the hippocampus, cingulate and other areas of interest. Functional neuroimaging assessments consisting of a single photon emission computed tomography (SPECT) were performed at baseline and at 6 months after the plasma exchange period to assess changes in brain perfusion (*Neurogam*™ software, Segami Corp., Columbia, MD, USA).[88] A final follow-up visit was scheduled at 1 year after the enrollment.

This pilot study was performed in a single center (ACE Foundation - Catalan Institute of Applied Neurosciences, Barcelona, Spain). Before participating, each patient and/or close relative and/or legal representative signed the corresponding informed consent. Previously, the study had been approved by the local Ethical Committee and by the Spanish Ministry of Health. In addition, the study was conducted according to the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association.

All patients fulfilled DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) criteria for dementia and were diagnosed according to the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and the Alzheimer's Disease and Related Disorders Association) criteria for possible and probable AD.[89] All patients received a thorough clinical and neurological examination and a comprehensive neuropsychological evaluation including tests for general cognition, memory, language, perceptual and constructional abilities and executive functions. Complete blood analysis and neuroimaging studies were performed in all subjects to exclude other potential causes of dementia following the guidelines for the diagnosis of AD from the Study group on Behavioral Neurology and Dementia of the Spanish Neurological Society.

The patient population consisted of male and female subjects aged between 55 and 85 years, diagnosed with mild to moderate AD (NINCDS-ADRDA criterion) and an MMSE score between 20 and 24. Moreover, patients had to be on stable treatment with donepezil (6 months) and had to have an MRI or CAT scan performed within 6 months prior to participation, with absence of cerebral-vascular findings.

Pilot study results

Ten patients were included in this pilot study following a single-arm, open-label design. Seven out of the 10 patients underwent

plasma exchanges with 5% *Human Albumin Grifols*. Out of these 7 patients, 3 underwent 5 plasma exchanges, 2 underwent 4 plasma exchanges and 2 underwent 3 plasma exchanges, during the planned 3-week period. The main reason why not all patients underwent the 6 plasma exchanges was that the hematology team responsible for the procedure followed the precautionary principle in this special patient population in relation with low coagulation parameters after each plasma exchange and with the mild anemia that is common in therapeutic plasma exchange programs. As will be stated later, based on the fact that the procedure was shown to be safe during the pilot study, an extension of the study was performed in which practically all patients underwent the 6 plasma exchanges within the planned 6 weeks.

Figure 2 shows the average plasma levels of $A\beta_{40}$ and $A\beta_{42}$ in the 7 patients that underwent plasma exchanges. Although there appears to be a slight variation of $A\beta_{40}$ within the plasma exchange period, no clear pattern can be seen. On the other hand, the lack of a variation pattern is even more evident for $A\beta_{42}$. At that moment, the investigators already realized that the lack of a reliable ELISA test for plasma Aβ determinations did not permit the adequate interpretation of plasma results. The method was improved during the study extension as shown later.

With respect to CSF $A\beta_{40}$ and $A\beta_{42}$, Figure 3 shows that both peptides follow a similar kinetics: a decrease is observed during the plasma exchange period followed by an increase after the plasma exchange period returning to baseline levels at 6 months of follow-up.

Figure 4 shows the changes from baseline of the scores corresponding to MMSE and ADAS-Cog tests measured at 3, 6 and 12 months after plasma exchanges. All scores (except obviously that measured at time 0) were assessed after the plasma exchange period (first 3 weeks). From the graphs it clearly appears that the cognitive status of the patients as measured by MMSE and ADAS-Cog remained stable after 1 year of follow-up.

Regarding MRI findings, the volume of the hippocampus measured at baseline, 3 and 6 months suggested a progressive volume increase. However, no clear pattern was

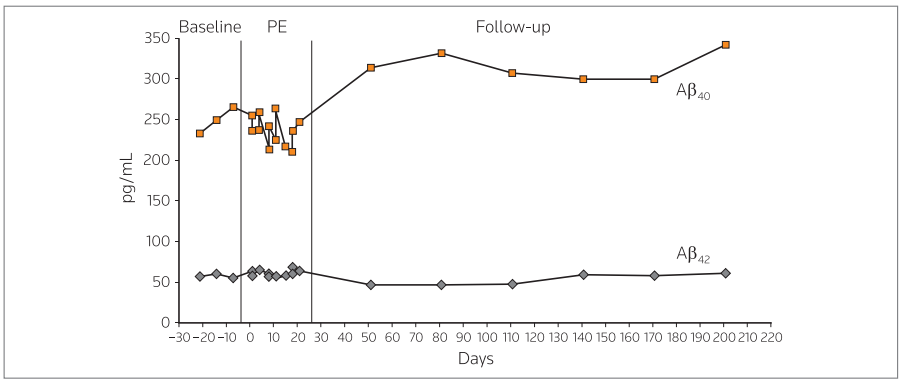

Figure 2. Mean plasma levels of $A\beta_{40}$ and $A\beta_{42}$ in the 7 patients that underwent plasma exchanges (PE) in the pilot study, determined at baseline, before and after each PE and once a month during 6 months of follow-up.

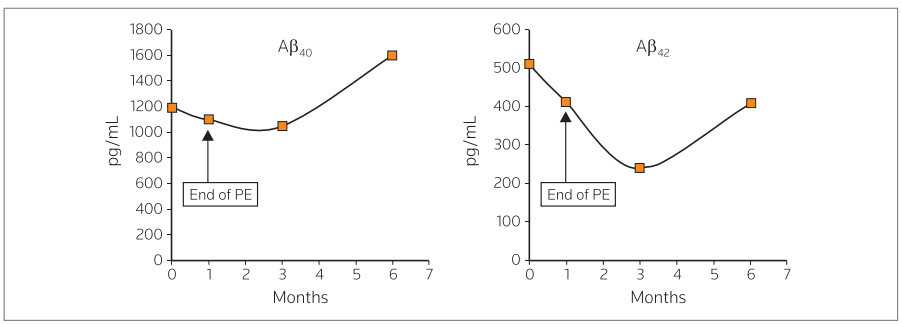

Figure 3. Mean cerebrospinal fluid levels of $A\beta_{40}$ and $A\beta_{42}$ in the 7 patients that underwent plasma exchanges (PE) in the pilot study, determined at baseline, at the end of the PE period and at 3 and 6 months after the PE period.

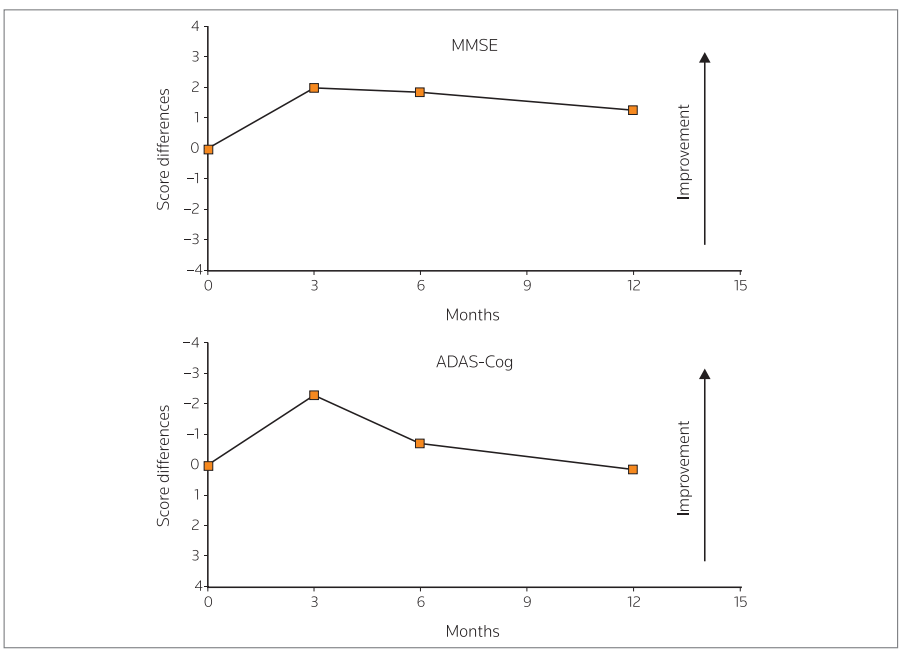

Figure 4. Changes from baseline scores (average from the 7 patients of the pilot study) of the Mini-Mental Status Examination (MMSE) and the Alzheimer's Disease Assessment Scale, cognitive subscale (ADAS-Cog) measured at 3, 6 and 12 months after plasma exchanges. For clarity, negative values of ADAS-Cog have been represented upwards.

observed for the posterior cingulate and the mid frontal gyrus (data not shown). With respect to functional neuroimaging (SPECT), 6 out of 7 patients showed a significant perfusion increase in the frontal and temporal areas (Fig. 5). At 6 months, statistical parametric mapping (SPM) analysis[88] also showed a significant perfusion increase in both the frontal and temporal areas (data not shown).

Pilot study conclusions

One of the principal conclusions of this pilot (proof-of-concept) study was that treatment with 5% *Human Albumin Grifols* through a therapeutic plasma exchange regimen was feasible in mild to moderate AD patients, a patient population in which, to our knowledge, this has been the first time that this therapeutic approach has been carried out. However, an area of uncertainty remained with respect to the number of plasma exchanges to be performed since not all patients completed the 6-exchange cycle.

Relative to plasma levels of $A\beta_{40}$ and $A\beta_{42}$, it was clear that the lack of a reliable ELISA test for plasma determinations made the knowledge that could be extracted from the data obtained very obscure. However, for CSF $A\beta_{40}$ and $A\beta_{42}$, a clear pattern of variation was observed for both peptides suggesting that CSF Aβ may be mobilized with 5% *Human Albumin Grifols* used in the plasma exchanges.

Regarding the neurocognitive scores, the fact that there was a tendency to stabilization after 1 year of follow-up was interpreted as a promising clinical result, in accordance to European Medicines Agency (EMEA) guidelines on medicinal products for the treatment of AD.[90] An obvious criticism is that since the study was open-label, the neurocognitive raters might have set up high expectations for the treatment leading to a bias in the cognitive assessment. Nevertheless, the objective of this pilot study was to uncover favorable tendencies which could be confirmed in subsequent randomized, controlled trials.

Very interestingly, once the study was completed, the patients and their families overtly expressed their satisfaction with their participation and requested an additional treatment cycle. At that moment, the researchers had improved the curve-fitting method to be used with the ELISA test and discovered that the manufacturer had launched an improved test (Innotest β-Amyloid (1-42) RUO, Innogenetics, Belgium) with a higher concentration of the reference peptide used in the kit. These circumstances, along with the observed trend to clinical stabilization at 1 year found in the study, were considered to be sufficient for offering the patients an extension of the original study.

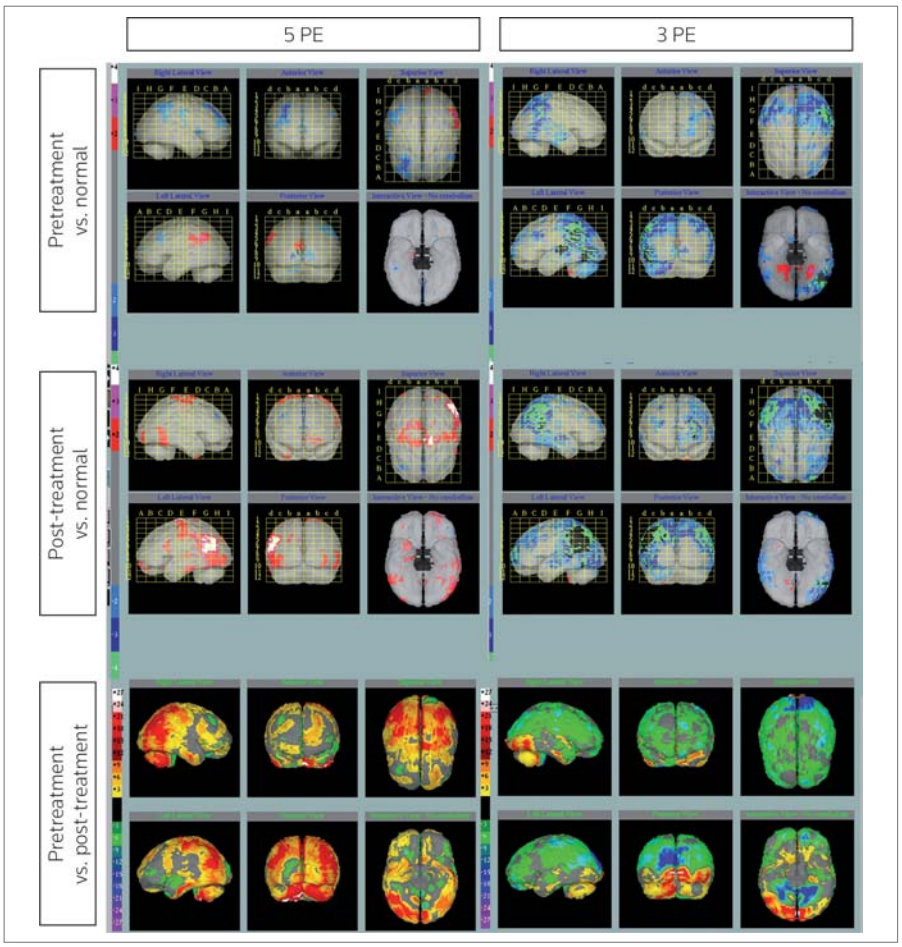

Figure 5. *Neurogam*™ results from 2 representative patients with a different number of plasma exchanges (PE) performed: patient with 5 PE (left panels) had an improved perfusion and patient with 3 PE had an impaired perfusion (right panels). Upper and middle images: before and after treatment compared with a database of healthy subjects (hot colors [red and white] mean hyperperfusion and cold colors [blue and green] mean hypoperfusion). Bottom images: differences observed before and after treatment for each patient (hot colors [yellow, red and white] mean improved perfusion and cold colors [green, blue and purple] mean impaired perfusion).

Extension study

The extension study was a replica of the pilot study with respect to the number and procedures of the plasma exchanges and spinal taps, cognitive and neuroimaging assessments. The follow-up period was also of 1 year. All the patients that participated in the pilot study were offered the opportunity to participate in the extension study. New informed consents were signed and new approvals from the local Ethical Committee and the Spanish Ministry of Health were obtained.

Six patients previously enrolled in the pilot study participated in the extension study. All patients except one completed the cycle of 6 plasma exchanges in 3 weeks in an outpatient regimen. The only patient that did not complete the entire cycle underwent 5 consecutive plasma exchanges. The subject did not undergo the last plasma exchange because the central catheter provided intermittent blood flow and had to be removed.

Extension study results

With the improved curve-fitting for the ELISA test (basically the improvement was that fitting was performed according to a 4-parameter-log model instead of a straight-line model) and the use of the new test with a higher concentration of the reference peptide, the levels of plasma $A\beta_{40}$ (hAmyloid b40 ELISA [HS], The Genetics Company, Switzerland) and $A\beta_{42}$ (Innotest β-amyloid [1-42] RUO, Innogenetics, Belgium) yielded the results shown in Figure 6. It is worth mentioning that the difference in plasma concentration between $A\beta_{40}$ and $A\beta_{42}$ is of about one order of magnitude as reflected in the graph. If one focuses on the plasma exchange period (central segment of the graph), a clear saw-tooth pattern is observed, although it is more apparent for $A\beta_{40}$ than for $A\beta_{42}$ due to the differences in concentration previously mentioned. This pattern was so regular and consistent for both peptides and so reproducible in relation with each plasma exchange that there is little doubt that it is related to the mechanism of action of albumin through plasma exchange on Aβ.

On the other hand, $A\beta_{40}$ and $A\beta_{42}$ in the CSF did not show the variation found in the pilot study but rather a tendency to remain stable and even a trend towards an increase in the case of $A\beta_{42}$ (data not shown).

Taking into account the total of 2 years of follow-up from both the pilot and extension studies, the neurocognitive scores yielded the results shown in Figure 7.

In both graphs, the upper line represents the patient's actual scores (changes from baseline) while the lower straight line represents the expected progression for this type of patient at 2 years of follow-up. Therefore, the surface lying in between can be considered a kind of "improvement area" and gives an idea of the tendency of the patients treated with 5% *Human Albumin Grifols* to remain more stable than what was expected. Finally, results of neuroimaging studies showed a similar trend to that observed in the pilot study.

After obtaining these results, it was clear that a phase II, randomized and controlled clinical trial was warranted to assess whether the behavior of one group of patients treated with albumin and plasma exchange was different from that of a group of nontreated patients in terms of biochemical, clinical and neuroimaging outcomes.

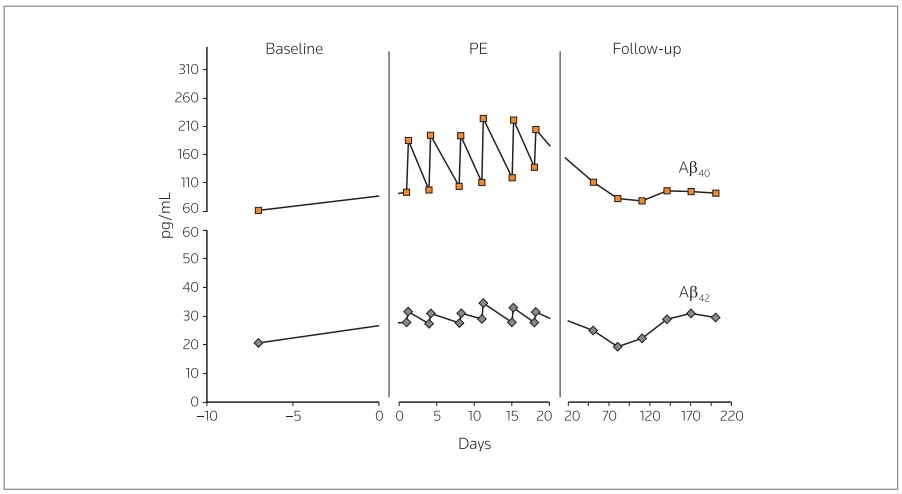

Figure 6. Mean plasma levels of $A\beta_{40}$ and $A\beta_{42}$ in the 6 patients that underwent plasma exchanges (PE) in the extension study, determined at baseline, before and after each PE exchange and once a month during 6 months of follow-up.

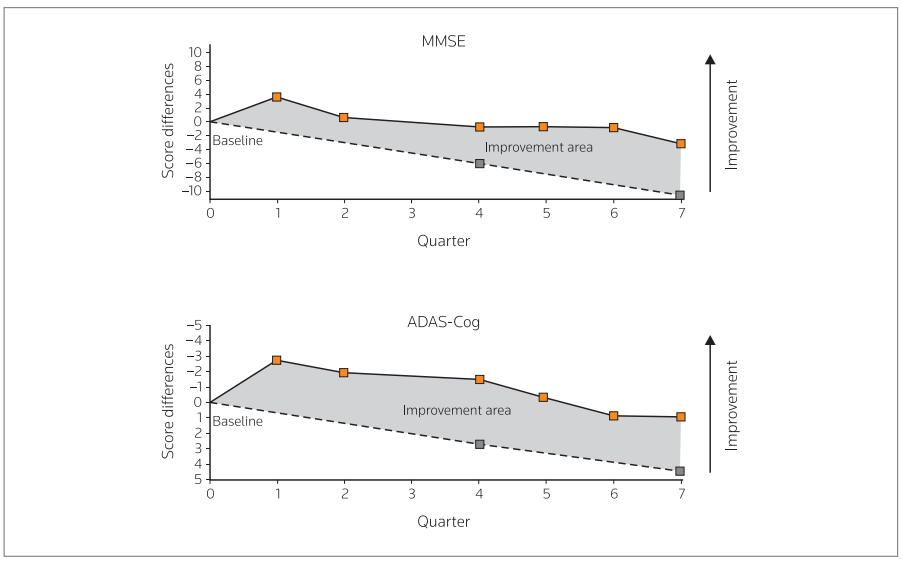

Figure 7. Changes from baseline scores (average from the patients included in both the pilot and the extension study) of the Mini-Mental Status Examination (MMSE) and the Alzheimer's Disease Assessment Scale, cognitive subscale (ADAS-Cog) measured during 2 years of follow-up at weeks 15, 27, 52, 60, 75 and 87. For clarity, the negative values of ADAS-Cog have been represented upwards. The dotted line represents the expected progression for this type of Alzheimer's disease patients at 2 years of follow-up. The surface lying between the two lines can be considered as an indicator of improvement.

Phase II clinical trial

A phase II, randomized, controlled, parallel, single-blind clinical trial was carried out to compare the mobilization of CSF and plasma Aβ, cognitive status and neuroimaging between a group of patients treated with 5% *Human Albumin Grifols* in a plasma exchange regimen and a group of nontreated patients. In addition, $A\beta_{40}$ and $A\beta_{42}$ determinations were planned to be carried out from the plasma removed from the treated patients.

The patient population consisted of male and female subjects, aged between 55 and 85 years, diagnosed with mild to moderate AD (NINCDS-ADRDA criterion) and an MMSE score between 18 and 26. Moreover,

patients were required to be on stable treatment with acetylcholinesterase inhibitors (3 months) and had to have an MRI or CAT scan performed within the 12 months prior to participation, with absence of cerebral-vascular findings.

In this phase II study, 36 evaluable patients were planned to be enrolled in 4 centers, 2 in Spain and 2 centers in the United States. However, the total sample size was increased to 42 patients in expectation of a dropout rate of 15% approximately.

Given the fact that there was approximately a 1-year delay in site initiations in the U.S. as compared with Spain, it was decided to perform an interim analysis with the first 29 patients (80% of the 36 evaluable patients planned) recruited in Spain (at that time, there was only one patient included in the U.S.). Out of the 29 patients included in Spain, 27 had completed the whole study. At the time of writing this interim analysis, the study continues including patients in one center in Spain and in the two U.S. centers.

As with the pilot and the extension studies, each patient and/or close relative and/or legal representative signed the corresponding informed consent before participation. Beforehand, the study had been approved by the corresponding local Ethical Committee and by the Spanish Ministry of Health. In the U.S. the study was approved by the local Independent Review Boards and was submitted to the Food & Drug Administration.

Patients were randomly assigned to either a plasma exchange (removal of one complete plasma volume with simultaneous substitution with 5% *Human Albumin Grifols*) group or a control group. The treatment group underwent the same plasma exchange procedure previously described for the pilot and extension studies. The control group did not undergo real plasma exchanges but a sham procedure consisting of first inserting a cut catheter under the skin at the same anatomical location as the treated patients. Through the sham central line, control patients were apparently connected to the plasma exchange machine which apparently worked in the same way as the real procedure by circulating a colored liquid in a close-circuit manner. Therefore, the plasma exchange procedure resembled that of the treated patients although no plasma was removed from the subject and no albumin was infused. The sham procedure was tested several times until the investigators reached the consensus that only an expert in the field could realize that the procedure was not real.

In this phase II study there were 3 plasma exchange periods: 1) an intensive period in which patients underwent 6 plasma exchanges in 3 weeks (2 per week, same as in pilot study); 2) a maintenance period I in which there were 6 plasma exchanges in 6 weeks (1 per week); and 3) a maintenance period II in which there were 6 plasma exchanges in 12 weeks (1 every 2 weeks). There were 1–2 weeks of rest between periods. Control group patients underwent the same number of sham procedures.

For both treatment and control groups, plasma $A\beta_{40}$ and $A\beta_{42}$ were determined at baseline, before and after each plasma exchange and at 3 and 6 months of follow-up. On the other hand, CSF $A\beta_{40}$ and $A\beta_{42}$ were determined through a regular spinal tap at baseline, between each plasma exchange period and at 3 and 6 months after the plasma exchange periods.

Cognitive status was evaluated through MMSE and the ADAS-Cog tests at baseline, between each plasma exchange period and at 3 and 6 months after the plasma exchange periods except for MMSE which had just 5 determinations since this test was not performed between the intensive period and the maintenance I period. Cognitive assessments were performed by independent neuropsychologists not directly involved in the trial and blinded to study treatment.

Neuroimaging assessments consisted of an MRI performed at baseline and at the end of the 3 plasma exchange periods and at 6 months after the plasma exchange periods. Functional neuroimaging (SPECT) was performed at baseline and at the end of maintenance I and maintenance II treatment period and at 3 and 6 months after the plasma exchange periods. A final follow-up visit was scheduled at 6 months after the plasma exchange periods (approximately 1 year after recruitment).

Phase II study interim results

Of the 29 patients included in this interim analysis, 14 were randomly assigned to the treatment group and 15 to the control group. In the treatment group, all except 2 patients underwent all the planned plasma exchanges corresponding to the 3 periods. One patient underwent only the 3 first plasma exchanges because the family decided to withdraw the patient from the study. Another patient also underwent only the 3 first plasma exchanges because of the presentation of transient aphasia with no ischemic findings on cerebral MRI. However, the investigators recommended this patient's withdrawal.

Figure 8 shows the average plasma levels of $A\beta_{40}$ and $A\beta_{42}$ for treated and control groups corresponding to the 23 patients with available data at the time of writing this report. Similarly to what occurred in the extension study, there is a clear saw-tooth pattern for $A\beta_{40}$ in the treated group which is not present in the control group, strongly suggesting that the changes found in the extension study that are now reproduced in the phase II study are associated with the plasma exchange procedure and related with its mechanism of action. Of note, once the plasma exchange period is over, there is a return to levels similar to those of the control group. For $A\beta_{42}$ there is also a saw-tooth pattern in the treated group, although the control group shows a similar behavior and an evident overlap exists between both groups. Again, since the plasma $A\beta_{42}$ concentration is much lower than that of $A\beta_{40}$ it lies near the detection limit of the technique. From these graphs it appears reasonable to think that $A\beta_{40}$ is the peptide that most reliably represents the kinetics of plasma Aβ during plasma exchange.

Very interesting information arose by working out the average rate of change of $A\beta_{40}$ for each plasma exchange period, that is, dividing the change of $A\beta_{40}$ concentration in each period by the time period in days. The result, shown in Figure 9, is a measure of the mobilization of $A\beta_{40}$ in pg/mL/day, indicating the average amount of $A\beta_{40}$ that is mobilized per unit volume and unit time.

The difference between the treatment and control groups in the graph is remarkable: While the control group does not present any change in $A\beta_{40}$ mobilization, the treatment group presents a clear pattern in relation with the plasma exchange periods. During the intensive period, there is a higher $A\beta_{40}$ mobilization (about 8 pg/mL/day) than that during the maintenance period I (about 4 pg/mL/day), and the lowest mobi-

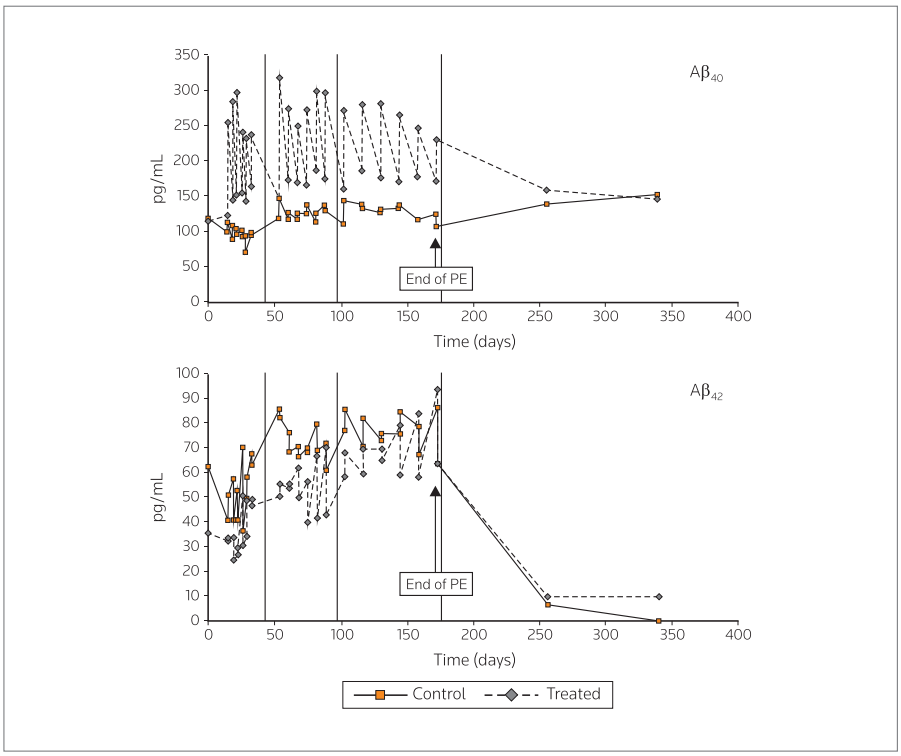

Figure 8. Average plasma levels of $A\beta_{40}$ and $A\beta_{42}$ of treated and control patients (N = 23) in the phase II study, determined at baseline, before and after each plasma exchange (PE) during the 3 treatment periods and at 3 and 6 months of follow-up. The three segments limited by vertical lines indicate the 3 PE periods: intensive period (2 PE per week), maintenance I period (1 PE per week) and maintenance II period (1 PE every 2 weeks). End of PE periods is indicated.

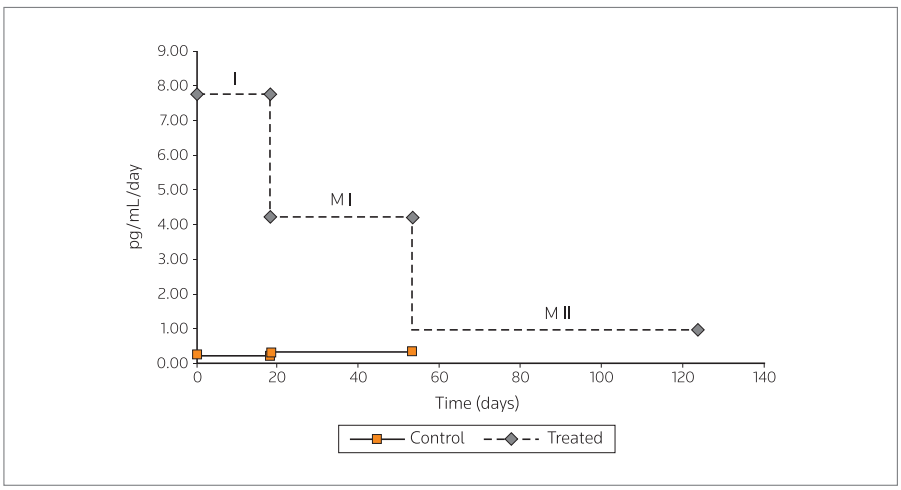

Figure 9. Plasma $A\beta_{40}$ mobilization (changes of average $A\beta_{40}$ concentration in pg/mL per day, during each plasma exchange period) for treated and control patients during the 3 plasma exchange periods (I: intensive period, M I: maintenance I period, M II: maintenance II period).

lization was during the maintenance period II (about 1 pg/mL/day). Beyond the figures, what is most important of this variable is the fact that in treated patients there appears to be a relation between the magnitude of $A\beta_{40}$ mobilization and the "plasma exchange dose" (2 plasma exchanges per week in the intensive period, 1 plasma exchange per week in the maintenance period I and 1 plasma exchange every 2 weeks in the maintenance period II). On the other hand, there is no variation observed in the control group regarding $A\beta_{40}$ mobilization.

However, in order to assess whether there is a plasma exchange period which is the best in terms of absolute change of $A\beta_{40}$ concentration, the authors subtracted the concentration at the beginning of each period from the concentration at the end of such period. These results are shown in Figure 10.

The interpretation of the results is very helpful in order to decide if a more favorable plasma exchange schedule could be selected: The change in plasma $A\beta_{40}$ concentration produced by 2 plasma exchanges per week for 3 weeks (intensive) is similar to that produced by 1 plasma exchange per week for 6 weeks (maintenance I) and twice the change produced by 1 plasma exchange every 2 weeks for 12 weeks (maintenance II). Therefore, if a therapeutic schedule were to be selected based on these data, 1 plasma exchange per week for 6 weeks should be the same as 2 plasma exchanges per week for 3 weeks.

Figure 11 shows CSF $A\beta_{40}$ and $A\beta_{42}$ for treated and control groups corresponding to the 23 patients with available data at the time of writing this report. While for $A\beta_{40}$ there is no overlap between both groups and levels of the treated group remain lower than those of the control group throughout the plasma exchange period, this pattern is not so clear for $A\beta_{42}$. However, it must be kept in mind that CSF $A\beta_{42}$ concentration is one order of magnitude lower than that of $A\beta_{40}$, making it difficult to reliably assess differences since $A\beta_{42}$ levels are closer to the detection limit of the technique.

Finally, results of cognitive scores are shown in Figure 12. Differences from baseline regarding the MMSE scores are represented on the first graph. The treated group scores on average are better than those of the control group during all the plasma exchange periods and during the follow-up (for MMSE higher values represent better cognitive status than lower values). There is no overlap between both groups and there appears to be a tendency towards impairment after the finalization of plasma exchanges. On the other hand, differences from baseline for the ADAS-Cog scores are represented in the second graph. Again, the treated group scores on average are better than those of the control group during all plasma

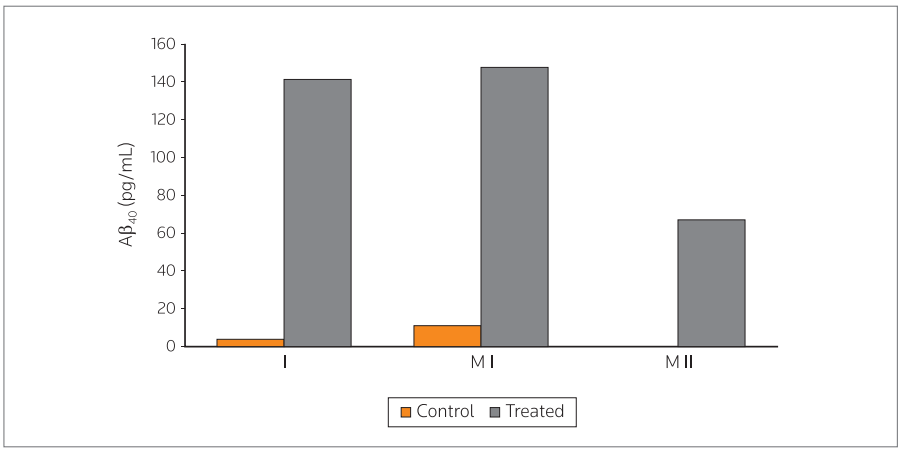

Figure 10. Intraperiod differences of plasma $A\beta_{40}$ (concentration at the beginning of each period subtracted from the concentration at the end of such period). I: intensive period, M I: maintenance I period, M II: maintenance II period.

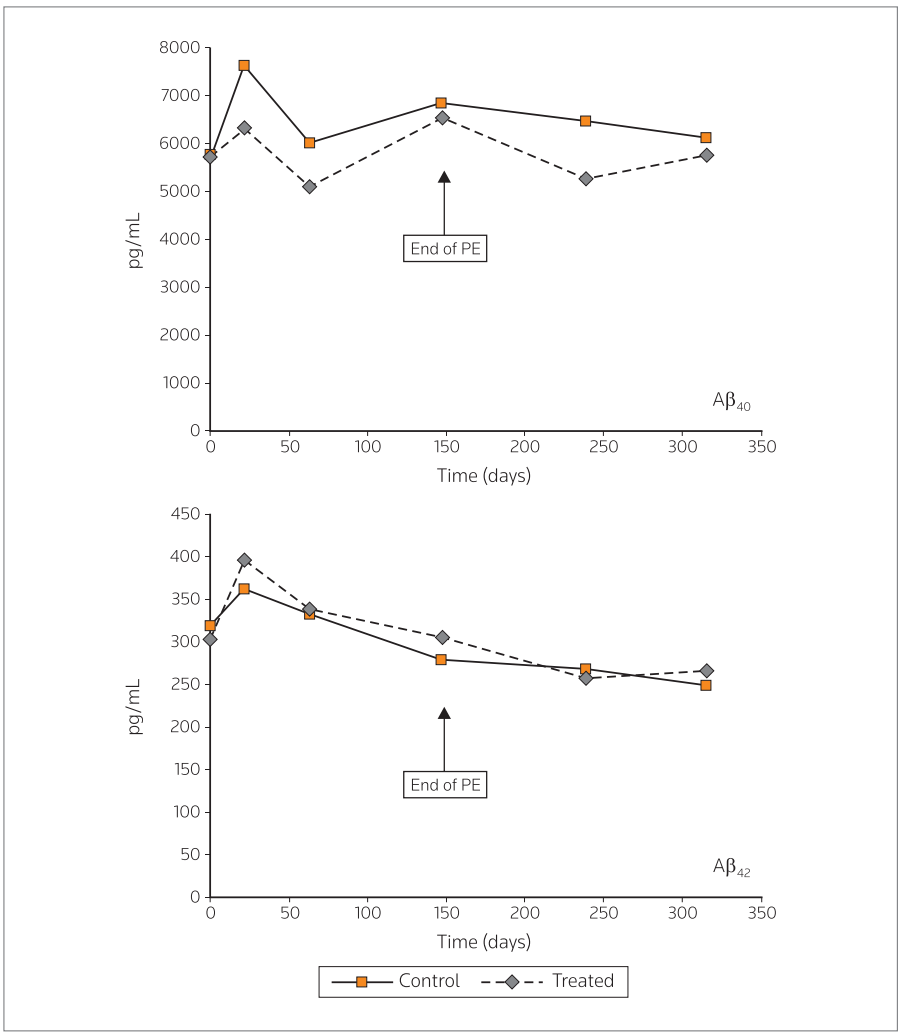

Figure 11. Average cerebrospinal fluid levels of $A\beta_{40}$ and $A\beta_{42}$ for treated (dotted line) and control (straight line) patients (N = 23) in the phase II study determined at baseline, after each plasma exchange (PE) period and at 3 and 6 months of follow-up. End of PE periods is indicated.

exchange periods and during the follow-up (for ADAS-Cog lower values represent better cognitive status than higher values). There is no overlap between both groups and there appears to be an approximation between them as the plasma exchange periods are over. It is important to note that after 1 year of follow-up, the differences between both groups in the ADAS-Cog scores still remained of about 2.5 units. Although the study was not statistically powered to find differences in the cognitive scores, the tendencies found in these interim results are very promising.

At the time of writing this report, neuroimaging variables are still being analyzed and determinations of the plasma removed from the patients are still being assessed.

CONCLUSIONS

The fact that about 90% of circulating Aβ is bound to albumin led to the hypothesis that if endogenous albumin were replaced by 5% *Human Albumin Grifols* through a plasma exchange schedule, the existing dynamic equilibrium set between the CSF and plasma may be altered and this may eventually lead to a decrease in the brain Aβ load.

In order to test this hypothesis, that authors initiated a clinical investigation program which included a proof-of-concept pilot study, an extension of the pilot study and a phase II clinical trial. The main questions that they tried to answer during the proof-of-concept studies (pilot study plus extension) were the following: i) whether plasma exchange procedures were feasible in this complex patient population in terms of management, safety and tolerability; ii) whether a tendency was observed in Aβ mobilization; and iii) whether a tendency was observed in neuropsychological evaluation.

During the pilot study, 7 patients with mild to moderate AD were planned to undergo 6 plasma exchanges in 3 weeks. Plasma and CSF $A\beta_{40}$ and $A\beta_{42}$ were monitored as well as cognitive status and neuroimaging (MRI and SPECT). The pilot study demonstrated for the first time that plasma exchange was feasible in this special patient population. On the other hand, plasma Aβ determinations were controversial since no reliable ELISA test was available at that moment while CSF Aβ determinations suggested a pattern of decrease during the plasma exchange period. Importantly, cognitive

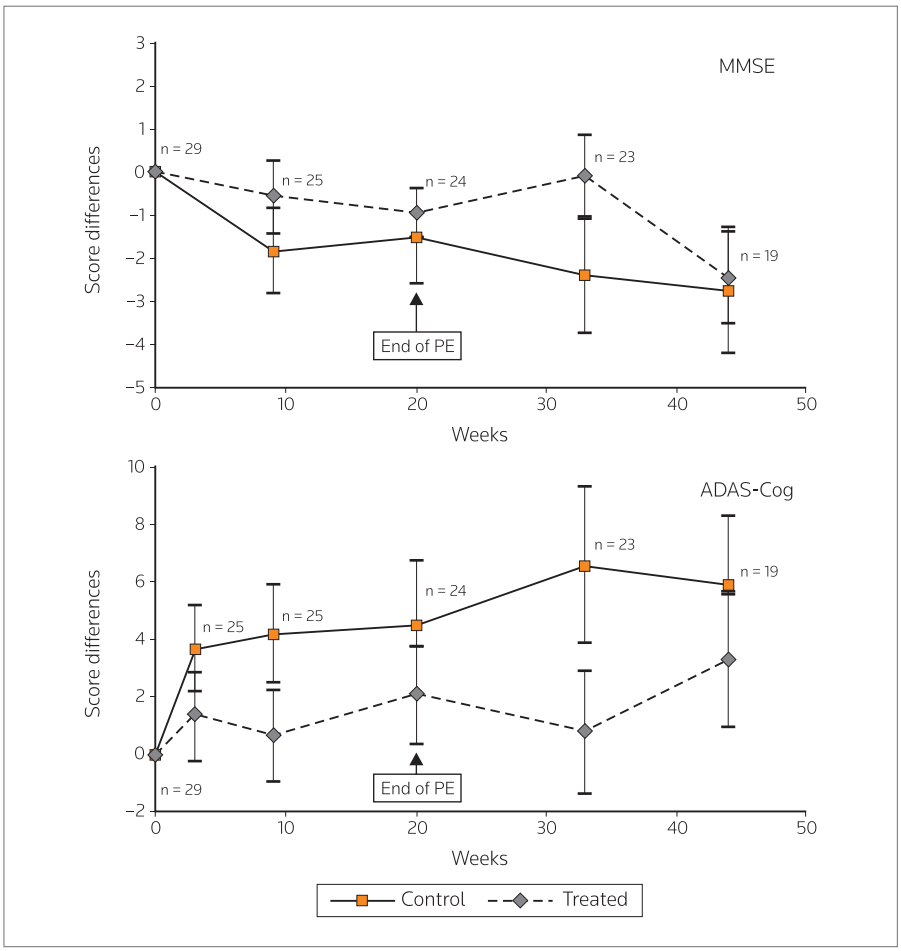

Figure 12. Differences from baseline scores (mean ± standard error) of the Mini-Mental Status Examination (MMSE) and the Alzheimer's Disease Assessment Scale, cognitive subscale (ADAS-Cog) in treated (dotted line) and control (straight line) patients, measured between each plasma exchange (PE) period and at 3 and 6 months of follow-up (MMSE was not determined between the intensive period and the maintenance I period). End of PE periods is indicated.

scores (MMSE and ADAS-Cog) remained stable at 1 year of follow-up.

Due to the tendencies observed in the pilot study and the requests formalized by the patients and their families, an extension study was performed following the same procedures as those of the pilot study and the patients were followed up for 1 more year. In the extension study a new and improved ELISA test was used and plasma Aβ determinations demonstrated that variations in levels were associated with the plasma exchanges, a pattern more apparent for $A\beta_{40}$ than for $A\beta_{42}$. However, CSF Aβ levels did not confirm the pattern found in the pilot study. When cognitive status was assessed at 2 years of follow-up, scores were much more stable than expected for this patient population.

Taken together, the results from the pilot study and its extension suggest that the replacement of endogenous albumin with 5% *Human Albumin Grifols* through plasma exchange was able to produce alterations in the plasma Aβ kinetics and that this finding may be related to a tendency towards the stabilization of cognitive scores.

The above conclusion led to a phase II, randomized and controlled clinical trial in which a group of patients treated with 5% *Human Albumin Grifols* through a plasma exchange program was compared with a control group of patients not treated with plasma exchanges. Thirty-six evaluable patients were planned for this trial but an interim analysis with the first 29 patients (80%) recruited has been performed. Three different plasma exchange schedules were assessed: 2 plasma exchanges per week for 3 weeks, 1 plasma exchange per week for 6 weeks and 1 plasma exchange every 2 weeks for 12 weeks. The total follow-up, including the treatment period, was of approximately 1 year. CSF $A\beta_{40}$ levels suggested differences between groups but this was not the case for $A\beta_{42}$. Plasma $A\beta_{40}$ levels were again found to be associated with plasma exchanges. Interestingly, $A\beta_{40}$ mobilization measured in pg/mL/day showed that the schedule of 1 plasma exchange per week for 6 weeks was similar to that of 2 plasma exchanges per week for 3 weeks, suggesting that both schedules may be equivalent. Changes from baseline in both cognitive scoring systems (MMSE and ADAS-Cog) measured at 1 year showed differences between treated and control groups, with patients belonging to the treatment group scoring better.

Although the phase II clinical trial is still ongoing, the interim results presented in this report together with the previous results obtained during the pilot study and its extension suggest that the novel approach of treating mild to moderate AD with the replacement of endogenous albumin with 5% *Human Albumin Grifols* through a plasma exchange program is not only feasible in this group of patients but it shows a tendency to arrest cognitive impairment at the time period studied. As a consequence, this approach may have a promising role in the future.

However, some uncertain areas still remain and will need further clarification: i) a more reliable method of measuring plasma and CSF $A\beta_{42}$ is needed; ii) the role of MRI and functional neuroimaging needs clarification in terms of their utility to compare treated with nontreated patients; iii) a consistent pattern of variation in CSF Aβ has not been observed, a result which needs further justification; and iv) the confirmation of the promising cognitive outcomes observed during our long-term follow-up studies is pending the finalization of the current study and will probably need larger randomized clinical trials.

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Dr. Becker has also collaborated in the MRI analyses.

DISCLOSURES

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