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Multifunctional liposomes delay phenotype progression and prevent memory impairment in a presymptomatic stage mouse model of Alzheimer disease

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Abstract

The failure of clinical trials largely focused on mild to moderate stages of Alzheimer disease has suggested to the scientific community that the effectiveness of Amyloid-β (Aβ)-centered treatments should be evaluated starting as early as possible, well before irreversible brain damage has occurred. Accordingly, also the preclinical development of new therapies should be carried out taking into account this suggestion. In the present investigation we evaluated the efficacy of a treatment with liposomes multifunctionalized for crossing the blood-brain barrier and targeting Aβ, carried out on young APP/PS1 Tg mice, taken as a model of pre-symptomatic disease stage.

Liposomes were administered once a week to Tg mice for 7 months, starting at the age of 5 months and up to the age of 12 when they display AD-like cognitive and brain biochemical/anatomical features. The treatment prevented the onset of the long-term memory

impairment and slowed down the deposition of brain Aβ; at anatomical level, prevented both ventricle enlargement and entorhinal cortex thickness reduction, otherwise occurring in untreated mice. Strikingly, these effects were maintained 3 months after treatment discontinuation. An increase of Aβ levels in the liver was detected at the end of the treatment, then followed also by reduction of brain Amyloid Precursor Protein and increase of Aβ-degrading enzymes. These results suggest that the treatment promotes brain Aβ clearance by a peripheral 'sink' effect and ultimately affects Aβ turnover in the brain.

Worth of note, the treatment was apparently not toxic for all the organs analyzed, in particular for brain, as suggested by the lower brain TNF-α and MDA levels, and by higher level of SOD activity in treated mice. Together, these findings promote a very early treatment with multi-functional liposomes as a well-tolerated nanomedicine-based approach, potentially suitable for a disease-modifying therapy of AD, able to delay or prevent relevant features of the disease.

Keywords: Liposomes, Alzheimer disease, Aβ peptide, Cognitive impairment, APP/PS1 Transgenic mice.

INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia, accounting for 60 to 80 percent of cases [1]. Although the cause and progression of AD are still not well understood, the central role of Amyloid-β (Aβ) peptide in AD pathogenesis is widely accepted, even if a variety of additional factors, either dependent or independent from Aβ, appears to contribute [2, 3]. In fact, Aβ is thought to directly damage the brain, disrupting the synaptic functionality, which strongly correlates with the cognitive deficits characteristic of the pathology. Given its pivotal role, many Aβ-centered strategies have been attempted and are still in progress; however, several clinical trials focused on

mild to moderate AD have been discontinued, suggesting that at that stage Aβ accumulation has already exerted substantial synaptic and neuronal loss, preventing a clinical recovery. As a matter of fact, accumulation and deposition of brain Aβ is a very early event in AD, and probably begins ~10–20 years prior to the onset of clinically detectable symptoms [4].

These evidences suggest that the effectiveness of Aβ-centered treatments should be evaluated starting as early as possible [5], well before irreversible brain damage has occurred and clinical trials are ongoing in this direction. Accordingly, also the preclinical development of new therapies should be carried out taking into account this suggestion.

Thus, in the present investigation we evaluated the efficacy of a treatment with Aβ-targeting liposomes carried out on young APP/PS1 Tg mice, taken as a model of presymptomatic stage of the disease, to prevent or slow down the onset of typical AD-like hallmarks, namely brain Aβ accumulation , cerebral anatomical abnormalities and memory impairment.

Within this frame, we utilized liposomes designed for AD treatment (mApoE-PA-LIP), dually functionalized with a synthetic peptide (mApoE) containing the receptor-binding domain of apolipoprotein-E for blood-brain barrier targeting and crossing, and with phosphatidic acid (PA) for Aβ binding [6,7]. These liposomes have been previously utilized for acute treatment of aged symptomatic Tg mice, taken as a severe AD model [8], while in the present investigation their efficacy for therapy of AD at presymptomatic stage has been tested, adapting the animal model, the frequency and duration of the treatment to this specific purpose.

MATERIALS AND METHODS

Liposomes preparation and characterization

Preparation and characterization of mApoE–PA–LIP was carried out as repeatedly reported [7-10]. Briefly, liposomes were prepared by extrusion procedure using polycarbonate filters (100 nm pore size diameter), and were composed of sphingomyelin (Avanti Polar Lipids) and cholesterol (Sigma) (1:1 molar ratio), containing 5 mol% of phosphatidic acid (PA; Avanti Polar Lipids) for Aβ binding, and 2.5 mol% of 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(-

poly(ethyleneglycol)-2000)] (mal-PEG-PE; Avanti Polar Lipids) for further surface functionalization with mApoE peptide for BBB targeting. mApoE peptide, carrying the amino acid sequence CWG-LRKLRKRLLR and containing residues 141–150 of the receptor-binding protein of human ApoE, was reacted to form a covalent thioether bond with mal-PEG-PE, resulting in formation of mApoE-PA-LIP [9,10].

Liposomes were freshly prepared and characterized for each round of administration, and size and ζ-potential were comparable to those described previously [7-10].

Animals and *Experimental design*

Twenty APP/PS1 5-month-old Tg male mice (Jackson Laboratory,USA), mean weight of 28-30g, and 20 non-Tg (WT) age-matched littermates were used. Mice were all drug and behavioral test naïve andno environmental enrichment was used because it notably improves AD pathology in mouse models of AD [11,12]. All procedures involving animals and their care were conducted according to European Union (EEC Council Directive 86/609, OJ L 358,1; December 12, 1987) and Italian (D.L. n.116, G.U., Suppl. 40, February 18, 1992) laws and policies, and in accordance with the United States Department of Agriculture Animal Welfare Act and the National Institutes of Health (Bethesda, MA, USA) policy on Humane Care and Use of Laboratory Animals. They were reviewed and approved by the Mario Negri Institute Animal Care and Use Committee that includes ad hoc members for ethical issues (1/04-D).

All animals (Tg or WT) were intraperitoneally injected with mApoE–PA–LIP (100 μl, 73.5 mg of total lipids/kg) or with vehicle (100 μl PBS) once a week for 7 months. Therefore, two experimental groups were treated with mApoE–PA–LIP (Tg and WT mice, n = 10 for each) and two control groups were treated with PBS (Tg and WT, n = 10 for each). The weight of the animals was recorded before each treatment. To minimize the effect of subjective bias, the treatment was performed in blind. Mice were treated always at the same time of the day (9:00–10:00 A.M.) in a specific room inside the animal facility, following a randomized order based on the draw of the animal identification code. At the end of treatment, five animals per group were sacrificed to assess

treatment effects. The rest of the animals was kept for other three months without any kind of treatment and then sacrificed to analyze the duration of the effects after treatment discontinuation.

Novel Object Recognition test (NORT)

NORT is a memory test that relies on spontaneous animal behavior without the need for stressful elements such as food or water deprivation or foot-shock [13]. In the NORT, mice are introduced into an arena containing two identical objects that they can explore freely. Twenty-four hours later, they are reintroduced into the arena, with two objects one of which had already been presented (familiar) and the other new and completely different (novel). The day before the beginning of the treatment, after 4 months from its start and at the end of treatment, mice were tested in an open-square grey arena (40 × 40 cm), 30 cm high, with the floor divided into Twenty-five squares by black lines, placed in a specific room dedicated to behavioral analysis and separate from the operator's room. The following objects were used: a black plastic cylinder (4 × 5 cm), a glass vial with a white cap (3 × 6 cm), and a metal cube (3 × 5 cm). The task started with a habituation trial during which the animals were placed in the empty arena for 5 min and their movements recorded as the number of line-crossings, which provide an indication of both the WT and Tg mouse motor activity. The next day, mice were again placed in the same arena containing two identical objects (familiarization phase). Exploration was recorded in a 10-min trial by an investigator blinded to genotype and treatment. Sniffing, touching, and stretching the head toward the object at a distance of not more than 2 cm were scored as object investigation. Twenty-four hours later (test phase), mice were again placed in the arena containing two objects: one of the objects presented during the familiarization phase (familiar object), and a new, different one (novel object), and the time spent exploring the two objects was recorded for 10 min. Mice were tested following a predefined scheme (five mice for each treatment group and the remaining mice by following the same scheme) so to precisely maintain the 24 h re-test for each mouse. Results were expressed as the percentage of time spent investigating objects in the 10 min or as a discrimination index (DI), i.e. (seconds spent on

novel – seconds spent on familiar)/(total time spent on objects). Animals with no memory impairment spent longer investigating the novel object, giving a higher DI.

MRI analysis

Animals were anesthetized with isoflurane in a mixture of O_2 (30%) and N_2O (70%). Body temperature was maintained at ~37°C by a warm water circulated heating cradle. Imaging was performed on a 7 T small bore animal Scanner (Bruker Biospec, Ettlingen, Germany). Two actively decoupled radio frequency coils were used: a volume coil of 7.2 cm diameter used as the transmitter and a surface coil as the receiver. A 3D RARE T2-weighted sequence was performed to assess anatomical changes. The morphological images were obtained with a voxel size of 117x147x147µm (matrix = 256x102x102 and Field of View = 3x1.5x1.5 cm); TR = 2500 ms, effective TE = 50 ms and a RARE factor of 16, for 1 average.

The volume measurements of structural MRI images were obtained using Java-based custom made software. ROIs were manually chosen by a trained expert following the Paxinos' atlas [14]. Total intracranial volume, whole brain, cortex, hippocampus, striatum and the ventricular system were measured. Data from each animal were obtained by the integration of averaged ROI area for slice thickness.

To measure thickness of the entorhinal cortex, nine coronal slices were selected at the level between Bregma -2.75 mm and Bregma -3.80 mm based on mouse brain atlases [14] and the thickness was measured below the rhinal fissure [15]. We visually inspected all the coronal acquisitions to choose a reference image. We then registered all the other images to the reference one in order to avoid bias due to bad head positioning during the acquisition. For the image registration we used a 3D to 3D rigid body registration with 6 degrees of freedom (FLIRT) [16,17]. The thickness of the entorhinal cortex was then measured six times (three for left and three for right) by a trained expert and the values were averaged to get the final values.

Blood and tissue collection

Animals were deeply anesthetized with an overdose of ketamine/medetomidine (1.5 and 1.0 mg/kg, respectively). The blood was collected from the heart for plasma separation and used for both Aβ level quantification and serum chemistry profile test. The latter test was performed using an automatic biochemistry analyzer (Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany). Afterward, liver, spleen, and brain were dissected, macroscopically analyzed and weighed. One brain hemisphere was fixed and processed for immunohistochemistry; the other hemisphere, liver, spleen and plasma were snap frozen in dry ice and stored at -80°C [18] until Aβ, APP, proteolytic degrading enzyme activity and inflammatory and oxidative stress marker dosage.

Brain immunohistochemistry

APP/PS1 plaque deposition was examined using the 6E10 monoclonal anti-Aβ antibody (Covance). Brain coronal cryostat sections (30 µm; three slices per mouse) were incubated for 1 h at room temperature with blocking solution [6E10: 10% normal goat serum (NGS)] and then overnight at 4°C with the primary antibody (6E10, 1:500). After incubation with the anti-mouse biotinylated secondary antibody (1:200; 1 h at room temperature; Vector Laboratories) immunostaining was developed using the avidin– biotin kit (Vector Laboratories) and diaminobenzidine (Sigma). Tissue analysis and image acquisition were done using an Olympus image analyzer and the Cell-R software. Plaques were quantified by an operator blind to genotype and treatment using Fiji software, through the application of a homemade macro.

Aβ quantification in animal organs

Mouse brains were treated as described previously [19] with some modifications. Mouse brain hemispheres were homogenized in a Tris buffer containing 50mM Tris-HCl, pH 7.4, 150mM NaCl, 50mM EDTA, 1% Triton X-100, and 2% protease inhibitor. After centrifugation (15.000 rpm, 21.000 × g, 4°C for 25 min), the supernatant was retained as the Triton-soluble fraction (soluble Aβ). The pellet was homogenized a second time in the presence of 70% formic acid (FA) (10% v/w) and ultracentrifuged (55.000 rpm, 100,000 × g, 4°C, 1 h), and the resulting FA-extracted supernatant was neutralized with 1 M Tris buffer, pH 11, representing the FA-extracted insoluble

fraction (insoluble Aβ). Levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in each fraction were quantified by sandwich ELISA (IBL international). Liver and spleen were homogenized as the brain and their Aβ levels were quantified by ELISA in the Triton-soluble fraction.

Levels of plasma $A\beta_{1-40}$ and $A\beta_{1-42}$were quantified by ELISA (Wako Chemicals GmbH). Each sample was assayed in triplicate.

Proteolytic enzyme, inflammatory and oxidative stress marker assay in mouse organs

The Triton-soluble fraction, obtained from brain, liver and spleen as described in the previous section, was also used to quantify the amount of active Neprilysin (NEP) and Insuline Degrading Enzyme (IDE) (SensoLyte Activity Assay Kit; AnaSpec, Inc.), IL-1β and TNF-α level (Quantikine ELISA kit; R&D Systems), the amount of MDA adducts (OxiSelect Competitive ELISA kit; Cell Biolabs, Inc.) and SOD activity (OxiSelect Activity Assay; Cell Biolabs, Inc). For active NEP and IDE quantification, the non-interference of the analyzed samples with the activity of the enzymes was verify.

Brain APP, IDE, LRP-1 and RAGE levels analysis

Aliquots of the Triton-soluble factions obtained from brains were run on a precast NuPAGE 4-12% bis-Tris gel (Invitrogen corporation, Milano, Italy), transferred to a nitrocellulose membrane, probed with anti-APP antibody (1:1000 dilution, Chemicon), anti-IDE antibody (1:1000 dilution, Invitrogen), anti-LRP-1 antibody (1:1000 dilution, Thermo Fisher) or anti-RAGE antibody (1:1000 dilution, Thermo Fisher) and visualized with enhanced chemiluminescence (ECL) by ImageQuant LAS4000. The protein load was controlled by β-actin immunoblotting using β-actin loading control antibody (1:2000 dilution, Invitrogen). The content of APP, IDE, LRP-1 or RAGE were quantified by intensity of the chemiluminescent bands using ImageJ Software.

Statistical analysis

Data were expressed as mean ± SEM. For western blot, Aβ dosage and plaque quantification, data were analyzed by Student's *t* test. For NORT, MRI analysis, proteolytic degrading enzyme activity and inflammatory and oxidative stress marker dosage, data were analyzed by a two-way ANOVA.

In the presence of a significant interaction between the factors Tg × treatment, the Tukey's *post hoc* test was applied. $p < 0.05$ was considered significant.

RESULTS

mApoE-PA-LIP treatment prevented memory impairment in APP/PS1 mice

5 month-old APP/PS1 Tg mice and age-matched WT littermates were intraperitoneally (IP) treated with mApoE-PA-LIP (treated mice; Tg n = 10, WT n = 10) or PBS (untreated mice; Tg n = 10, WT n = 10) once a week for 7 months and periodically submitted to the Novel Object Recognition memory test (NORT). The day before the first injection (Figure 1A and B), as assessed by NORT, 5 month-old WT were indistinguishable from Tg mice in their memory performance, as expected at this age [20]. At 9 months, which is after 4 months treatment, untreated Tg mice displayed memory impairment with respect to WT mice (Figure 1A and C). On the contrary, Tg mice receiving mApoE-PA-LIP showed a significant memory preservation, since their performance was comparable to that of WT. The same pattern was observable at the end of treatment, after 7 months (Figure 1A and D). As a further control, the treatment of WT mice with mApoE-PA-LIP exerted no negative effect on their memory at any age.

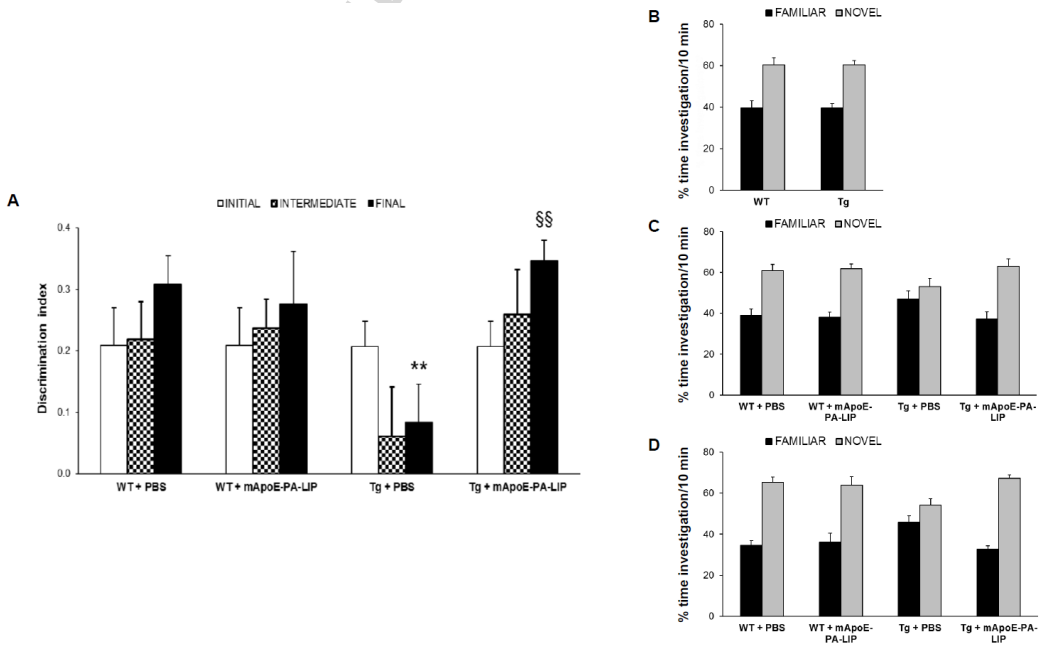

Figure 1.
Effect of mApoE-PA-LIP treatment on long-term recognition memory. 5 month-old APP/PS1 Tg or WT mice were intraperitoneally treated with mApoE-PA-LIP (100 µl, 73.5 mg of total lipids/kg) or vehicle (100 µl PBS) once a week for 7 months and periodically submitted to NORT. (**A**) Histograms show the discrimination index of the experimental groups the day before the first injection (white bars), after 4 months from beginning the treatment (checked bars) and at its end (black bars). (**B**) Histograms show the time percentage of investigation of the familiar (black bars) and novel (grey bars) objects of the experimental groups tested the day before the first injection. (**C**) Histograms show the time percentage of investigation of the familiar (black bars) and novel (grey bars) objects of the experimental groups tested after 4 months from the beginning of the treatment. (**D**) Histograms show the time percentage of investigation of the familiar (black bars) and novel (grey bars) objects of the experimental groups tested at the end of treatment. Data are presented as mean ± SEM (one-way ANOVA, Tukey's *post hoc* test, **$p<0.01$ untreated Tg *versus* untreated WT mice; §§$p<0.01$ treated *versus* untreated Tg mice; n = 10/group).

mApoE-PA-LIP treatment prevented the occurrence of AD cerebral anatomical abnormalities

At the end of treatment, a magnetic resonance imaging (MRI) analysis was carried out on treated and untreated mice to investigate eventual changes in volume or thickness of different brain areas. The ventricle volume of treated Tg mice was smaller (-22%), while entorhinal cortex thickness was higher (+10%) than untreated Tg mice and were comparable to WT (Figure 2A and B). The treatment did not exert any effect on whole brain, hippocampus, cortex or striatal volumes (data not shown).

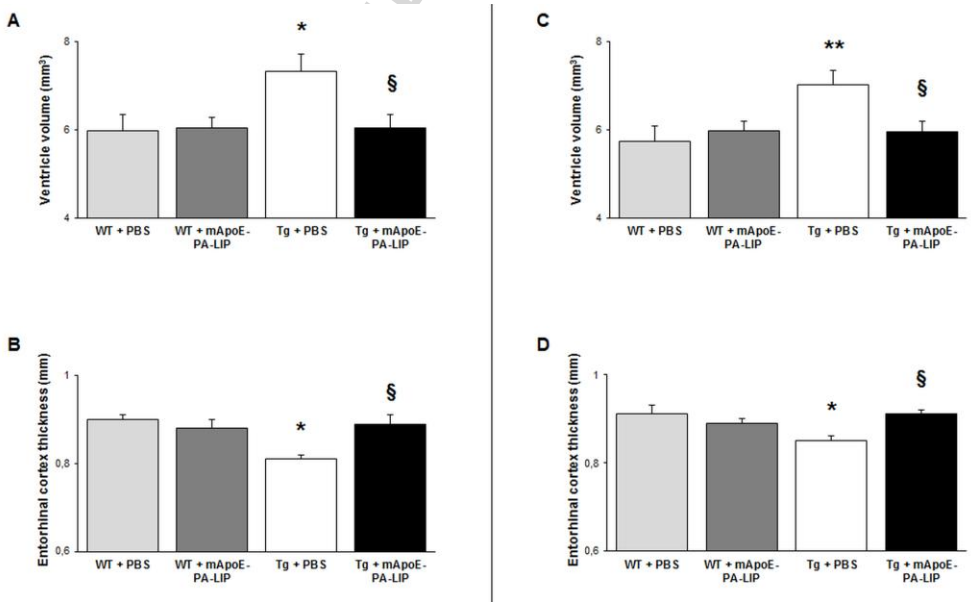

Figure 2. Effect of mApoE-PA-LIP treatment on brain structures. At the end of treatment, a MRI analysis was performed (**A**, **B**). After 3 months from treatment interruption, the MRI analysis

was repeated on remaining animals and the same brain structures quantified in volume or thickness (**C**, **D**). (**A** and **C**) Histograms show the change of ventricular volume in the brain of untreated or treated Tg mice with respect to untreated WT mice. (**B** and **D**) Histograms show the change of entorhinal cortex thickness in untreated or treated Tg mice with respect to untreated WT mice. Data are presented as mean ± SEM (one-way ANOVA, Tukey's *post hoc* test, *p<0.05, **p<0.01 untreated Tg *versus* untreated WT mice; § p<0.05treated *versus* untreated Tg mice; n = 10/group in A and B; n = 5/group in C and D).

mApoE-PA-LIP treatment slows down brain Aβ accumulation in APP/PS1 mice

At the end of treatment animals (n = 5 per group) were sacrificed and brains collected and analyzed to quantify plaque load and to measure Aβ levels. As shown in Figure 3A, untreated Tg mice exhibited important plaque load, as expected at 12 months of age [21]. Notably, the brains of mApoE-PA-LIP-treated Tg mice displayed a lower content of plaques, both in area (-29%) and in number (-31%) in both the cortex and the hippocampus (Figure 3B and C). In parallel, the content of insoluble Aβ species in the brain of treated mice was lower than in untreated ones (Figure 4A). In contrast, no difference was detected in soluble Aβ species (Figure 4B).

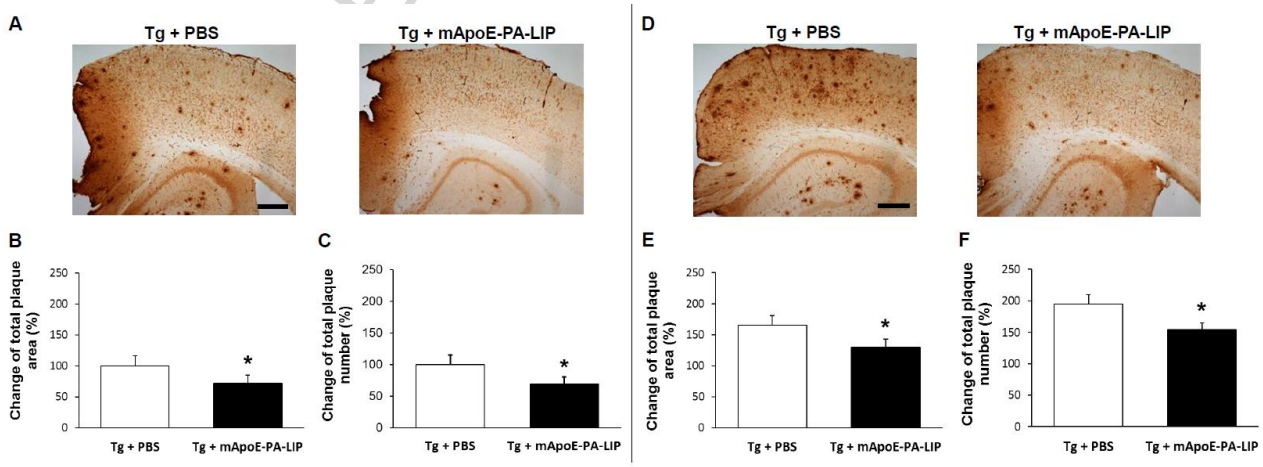

Figure 3. Effect of mApoE-PA-LIP treatment on Aβ plaque deposition. At the end of treatment, half of the animals was sacrificed and half brain was immunostained with the anti-Aβ 6E10 monoclonal antibody for plaque quantification (**A, B, C**). After three months from treatment

interruption, the rest of the animals was sacrificed and their brain processed for plaque quantification (**D, E, F**). (**A** and **D**) Representative brain sections of untreated and treated Tg mice, stained with the anti-Aβ 6E10 antibody, are shown. Scale bar = 250 μm. (**B** and **E**) Histograms report the percentage difference in the total plaque area in treated and untreated Tg mice with respect to untreated Tg mice at the end of treatment. (**C** and **F**) Histograms report the percentage difference in the number of plaques in treated and untreated Tg mice with respect to untreated Tg mice at the end of treatment. Data are presented as mean ± SEM (Student's *t* test, **p*<0.05; n = 5/group).

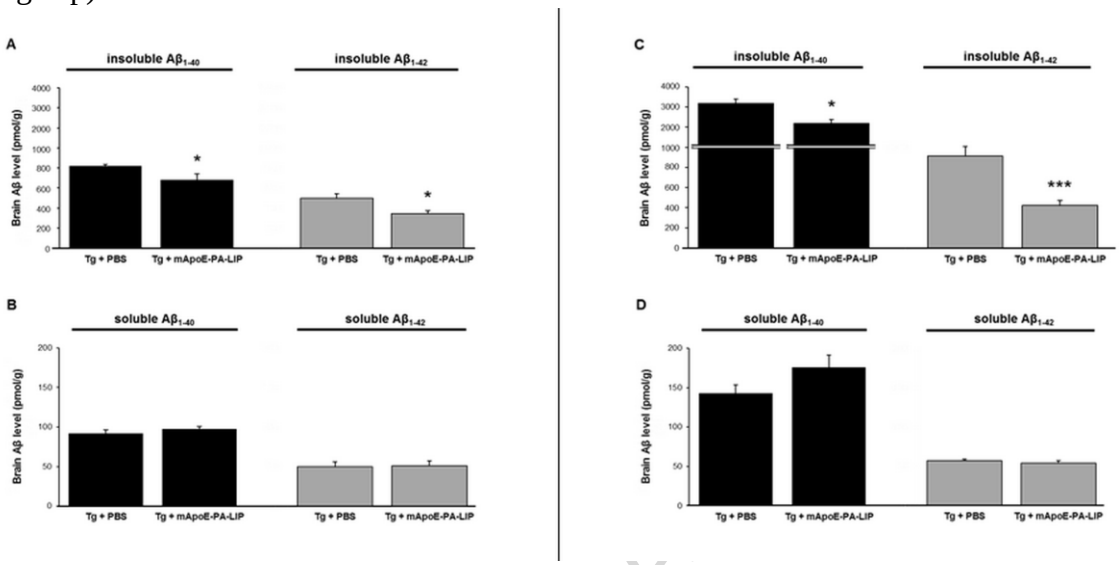

Figure 4. Effect of mApoE-PA-LIP treatment on brain Aβ burden. At the end of treatment, half of the animals was sacrificed, half brain was homogenized and insoluble and soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ amounts were measured by ELISA (**A**, **B**). After three months from treatment interruption, the rest of the animals was sacrificed and their brain analyzed for Aβ content (**C**, **D**). Histograms display the level of insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in the brain of treated and untreated Tg mice (**A** and **C**). Histograms display the level of soluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in the brain of treated and untreated Tg mice (**B** and **D**). Data are presented as mean ± SEM (Student's *t* test, **p*<0.05, ***p<0.001; n = 5/group).

mApoE-PA-LIP treatment increased peripheral Aβ levels

At the end of treatment, we assessed the amount of brain Neprilysin (NEP) and Insulin degrading enzyme (IDE) [22]. As shown in Figure 5A and B, the amount of active NEP and IDE in untreated Tg mice was lower (-60% and -35% respectively) than in WT, and was not affected by the treatment. As regards to brain APP, no difference between treated or untreated Tg mice was observed (Figure 6C dotted bars).

Finally, after completion of the treatment, Aβ levels were found significantly increased (+36%) in the liver of treated Tg mice with respect to untreated Tg (Supplementary Information, S1).

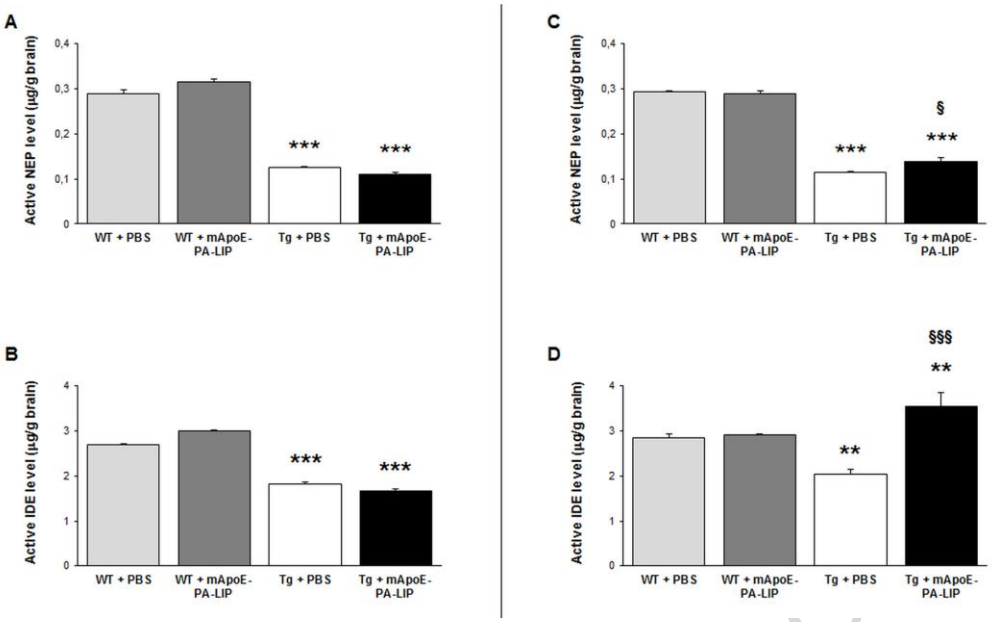

Figure 5. Effect of mApoE-PA-LIP treatment on the amount of active proteolytic degrading enzymes. At the end of treatment, half of the animals was sacrificed, half brain was homogenized and the amount of active Neprilysin (NEP) and Insuline degrading enzyme (IDE) measured (**A**, **B**). After three months from treatment interruption, the rest of the animals was sacrificed and their brain analyzed for active NEP and IDE quantification (**C**, **D**). (**A** and **C**) Histograms report the level of active NEP in the brain of treated and untreated WT or Tg mice. (**B** and **D**) Histograms report the level of active IDE in the brain of treated and untreated WT or Tg mice. Data are presented as mean ± SEM (one-way ANOVA, Tukey's *post hoc* test, ***p*<0.01, ****p*<0.001 *versus* untreated WT mice; §*p*<0.05, §§§*p*<0.001 treated *versus* untreated Tg mice; n = 5/group).

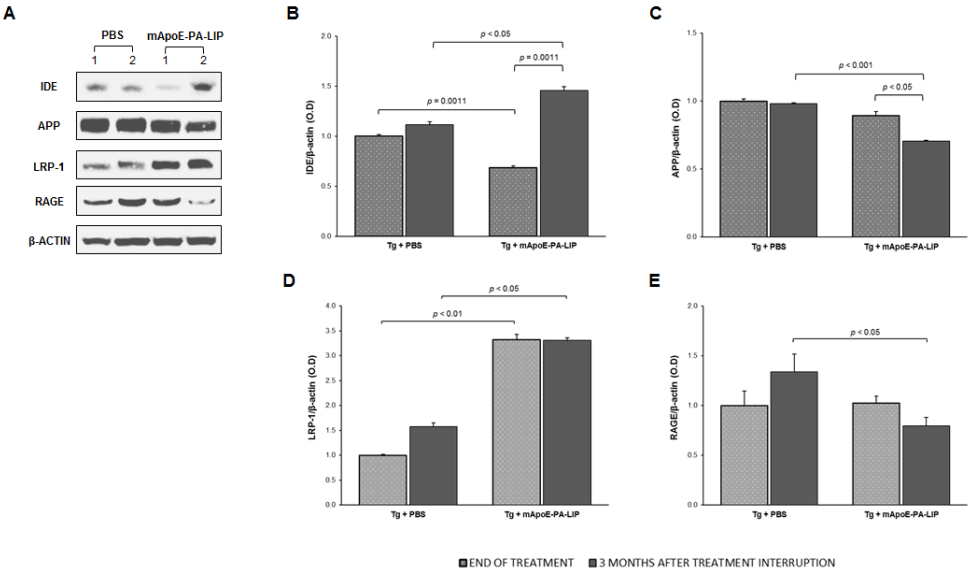

Figure 6. Effect of mApoE-PA-LIP treatment on brain APP, IDE, LRP-1 and RAGE levels. At the end of treatment and three months after its interruption, animals were sacrificed and half brains homogenized and submitted to SDS-PAGE and Western blot (**A, B, C, D** and **E**). (**A**) Immunoblots of brain homogenates from untreated (PBS) and treated (mApoE-PA-LIP) Tg mice. Lanes 1, at the end of the 7-month treatment; lanes 2, three months after treatment interruption. (**B**) Relative quantification of the immunoblot bands of IDE on β-actin. (**C**) Relative quantification of the immunoblot bands of APP on β-actin. (**D**) Relative quantification of the immunoblot bands of LRP-1 on β-actin. (**E**) Relative quantification of the immunoblot bands of RAGE on β-actin. Dotted bars, at the end of treatment; grey bars, 3 months after treatment interruption. Data are presented as mean ± SEM (Student's *t* test; n = 5/group).

mApoE-PA-LIP treatment effects persisted 3 months after discontinuation

To investigate whether the effects exerted by the treatment would persist after its discontinuation, five animals per group were kept for 3 further months after the end of treatment. The memory test at the end of this period was not performed, due to the small number of animals, not suitable for a statistically significant evaluation. Brain MRI analysis showed that, after three months, both ventricle volume and entorhinal cortex thickness of treated Tg mice remained stable and comparable to WT, while untreated Tg mice still displayed enlarged ventricle volume (+23%) and thinner entorhinal cortex (-7%) (Figure 2C and D). No changes were detected on the other brain areas.

After MRI, animals were sacrificed and organs analyzed. Tg mice having received the mApo-PA-LIP treatment were compared with untreated Tg mice and the results, 3 months after treatment discontinuation, can be summarized as follows:

i) the amount of plaques in both the cortex and the hippocampus as well as the total amount of brain insoluble and soluble Aβ species were still lower in treated Tg mice (Figure 3D, E and F and Figure 4C and D).

ii) Aβ levels in the liver remained significantly higher (+43%). (Supplementary Information, S1)

iii) Aβ levels in the plasma significantly decreased (-23%)

iv) The amount of NEP and IDE was higher (+21% and +73% respectively) (Figure 5C and D; Figure 6A and B).

v) APP brain levels were significantly lower (-21%).(Figure 6A and C).

vi) LRP-1 and RAGE brain levels were significantly different (+111% and -41%, respectively) (Figure 6A, D and E).

mApoE-PA-LIP treatment did not induce toxic effects either in the brain or in peripheral organs

The potential toxicity of mApoE-PA-LIP treatment was investigated both at the end of treatment and after its discontinuation. Body weight and motor activity of all mice were monitored throughout the 7 months of treatment and no significant changes among the groups was observed (data not shown). The serum values of total cholesterol, triglycerides, lipoproteins (high-density lipoproteins, HDL; low density lipoproteins, LDL) and transaminases (alanine transaminases, ALT; aspartate transaminases, AST) were not statistically different between experimental groups (data not shown), suggesting no effect of mApoE-PA-LIP treatment on hepatic function. In addition, macroscopic examination of livers, spleens and brains revealed a normal aspect, without hyperplasia or necrosis, and the comparison of organ weights between treated and control groups did not show any significant difference (data not shown). At a molecular level, brains, livers and spleens also underwent a biochemical analysis for inflammation and reactive oxygen species (ROS) production.

As first, the effects on CNS were evaluated. Regarding inflammation, IL-1β and TNF-α were chosen as representative inflammatory markers [23]. Brain TNF-α levels of treated Tg mice were lower than untreated Tg, and comparable with those of WT, both at the end of treatment (Figure 7B) and 3 months after its discontinuation (Figure 7D). The treatment of Tg mice, with respect to untreated, did not induce any change of brain IL-1β levels, which were higher than in WT, both at the end of treatment (Figure 7A) and 3 months after its discontinuation (Figure 7C).

Concerning oxidative stress, MDA decreased and SOD activity increased in the brain of treated Tg mice with respect to untreated ones also after its discontinuation (Figure 8).

Successively, peripheral organs were analyzed. TNF-α in the spleens was higher (+69%) in Tg mice at the end of the treatment with respect to untreated and WT, and decreased down to the values of other groups after treatment discontinuation (data not shown). On the contrary, MDA adducts were found decreased (-28%) in the livers both at the end of treatment and after its discontinuation (data not shown).

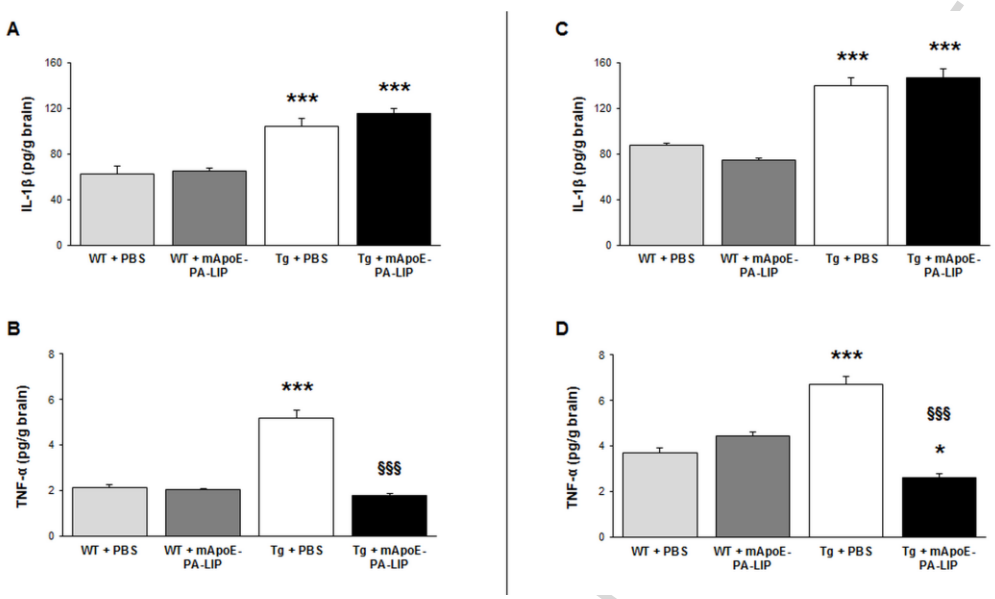

Figure 7. Effect of mApoE-PA-LIP treatment on brain inflammation. At the end of treatment, half of the animals was sacrificed, half brain was homogenized and inflammatory cytokine levels were measured (**A**, **B**).After three months from treatment interruption, the rest of the animals was sacrificed and their brain analyzed for inflammatory cytokine levels (**C**, **D**). (**A** and **C**) Histograms indicate the level of IL-1β in the brain of treated and untreated WT or Tg mice. (**B** and **D**) Histograms indicate the level of TNF-α in the brain of treated and untreated WT or Tg mice. Data are presented as mean ± SEM (one-way ANOVA, Tukey's *post hoc* test, **p*<0.05, ****p*<0.001 *versus* untreated WT mice; §§§*p*<0.001 treated *versus* untreated Tg mice; n = 5/group).

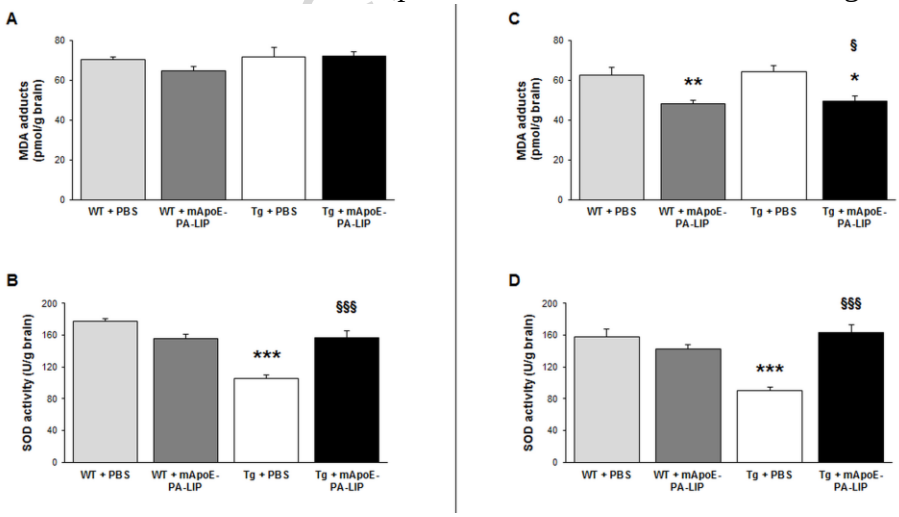

Figure 8. Effect of mApoE-PA-LIP treatment on brain oxidative stress. At the end of treatment, half of the animals was sacrificed, half brain was homogenized and oxidative stress

indicator levels were measured (**A**, **B**). After three months from treatment interruption, the rest of the animals was sacrificed and their brain analyzed for oxidative stress indicator levels (**C**, **D**). (**A** and **C**) Histograms show the level of MDA adducts in the brain of treated and untreated WT or Tg mice. (**B** and **D**) Histograms show the level of SOD activity in the brain of treated and untreated WT or Tg mice. Data are presented as mean ± SEM (one-way ANOVA, Tukey's *post hoc* test, *p<0.05, **p<0.01, ***p<0.001*versus* untreated WT mice; §p<0.05, §§§p<0.001 treated *versus* untreated Tg mice; n = 5/group).

DISCUSSION

It is nowadays recognized that brain Aβ pathophysiological alterations leading to AD take place decades before the appearance of the first signs of dementia, providing a wide pre-symptomatic time window for intervention with Aβ-targeted therapies [4,5,25]. Within this frame, in the present investigation we evaluated the possibility to use a nanomedicine tailored for treatment of AD, mApoE-PA-LIP, to hinder the progression of brain Aβ accumulation and/or memory impairment. We tested this possibility by treating APP/PS1 mice of age 5 months, that can be taken as a model of presymptomatic stage of AD. In fact, at this age APP/PS1 mice display detectable brain extracellular Aβ deposition, but dysfunction of learning and memory is not yet apparent.

The treatment ended at 12 months of age, when untreated mice display AD-like cognitive, anatomical and biochemical feature [20,26,27].

A remarkable outcome of this investigation is that an early treatment with mApoE-PA-LIP can prevent memory impairment, otherwise occurring in untreated mice. In fact, the cognitive performance in terms of memory evaluation of treated Tg animals remained comparable to that of WT for the entire duration of the treatment.

Another relevant result of this study is that brain Aβ accumulation, both as plaques and total insoluble peptide, was strongly delayed in treated Tg mice. Looking for a possible explanation of these striking results, it should be pointed out that no difference in NEP, IDE and APP brain levels were observed in treated Tg mice with respect to untreated, thus ruling out an effect on CNS Aβ metabolism exerted by the treatment. Instead, the increase in liver Aβ levels of treated Tg mice suggests that the peptide is hauled from the brain to this organ. Thus, it is possible that mApoE-PA-

LIP enter the brain and slow down Aβ aggregation, as previously demonstrated [7,8], maintaining soluble Aβ species available for efflux to blood withdrawn by circulating liposomes - the so called 'sink effect' [28,29,30]. This hypothesis is supported by the observation that, in spite of a reduced plaque deposition, no changes in soluble brain Aβ levels were detected, and also by previous investigations showing that liposomes mono-functionalized only to target Aβ (with PA) or only the BBB (with mApoE) did not exert significant improvement of memory and did not reduce brain plaques [8,29]

Finally, the present study showed that 3 months after discontinuation of the treatment, mice still displayed a lower brain Aβ burden than untreated animals, suggesting that the treatment is effective also for a certain time after its discontinuation. The long-lasting effect of treatment on brain Aβ levels could be linked to a later involvement of NEP/IDE and APP, whose levels significantly increased or decreased, respectively, after the end of treatment.

Taking into account the relatively short half-life of mApoE-PA-LIP in the blood [7], the fact that Aβ levels were still high in the liver of treated animals three months after discontinuation of the treatment suggests that the treatment could affect the Aβ transport pathways across the BBB. This possibility is partly supported by the observation that the brain levels of the two main transporters in either direction of Aβ across the BBB, LRP-1 and RAGE [31], were found increased and decreased, respectively, in the brains of treated Tg mice. Of course, the mechanism by which mApoE-PA-LIP treatment may influence the expression of those transporters needs to be clarified. However, it could be speculated that these changes in the expression of the transporters might reflect the levels of their substrate (soluble Aβ) both in the brain (increasing) or in the blood (decreasing).

Concerning the effects of the treatment on cognitive impairment, a possible explanation could be found in the lower TNF-α level and in the higher level of SOD activity detected in the brain of treated Tg mice, suggesting an anti-inflammatory action and a protective effect against ROS, persisting after the end of treatment. These features could prevent damage at synapses, which are

considered as the primary Aβ target in AD pathogenesis [32]. The hypothesis of neuronal protection is also supported by the MRI analysis, showing that treated Tg mice displayed brain ventricle volume and entorhinal cortex thickness comparable to WT.

Despite its long duration, the current treatment proved that mApoE-PA-LIP are well-tolerated, without significant toxic effects either on brain or on peripheral organs. Also previous studies [7,8,10, 33], carried out in vitro assessing complement activation and cytotoxicity and oxidative stress markers , or in vivo, assessing mouse weight and motor activity, did not show negative outcomes.

Since this investigation has been carried out using the i.p. route that is largely utilized especially in mice for practical reasons, representing a convenient way for repeated administrations, it could be questioned how liposomes reach the brain. A first possibility is that they enter the mesenteric vessels, are drained into the portal vein and pass through the liver. Another possibility is that vesicles can drain through the stomata in the diaphragm to reach the lymphatic system, and eventually the bloodstream. Of course, this is an interesting hypothesis that neither has been evaluated for liposomes nor for other types of nanoparticles and deserves further investigations.

Finally, it should be also taken into account that i.p. administration for a long time, even if convenient for mice, could be clinically not practical. Thus, we performed comparative experiments by administering radiolabelled liposomes, i.p. or i.v. to healthy mice, searching for indications that another route of administration could be pursued. The results (Supplementary information, fig. S2) suggest that liposomes reach the brain intact and in comparable amounts, independently on the route of administration, thus opening the possibility that the treatment with mApoE-PA-LIP is potentially transferrable to clinics. Of course, the exact timing and dosing are still to be defined.

CONCLUSIONS

Currently no cure is available for Alzheimer's disease (AD) and the only drugs approved may alleviate some symptoms without delaying or stopping the progression of the disease. Considering that Aβ-targeted clinical trials were discontinued on overt AD patients, early treatment strategies are envisaged. Our findings indicate mApoE-PA-LIP as a new all-in-one multitask approach potentially suitable as disease-modifying therapy for treatment of AD at presymptomatic stage, avoiding the onset of cognitive deficit and brain structural changes, and slowing down the progression of Aβ accumulation. Moreover, they suggest that strategies targeting abeta peptide accumulation, until now not clinically successful, are worth to be re-approached at this stage of the disease.

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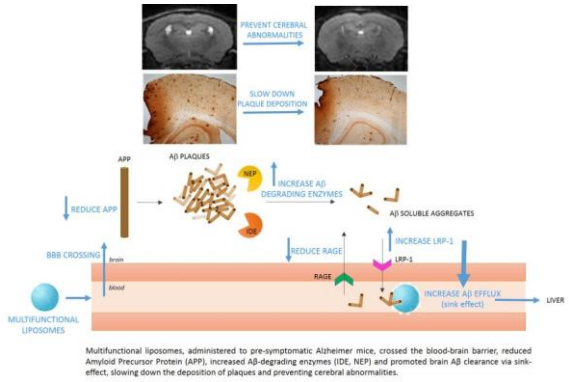
Graphical abstract



Multifunctional liposomes, administered to pre-symptomatic Alzheimer mice, crossed the blood-brain barrier, reduced Amyloid Precursor Protein (APP), increased Aβ-degrading enzymes (IDE, NEP) and promoted brain Aβ clearance via sink-effect, slowing down the deposition of plaques and preventing cerebral abnormalities.