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Supplementary Methods

Determination of aducanumab serum concentrations and anti-aducanumab antibodies (clinical study)

Blood samples were collected for aducanumab concentration assessment on Day 1, and at Weeks 4, 6, 8, 12, 16, 20, 24, 26, 28, 32, 36, 40, 42, 44, 48, 52, and end of study or termination (pre- and ≤10 min post-infusion on Day 1 and at Weeks 4, 24, and 40). Microtitre plates were coated with anti-idiotype aducanumab antibody, followed by blocking, washing, and incubation with 1/100 diluted calibrators, controls, and samples. A horseradish peroxidase (HRP)-conjugated anti-human IgG and tetramethyl benzidine (TMB) substrate were used to detect bound aducanumab. The standard curve range was 0.2–10 μg/mL for aducanumab in human serum. Samples measuring >10 μg/mL were reanalysed at an appropriate dilution. Sample results <0.2 μg/mL were reported as below the limit of quantitation.

Blood samples collected on Day 1, and at Weeks 4, 8, 12, 20, 28, 36, 44, 52, and end of study or termination, were analysed for the presence of anti-aducanumab antibodies. ADAs were measured in serum using a screening assay based on a bridging solution ELISA format. Samples (including positive and negative controls) were diluted 1:100 with phosphate-buffered saline/Casein containing an equimolar mixture of biotin-aducanumab and digoxigenin (DIG)-aducanumab, and incubated overnight at 2–8ºC. The bridging complexes formed of ADA, biotin-aducanumab, and DIG-aducanumab molecules were captured on a streptavidin-coated Nunc™ plate and detected using anti-DIG-HRP conjugate and TMB substrate. Enzymatic reaction was stopped by the addition of sulphuric acid. Absorbance read at 450 nm was

proportional to the amount of ADA. A sample was considered positive when: average (sample signal) ≥ screening assay cut-point (1.16 x mean negative control signal). Antibody-positive samples in the screening assay were further confirmed in the confirmation assay by using the same assay format as for the screening assay but in a competitive assay format by adding excess unlabelled drug to the labelled drug molecule mixture and measuring specific signal inhibition. A sample was confirmed positive if signal inhibition was ≥ confirmation assay cut-point (6.6%). Titres of the confirmed antibody-positive samples were determined by testing 2-fold serially diluted samples in the screening assay. Antibody titre represents the highest dilution of a sample yielding the assay signal ≥ screening assay cut-point (1.16 x mean negative control signal).

Biochemical measurements in preclinical mouse models

For the 'Target engagement' and 'Chronic efficacy' studies, frozen hemi-brains were homogenised in 10 volumes (10 mL/g of wet tissue) of a solution containing 50 mM NaCl, 0.2% DEA[42] with protease inhibitors, and sonicated for 15–20 sec on ice. Samples were then centrifuged at 100,000 x g for 30 min at 4°C. The supernatant was retained as the DEA extracted soluble Aβ fraction. The remaining pellets were resuspended in 10 volumes of 5 M GuHCl, sonicated, and centrifuged as above. The resultant supernatant was retained as the GuHCl-extracted insoluble Aβ fraction. The concentrations of individual Aβ species ending at positions 40 and 42 were determined in brain and plasma using the Multi-Spot Human (4G8) Abeta Triplex Assay (Meso Scale Discovery, Gaithersburg, MD, USA), according to the manufacturer's instructions. To determine plasma and brain drug concentrations, 96 well microplates (Nunc Maxisorp, Corning Costar) were coated with $A\beta_{42}$ peptide at a

concentration of 5 µg/mL in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 overnight at 4°C. Plasma or DEA-extracted brain homogenate samples were diluted to final working concentrations and incubated for 2 hours at room temperature. Binding was determined using a HRP-conjugated goat anti-human or goat anti-mouse polyclonal antibody (Jackson ImmunoResearch, West Grove, PA, USA) followed by measurement of HRP activity using the substrate TMB. Concentrations were determined by comparison to a standard curve generated using purified antibodies.

Histological assessment

Brains were dissected and fixed by immersion in 10% neutral buffered formalin for 48 to 72 hours. Fixed brains were then processed and paraffin-embedded in a horizontal orientation. Each block was sectioned until the hippocampus was identified, at which point 300 consecutive 5 µm sections (3 sections per slide) were prepared. For both 6E10 and ThioS staining, every 14th slide was stained (approximately 1 section every 225 µm).

Immunohistochemistry to define total brain β-amyloid load used the mouse monoclonal antibody 6E10 (BioLegend, Dedham, MA, USA) as the primary antibody (1.3 µg/mL) and the UltraMap anti-mouse Alkaline Phosphatase Kit (Ventana Medical Systems, Tucson, AZ, USA). Slides were pretreated with 88% formic acid solution, prior to being placed on a *Ventana Discovery XT* immunostainer. Slides were counterstained with Ventana Hematoxylin, coverslipped, and air-dried overnight.

After immunostaining, slides were scanned with an *Aperio XT* (Aperio Technologies, Inc., Vista, CA, USA) whole-slide imaging system at 20x magnification following the

manufacturer's instructions. Digital images were then reviewed and the section with the best morphology out of the 3 on each slide was selected. Hippocampus and cortex from this section were manually annotated as separate regions of interest (ROI) and analysed using an algorithm written with Visiopharm software (Visiopharm A/S, Hörsholm, Denmark) that determined the total area of the annotated hippocampus or cortex and the areas of parenchymal and vascular β-amyloid in these anatomic regions at 10x virtual magnification. Validation of the algorithm was performed on a set of 50 slides.

Slides were also stained with ThioS as described previously,[43] and were coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). After ThioS staining, slides were reviewed and the section with the best morphology out of the 3 on each slide was selected and scanned with an *Aperio FL* fluorescent whole-slide imaging system as described above. Dunnett's post-hoc test following Kruskall–Wallis one-way analysis of variance based on ranks was used to assess significance of treatment effects between groups. For detection with 6E10, the ROIs were manually annotated and analysed using an algorithm written with Visiopharm software and adapted for fluorescence. This algorithm determined the total area of the hippocampus and the cortex and the areas of parenchymal and vascular β-amyloid in these anatomic regions at 10x virtual magnification. Validation of the algorithm was performed on a set of 10 slides. All histological assessments were conducted in a blinded manner.

Biophysical characterisation of high molecular weight Aβ aggregates

The high molecular weight Aβ oligomers and β-amyloid fibrils were characterised by transmission electron microscopy (TEM), size exclusion chromatography, and SDS

polyacrylamide gel electrophoresis (SDS-PAGE). Using TEM, β-amyloid fibrils appeared as long, straight, and unbranched structures with a characteristic diameter of 70–80 Å, as described previously.[44] Oligomers appeared as segments shorter than fully formed fibrils, with a curved appearance, ~30 Å in diameter. Using size exclusion chromatography on a Superdex-200 column with PBS as the mobile phase, the monomeric preparation eluted at an apparent molecular weight of ~4–10 kDa, as expected, while the oligomeric preparation contained a major peak at >500 kDa (i.e. in the void volume of the column) with a minor amount of monomer. On SDS-PAGE, monomeric Aβ was characterised primarily by the predicted band at 4 kDa, with smaller amounts of a band at ~8 kDa that is most likely an artefact,[45] while oligomeric and fibrillar preparations showed significant quantities of high molecular weight species (>50 kDa) and, particularly in the case of the fibrillar preparations, insoluble material that failed to enter the gel.

ELISA

For the direct-binding ELISA, 96-well microplates (Nunc Maxisorp or Corning Costar) were coated with fibrillar $A\beta_{42}$ peptide at 2.5 µg/mL in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 overnight at 4°C. Plates were washed and blocked for 1 hour at 25°C with PBS containing 2% BSA (blocking buffer). Antibodies were diluted to the indicated concentrations in blocking buffer, added to the plate, and incubated for 2 hours at room temperature. Binding of antibodies was detected using a HRP-conjugated goat anti-human (for studies with aducanumab) or a goat anti-mouse (for studies with ^{ch}aducanumab) polyclonal antibody (Jackson ImmunoResearch, West Grove, PA, USA), followed by measurement of HRP activity using TMB. For the capture ELISA, plates were coated with 50 µg/mL antibody, blocked as above, and

incubated with dilutions of biotin-conjugated $A\beta_{40}$ ($A\beta_{40}$-Lys-Biotin; AnaSpec, Fremont, CA, USA). Binding was detected using HRP-conjugated streptavidin (Thermo Scientific, Rockford, IL, USA) followed by TMB.

Ex vivo phagocytosis assay

The BV-2 microglia cell line (obtained from Dr. V. Bocchini, University of Perugia, Italy; tested negative for mycoplasma contamination; not independently authenticated) was maintained in cell culture media containing DMEM, 5% FBS, Penicillin/Streptomycin 10 µg/mL, glutamine. Cells were trypsinised and 120,000 BV-2 cells/well were seeded in 24-well plates. After 14 hours, media was replaced in each well with DMEM/F12 supplemented with 20 mM HEPES (pH 7.3), 1% BSA, and Penicillin/Streptomycin. 100 µg/mL Fucoidan, an inhibitor of the scavenger receptor, was added 30 min prior to the experiment. 50 µM FITC-labelled $A\beta_{42}$ fibrils (1:4 ratio mix of FITC-labelled and non-labelled $A\beta_{42}$, incubated for 1 day at 37°C , shaking at 500 rpm) were pre-incubated with the indicated concentrations of antibodies for 30 min at 37°C, washed twice followed by centrifugation for 5 min at 14,000 x g. This antibody-$A\beta_{42}$ fibril suspension was added to the tissue culture medium by diluting 1:25. After 30 min, BV-2 cells were washed twice with HBSS to remove unassociated fibrillar Aß. Cells were treated with 250 µg/mL trypsin/EDTA for 20 min at 4°C and washed twice by centrifugation at 500 x g for 5 min at 4°C. Cells were fixed for 20 min in FACS-Fix (PBS, 2%FA, 2% Glucose, 5 mM NaN) and washed twice (PBS, 5 µM EDTA, 0.2% BSA). Fluorescence of 10,000 cells was determined by FACS analysis (based on Webster *et al*[46]).

Primary mouse microglia cultures were isolated from 1–2 postnatal day mouse cortices and established as previously described.[47] They were >95% CD11b and

CD45 positive. Microglial experiments were carried out in serum-free DMEM/F12 media as described above, 24 hours after the microglia had been isolated.

Supplementary Discussion

Possible pathogenesis of ARIA

The underlying cause of ARIA is still not understood. In addition to the risk factors of dose and ApoE ε4 genotype, underlying increased vascular Aβ (CAA) and the higher vascular burden of carriers compared with non-carriers[48] may play an important role in the pathogenesis of ARIA. Aβ is normally cleared via multiple pathways in the brain, including perivascular drainage; however, increased vascular Aβ deposition in AD may reduce arterial wall integrity. Removal of vascular Aβ by anti-Aβ monoclonal antibodies, including aducanumab, may exacerbate vascular integrity leading to fluid or micro-haemorrhage in the parenchyma, detectable on MRI. In addition, rapid removal of brain parenchymal amyloid with Aβ-removing therapy may saturate the perivascular clearance pathway, resulting in transient ARIA during the induction phase of treatment.[49] Local inflammatory responses may also contribute to the pathogenesis of ARIA.

Supplementary Notes

Clinical course of ARIA-E cases and ARIA-H

ARIA-E was generally observed early in the course of treatment, with the onset of ARIA-E (by MRI) observed within the first five doses in 24/27 (89%) of cases. Of the 9 (33%) subjects with symptoms, most subjects (7/9 [78%]) had symptoms that were rated as mild to moderate. Symptoms, which included headache, visual disturbances, and confusion, were generally transient, typically resolving within 4 weeks, and MRI

findings typically resolved within 4–12 weeks. Of the 27 subjects in the aducanumab treatment groups who developed ARIA-E, 15 (56%) continued treatment (2 at the same dose and 13 at a reduced dose), and 12 (44%) discontinued treatment (shown by dose and ApoE ε4 carrier status in Extended Data Table 2). No subjects developed recurrent ARIA-E. The incidences of isolated ARIA-haemorrhage or haemosiderosis (ARIA-H) abnormalities by MRI were similar across the placebo and active treatment groups. Of the 27 subjects with ARIA-E, 15 (56%) also had ARIA-H. Symptoms, which included headache, visual disturbances, and confusion, were generally transient, typically resolving within 4 weeks and confounded by underlying AD.

Deaths

One patient in the placebo group died due to cardiac arrest. Two subjects died after withdrawal from the study, one due to malignant melanoma (placebo group) and one due to complications from a fall after a total hip revision (10 mg/kg group); these events were not considered related to study drug.

Plasma and brain drug concentrations in transgenic mice

Some animals in the control group displayed detectable signal in plasma (data not shown) that might be explained by the presence of anti-Aβ autoantibodies, as previously described in APP transgenic models.[50] Conversely, some animals in the drug-treated groups had plasma drug levels that were below the limit of detection, and the presence of anti-drug antibodies (ADA) was confirmed in those animals. An inverse correlation was defined between the plasma drug levels and the levels of mouse ADAs. Excluding the animals with measurable levels of mouse ADAs from the analysis did not affect the overall size of the treatment effect (data not shown).

The average brain drug concentrations of ^{ch}aducanumab measured in the DEA-extracted fraction were proportional to both the administered doses and to the corresponding plasma drug concentrations (Extended Data Fig. 5c). The ratio of the average brain drug concentration to the average plasma concentration for the dose groups at which brain concentrations could be accurately quantitated (3–30 mg/kg) was 0.7–1.0%.

Role of microglia in aducanumab-mediated plaque clearance

Immunostaining of fixed brain tissues from the 'Chronic efficacy study' with the Aβ antibody 6E10 revealed different amyloid plaque morphologies in the ^{ch}aducanumab- and vehicle-treated groups. In vehicle-treated animals, the contour of a typical amyloid plaque was irregular with indistinct 'fuzzy' edges (Extended Data Fig. 7a), whereas in ^{ch}aducanumab-treated animals, most plaques remaining in the brain after 6 months of dosing comprised small compact dense cores with a sharply defined border (Extended Data Fig. 7b). Co-staining with microglia marker Iba-1 revealed that microglial cells were tightly associated with the compact plaques, often completely surrounding the plaques. In the ^{ch}aducanumab-treated group, these microglia were amoeboid in morphology (Extended Data Fig. 7b). In contrast, in the PBS control group, microglia rarely surrounded plaques and often presented with abundant ramifications that extended into the neuropil (Extended Data Fig. 7a). Quantification of plaques that were surrounded by microglia for at least 70% of their circumference was performed for the 3 mg/kg treatment group (minimal efficacious dose), demonstrating that a larger proportion of these plaques were found in the ^{ch}aducanumab-treated group, independently of the plaque surface area (Extended Data Fig. 7c). These findings suggest that, upon binding to amyloid plaques, ^{ch}aducanumab

could recruit phagocytic cells, leading to microglia-mediated clearance of the plaques. An additional dosing study (the 'Chronic efficacy study with Agly') comparing the plaque-clearing ability of ^{ch}aducanumab to that of an effector function-impaired variant (^{ch}aducanumab-Agly) with equivalent binding selectivity and affinity toward Aβ species, was conducted. Treatment with ^{ch}aducanumab led to significant reductions in the same endpoints as measured in the 'Chronic efficacy study', whereas no significant differences between the ^{ch}aducanumab-Agly and vehicle control groups were observed (data not shown).

Ex vivo phagocytosis of Aβ fibrils pre-incubated with aducanumab by BV-2 microglial-derived cells or primary microglia was shown to be dose-dependent (Extended Data Fig. 7d, e).

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