Chapter 1
Materials and Methods

The Materials and Methods section is divided in three different sections, corresponding to the following results section.s To avoid redundancy, previously described methods will be omitted from subsequent sections.

1. First section

1.1 Patients

Between October 2020 and May 2023, we collected 52 CSF samples from patients referred to the Centre for Alzheimer's Disease and Adult Cognitive Disorders of Careggi Hospital in Florence. Patients met the following inclusion criteria: 29 patients who received a diagnosis of AD dementia according to the NIA-AA criteria (McKhann *et al*. 2011) or a diagnosis of MCI according to NIA-AA criteria (Albert *et al*. 2011).23 patients with diagnosis of other diseases affecting the CNS but without having a clear involvement of protein misfolding or aggregation (non-AD cases) have participated to this study: 19 with idiopathic normal pressure hydrocephalus (iNPH) according to international guidelines (Relkin *et al*. 2005), 1 patient with multiple sclerosis (MS), 2 with frontotemporal dementia (FTD), 1 with DLB and 1 with vascular dementia (VD), all diagnosed according to international guidelines (Thompson *et al.* 2018; Neary *et al*. 1998; McKeith *et al*. 2017; Sachdev *et al*. 2014).

1.2 Neuropsychological examination

Neuropsychological examination included global measurements of Mini-Mental State Examination (MMSE), Digit and Visuo-spatial Span forward, Rey

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Referee List (DOI 10.36253/fup_referee_list)
FUP Best Practice in Scholarly Publishing (DOI 10.36253/fup_best_practice)

Liliana Napolitano, *Materials and Methods*, © Author(s), CC BY 4.0, DOI 10.36253/979-12-215-0993-9.03, in Liliana Napolitano, *A multidisciplinary approach for the early diagnosis of Alzheimer's disease and potential therapeutic applications*, pp. 47-64, 2026, published by Firenze University Press, ISBN 979-12-215-0993-9, DOI 10.36253/979-12-215-0993-9

Book References DOI 10.36253/979-12-215-0993-9.references

Auditory Verbal Learning Test, Trail Making Test A, attentional matrices, language and phonemic fluency task, constructional praxis, such as Rey-Osterrieth complex figure copy and Clock test, and executive function tests, such as Trail Making test B, Stroop Test, and Frontal Assessment Battery (Giacomucci *et al*. 2022).

1.3 CSF collection and biomarkers analysis

The CSF samples were collected by lumbar puncture, then immediately centrifuged at 200 g for 1 min, 20 °C, then at 4000 x g for 10 min at 4 °C, and the supernatant was further used for the analyses of the novel biomarkers. The pellet contained generally tiny amounts of proteins. An aliquot of the samples was stored at − 80 °C until analysis for classical biomarkers. $A\beta_{42}$ levels, $A\beta_{42}/A\beta_{40}$ ratio and T-tau and P-tau levels were measured using a CLEIA analyzer LUMIPULSE G600: LumipulseG $A\beta_{40}$, LumipulseG $A\beta_{42}$, LumipulseG t-tau, and LumipulseG p-tau 181, with all reagent kits obtained from Fujirebio.

1.4 Apolipoprotein E ε4 genotyping

A standard automated method (QIA cube, QIAGEN) was used to isolate DNA from peripheral blood samples. APOE genotypes were investigated by high-resolution melting analysis (HRMA) (Sorbi *et al*. 1994). Two sets of PCR primers were designed to amplify the regions encompassing rs7412 [NC_000019.9: g[M13] [GG14] 0.45412079C > T] and rs429358 (NC_000019.9:g.45411941 T > C). The samples with known APOE genotypes, which had been validated by DNA sequencing, were used as standard references.

1.5 Bradford assay

Protein concentration of the supernatant was determined by the Bradford assay (Bradford 1976). In brief, 50 µl solutions containing increasing concentrations of bovine serum albumin (BSA, Sigma-Aldrich), ranging from 0 to 2 mg/ml, were used to develop a standard curve; 1:5 diluted Protein Assay Dye Reagent (Bio-Rad) was added to these solutions and the measured absorbance at 595 nm was then plotted versus the corresponding BSA concentration. The resulting equation was used to calculate CSF protein concentration after measuring the absorbance at 595 nm of 3 µl of CSF supernatant diluted in 47 µl of H_2O in the presence of the Protein Assay Dye Reagent.

1.6 Far-UV CD

CSF samples were analysed at 25 °C using a Jasco J-810 Spectropolarimeter equipped with a thermostated cell holder attached to a Thermo Haake C25P water bath and using a 0.1 cm path-length cell. Far-ultraviolet circular dichroism (far-UV CD) spectra and the high tension (HT) spectra were collected over the 200-260 nm wavelength range. It was not possible to record the spectra at wavelength values lower than 200 nm, because of the high absorbance values (HT > 700 V) recorded at these wavelength values. All spectra were blank subtracted and plotted as non-normalised ellipticity (θ) values (mdeg units) versus wavelength (nm). Spectra were not normalised to mean residue ellipticity ($[\theta]_{res}$) because CSF samples are mixtures of proteins, each with a well-defined molecular weight.

1.7 DLS measurement

The size distribution profiles of CSF samples were recorded at 25 °C using a Zetasizer Nano S dynamic light scattering (DLS) device (Malvern Panalytical) thermostated with a Peltier temperature controller and using a 10 mm reduced-volume plastic cell (50 µL volume). The refractive index and viscosity were 1.45 and 0.82 cp, respectively. These values were determined from the Malvern analysis software after entering the typical CSF composition previously reported (Robertson 2010). The measurements were acquired with cell position 3.00 and attenuator index 10.

1.8 Intrinsic Tryptophan Fluorescence Assay

Intrinsic tryptophan fluorescence spectra of CSF samples were acquired at 25 °C from 300 to 500 nm after excitation at 280 nm (slits of 5 and 5, respectively), using a 0.3 × 0.3 cm black wall quartz cell (50 µl volume) on an Agilent Cary Eclipse spectrofluorometer (Agilent Technologies) equipped with a thermostated cell holder attached to an Agilent PCB 1500 water Peltier system.

1.9 Neuroblastoma cell culture

Authenticated human SH-SY5Y neuroblastoma cells were purchased from A.T.C.C. and cultured in Dulbecco's modified Eagle's medium (DMEM), F-12 Ham with 25 mM 4-(2- Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and $NaHCO_3$ (1:1) supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine and 1% antibiotics, as reported previously (Capitini *et al*. 2014; Bigi *et al*. 2020). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C and grown until 80% confluence for a maximum of 20 passages.

1.10 Measurement of intracellular Ca^{2+}

SH-SY5Y cells were plated on 24-well plates containing glass coverslips at a density of 40.000 cells per well. After 24 h, they were washed with PBS and then treated for 5 h with 75 µl of CSF samples mixed 1:1 with 75 µl of cell culture medium (150 µl final volume), or for 2 h with 1 µM ionomycin in a culture medium (75 µl) mixed 1:1 with PBS (75 µl), or with culture medium (75 µl) mixed 1:1 with PBS (75 µl) without additives (referred to as "untreated"). Cells were then washed with PBS and loaded with 4.5 µM Fluo-4 AM (Thermo Fisher Scientific) for 10 min. Following incubation, the cytosolic Ca^{2+} levels were detected after excitation at 488 nm by a TCS SP8 scanning confocal microscopy system (Leica Microsystems) equipped with an argon laser source. A series of 1-µM-thick optical sections (1024 × 1024 pixels) was taken through the cell depth for each sample using a Leica Plan Apo 63× oil immersion objective, and all sections were projected as a single composite image by superimposition. Pinhole diameters, detector gain and laser powers were optimized and settings were maintained constant. Images were analyzed using the ImageJ (NIH) software (Rasband 1997–2018). Fluorescence intensities were expressed as the percentage of that measured in untreated cells.

1.11 MTT reduction assay

SH-SY5Y cells were seeded on 96-well plates at a density of 10.000 cells per well and the MTT reduction was evaluated by the MTT assay, as reported previously (Capitini *et al*. 2014; Bigi *et al*. 2023). Briefly, cells were treated for 24 h with solutions containing CSF samples (50 µl) diluted 1:1 in complete cell culture medium (50 µl) or with culture medium (50 µl) mixed 1:1 with PBS (50 µl) without additives (referred to as "untreated"). Then, they were washed with PBS and the MTT solution was added to the culture medium for 4 h. After this time, a cell lysis buffer (20% sodium dodecyl sulfate and 50% N,N-dimethylformamide, pH 4.7) was added to each well to solubilize formazan crystalline precipitates; 1 h later,the absorbance values of blue formazan were measured at 595 nm by using an enzyme-linked immunosorbent assay plate reader (Bio-Rad). MTT reduction values were expressed as the percentage of those measured in untreated cells.

1.12 Receiver operating characteristic curve and area under the curve analysis

The values of two given parameters X and Y were plotted in a scatter plot for both non-AD and AD CSF samples. For each of the two parameters the Youden's index J was determined as:

$$J = \text{sensitivity} + \text{specificity} - 1 = q(t) + 1 - p(t) - 1 = q(t) + p(t) \qquad (1)$$

where t is a given mobile threshold, q(t) is the "sensitivity" and corresponds to the probability of a true positive, 1- p(t) is the "specificity" and correspond to the probability of a true negative. Equation 1 corresponds to:

$$J = [n_{\text{true positives}} / (n_{\text{true positives}} + n_{\text{false negatives}})] + [n_{\text{true negatives}} / (n_{\text{true negatives}} + n_{\text{false positives}})] - 1 \qquad (2)$$

where $n_{\text{true positives}}$ is the number of true positives and so on. The optimal threshold t* of a given parameter in a test is provided by moving t so that J is maximum:

$$J = \max_{t}\,[q(t) + p(t)] \quad (3)$$

The Receiver operating characteristic (ROC) curve was edited by plotting J for a moving t value in a graph of *q(t)* (sensitivity) versus *p(t)* (1-specificity), so that the diagnostic value of the analysed parameters could be evaluated, and the AUC, representing their ability to discriminate between non-AD and AD CSF samples and resulting from the area under the broken line (or polygonal chain) traced by the J values, was calculated using the GraphPad Prism 5.0 software.

1.13 Statistical analysis

Values of each parameter in non-AD and AD groups were expressed as means ± standard deviation (S.D.). An unpaired one-tailed Student t test was performed to compare non-AD versus AD values for each parameter (GraphPad Prism 5.0 software). P values lower than 0.05, 0.01 and 0.001 were considered to be statistically significant (*), highly statistically significant (**) and very highly statistically significant (***), respectively. Populations of non-AD and AD CSF samples were categorised in two groups each, on the grounds of whether their individual values for a given parameter were below or above the threshold value (t*) or occupied a given quadrant in scatter plots. This produced a total of four groups for each analysis, two for AD and two for non-AD CSF samples. The significance of the separation between non-AD and AD CSF samples was evaluated using the Fisher's exact test and the Chi-square test (GraphPad Prism 5.0 software). P values lower than 0.05, 0.01 and 0.001 were considered as above.

2. Second section

2.1 Preparation of Aβ_{42} aggregates

Aβ_{42} conformers were prepared as previously reported (Lambert *et al*. 1998; Dahlgren *et al*. 2002; Ladiwala *et al*. 2012). Briefly, the lyophilized peptide (Bachem) was dissolved in 100% hexafluoro-2-isopropanol (HFIP) to 1 mM and the solvent was then evaporated under nitrogen. To obtain Aβ_{42} oligomers, the peptide was resuspended in 50 mM NaOH at 1 mg/ml and diluted in PBS to a final concentration of 25 µM. Then, the sample was centrifuged at 22,000 g for 30 min, the pellet discarded and the supernatant incubated at 25 °C without agitation for 1 day to obtain A+ oligomers or for 4 days to obtain A- oligomers (Ladiwala *et al*. 2012). F1 were obtained, with the same procedure, at a final concentration of 50 µM after 1 day of incubation. ADDLs were obtained by dissolving an aliquot of the peptide in anhydrous dimethyl sulfoxide (DMSO) to 5 mM and then diluting in ice-cold F-12 medium to a final concentration of 100 µM. This solution was incubated at 4 °C for 1 day and then centrifuged at 14,000 × g for 10 min. Finally, F2 were prepared by dissolving the peptide in DMSO to 5 mM and then diluting it in 10 mM HCl to a final concentration of 100 µM. The sample was incubated at 37 °C without agitation for 1 day (Dahlgren *et al.* 2002).

2.2 CSF samples

CSF samples from human aged controls (n = 4) or AD (n = 9) samples were obtained from BioIVT (United Kingdom). Each CSF sample was received in 0.5-1 ml aliquot and stored at -80 °C. Samples were centrifuged at 4000 x g for 10 min, obtaining a pale pellet that was separated from the supernatant. The supernatant was then analyzed. Protein concentration in the supernatants of the CSF samples was determined by the Bradford colorimetric method (Bradford 1976).

2.3 DesAb-O and DesAb$_{18–24}$ purification

DesAb-O and DesAb$_{18–24}$ were purified a previously described (Aprile et al, 2017). Briefly, DesAb-O and DesAb$_{18–24}$ were expressed in Eschirichia Coli (*E.Coli*) OrigamiTM (DE3) pLysS cells (Merck Millipore), and grown for at least 15 hours at 30°C in Overnight Express Instant TB Medium (Merck Millipore) supplemented with 100 µg/ml ampicillin. Cells were collected by centrifugation, resuspended in PBS (8 mM Na_2HPO_4, 15 mM KH_2PO_4, 137 mM NaCL and 3 mM KCl, pH 7.3) with an EDTA-Free Complete Protease Inhibitor Cocktail tablet (Roche). Cells were lysed by sonication and cellular debris was removed by centrifugation. The supernatant was applied to a HisTrap HP 5 ml column (Cytiva) that has been pre-equilibrated with PBS supplemented with 15 mM imidazole. The column was then washed with several column volumes of PBS supplemented with 15 mM imidazole after which the antibody was eluted in PBS with 300 mM imidazole. The sample was then dialysed against PBS overnight at 4°C to remove imidazole, after which it was applied to a HiLoad Superdex 75 16/600 pg (Cytiva) column for size exclusion chromatography. Protein concentration was determined by measuring the absorbance at 280nm and using the molecular coefficient of DesAb-O or DesAb$_{18–24}$.

2.4 Dot-blot analysis

Dot-blot analysis was performed by spotting 2.0 µl (corresponding to 0.1 µg) of each Aβ$_{42}$ conformer onto a nitrocellulose membrane. After 30 min blocking (1.0% bovine serum albumin, BSA, in TBS/TWEEN 0.1%), Aβ$_{42}$ species were probed with 2 µM DesAb-O or with 1:15.000 human monoclonal anti-ADDLs (19.3) Ab (Creative Biolabs), or with 1:1000 rabbit polyclonal anti-oligomer

(A11) Ab (Thermo Fisher Scientific), or with 1:1000 rabbit polyclonal anti-amyloid fibrils (OC) Ab (Sigma-Aldrich, SA), or 1:1000 with mouse monoclonal anti-Aβ (6E10) Ab (Biolegend Way) and then with 1:2000 goat anti-6X His tag (Abcam), or goat anti-human (Sigma Aldrich), or goat anti-rabbit (Abcam) or rabbit anti-mouse (Abcam) the appropriate horseradish peroxidase- conjugated secondary Abs. In another set of experiments, decreasing quantities (0.1, 0.05, 0.0025, 0.001 and 0.005 µg) of Aβ_{42} aggregates were spotted onto the nitrocellulose membrane, and then probed with DesAb-O and 19.3 Abs, as described above. The immunolabeled dots were detected using a Super Signal West Dura (Pierce) ImageQuant™ TL software (GE Healthcare UK Limited version 8.2).

In a set of experiments, sandwich dot-blot was performed. Briefly, 2 µl of 6E10 and DesAb-O Abs (1:500 diluted and 20 µM, respectively) were spotted onto nitrocellulose membranes. After 20 min, the blots were blocked in TBS-Tween-20 0.2% and 2.5% BSA IgG free for 40 min. The membranes were incubated with different Aβ_{42} species at 1.9 µM (monomeric Aβ_{42}, A+ oligomers, F1 fibrils (Ladiwala *et al*. 2012) and the CSF of AD patients and control subjects at 0.1 mg/ml. Then, the membranes were probed with 1:1000 6E10 Ab overnight at 4 °C under constant shaking. The following day, the membranes were washed three times in TBS-Tween-20 0.2% and incubated with 1:3000 rabbit anti-mouse (Abcam) HRP-conjugated secondary Abs for 1 h. After three additional washes, the immunolabeled dots were detected as reported above.

2.5 ELISA assay

For indirect ELISA assay, increasing concentrations (0, 1, 5, and 10 µM, monomer equivalents) of each Aβ_{42} conformer, prepared as reported above, were immobilised on a 96-well Maxisorp ELISA plate (Nunc) without shaking for 1 h at room temperature (RT). The plate was then washed three times with 20 mM Tris, pH 7.4, and 100 mM NaCl and incubated in 20 mM Tris, pH 7.4, 100 mM NaCl, and 5% BSA under constant shaking overnight at 4 °C. The day after the plate was washed six times with 20 mM Tris, pH 7.4, and 100 mM NaCl and then incubated with 40 µL solutions of 2.0 µM DesAb-O, or with 1:20.000 human monoclonal anti-oligomer (19.3) Ab (Creative Biolabs), or with 1:8000 mouse monoclonal anti-Aβ (6E10) Ab (Biolegend Way), under constant shaking for 1 h at RT. The plate was then washed six times with 20 mM Tris, pH 7.4, and 100 mM NaCl and incubated with solutions of the appropriate horseradish peroxidase-conjugated secondary Abs diluted 1:2000 goat anti-6X His tag (Abcam) for DesAb-O, 1:5000 goat anti-human (Sigma Aldrich) for 19.3 and 1:4000 rabbit anti-mouse (Abcam) for 6E10 HRP-conjugated secondary Abs, in 20 mM Tris, pH 7.4, 100 mM NaCl, and 5% BSA under shaking for 1 h at RT. Finally, the plate was washed three times with 20 mM Tris, pH 7.4, and 100 mM NaCl, then twice with 20 mM Tris, pH 7.4, 100 mM NaCl, and 0.02% Tween-20, and again three times with 20 mM Tris, pH 7.4, and 100 mM NaCl. Finally, the amount of bound Abs was quantified by using 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific), according to the manufacturer's instructions, and the reaction was stopped by adding 40 µl of H_2SO_4. Then, the absorbance was measured at 450 nm by means of a CLARIOstar plate reader (BMG Labtech).

For sandwich ELISA assay, 1 µM DesAb-O or 0.5 µM $DesAb_{18–24}$ Abs were immobilised on a 96-well Maxisorp ELISA plate (Nunc) without shaking for 1 h at RT. After three washes in PBS, the plate was blocked with 5% BSA IgG free overnight at 4 °C under constant shaking. The day after, the plate was washed six times in PBS and different $Aβ_{42}$ species (M, A+ oligomers, F1 at decreasing concentrations (4500, 2250, 450, 45, 4.5 and 2.25 pg/ml for DesAb-O and 4500·103 pg/ml, 4500 pg/ml and 4.5 pg/ml for $DesAb_{18–24}$) and the CSF of AD patients (n = 9) and control subjects (n = 4) at 0.25 mg/ml were loaded into the plate overnight at 4 °C under constant shaking. In the DesAb-O plate, we loaded 4500 pg/ml of monomeric αSyn as negative control. The following day after six additional washes in PBS, the plate was incubated with 1:4000 6E10 Ab for 2 h at RT with no shaking, while αSyn was incubated with 1:4000 mouse monoclonal anti-αSyn (211) Ab (Santa Cruz Biotechnology, INC). The plate was washed six times in PBS- Tween-20 0.2% and incubated with 1:5000 rabbit anti-mouse (Abcam) HRP-conjugated secondary Ab for 1 h at RT. The plate was then washed six additional times in PBS- Tween-20 0.2% and the amount of $Aβ_{42}$ species bound was quantified as reported above.

2.6 Cell cultures

Authenticated human SH-SY5Y neuroblastoma cells were purchased and maintained as previously reported in *section 1.9*. Furthermore, Primary rat cortical neurons (Thermo Fisher Scientific) were plated in 12-well plate containing glass coverslips and maintained in neuronal basal plus medium (Thermo Fisher Scientific) supplemented with GlutaMAX (Gibco) at the concentration of 0.5 mM and 2% (v/v) B-27 serum-free complement (Gibco), in a 5% CO_2 humidified atmosphere at 37 °C (Fani *et al*. 2022). All the experiments were performed 14 days after plating (Fani *et al*. 2022).

2.7 Stimulated emission depletion (STED) microscopy

$Aβ_{42}$ assemblies were added to the culture medium of SH-SY5Y cells seeded on glass coverslips for 1 h at 3 µM (monomer equivalents). After incubation, the cells were washed with PBS, the plasma membranes were counterstained with 0.01 mg/ml tetramethylrhodamine conjugate wheat germ agglutinin (Thermo Fisher Scientific) (Cascella *et al*. 2021) for 15 min at 37 °C and cells were then fixed with 2.0% (w/v) paraformaldehyde. After washing with PBS, the plasma membranes were permeabilized with a 3.0% (v/v) glycerol solution for 5-10 min. $Aβ_{42}$ species were then detected with 4 µM DesAb-O and 1:800 FITC anti-6X tag secondary Abs (Abcam) or with 1:250 diluted human monoclonal (19.3) anti-oligomer Ab (Creative Biolabs) and 1:1000 Alexa Fluor 488-conjugated anti-human secondary Abs or with 1:400 diluted rabbit polyclonal anti- oligomer A11 Ab (Thermo Fisher Scientific), or with 1:800 diluted rabbit polyclonal anti-amyloid fibrils OC Ab (Sigma-Aldrich), or with 1:400 diluted mouse monoclonal anti- Aβ 6E10 Abs (Biolegend Way) and 1:500 Alexa Fluor 514-conjugated anti-rabbit or anti- mouse secondary Abs (Thermo Fisher Scientific). STED xyz images (i.e., z-stacks acquired along 3 directions: x, y, and z axes) were acquired as previously reported (Cascella *et al.* 2021). In a set of experiments, SH-SY5Y cells seeded on glass coverslips were experienced for 24 h with ADDLs oligomers 3.0 µM (monomer equivalents) or CSF samples from AD patients or controls (n = 4 for both AD and control CSF samples) diluted 1:1 with cellular medium. In another set of experiments, primary rat cortical neurons were treated with CSF samples from AD patients or controls (n = 4 for both AD and control CSF samples) diluted 1:1 with cellular medium. After the incubation, the cells were counterstained and analysed by STED microscopy as reported above. In another set of experiments, A+ oligomers, F1 and a mixture containing both $Aβ_{42}$ species (1:1 molar ratio) were incubated on a glass coverslip at 25 µM in cell culture medium without cells while the CSF samples (n = 9 and n = 4 for AD and controls, respectively) were spotted at a concentration of 0.5 mg/ml. After 30 min of incubation, the samples were blocked in Casein 1X with TBS-Tween-20 0.2% for 30 min. Once washed with TBS-Tween-20 0.2%, $Aβ_{42}$ species and the CSF samples were detected with 1:800 6E10 (Biolegend Way), 2.0 µM DesAb-O or 4 µM $DesAb_{18–24}$ Ab for 1 h and then and with 1:500 Alexa Fluor 514-conjugated anti-mouse secondary Abs (Thermo Fisher Scientific) or with 1:500 FITC anti-6X tag secondary Abs (Abcam). The acquisition was performed as reported above.

2.8 Microscopy analysis of $Aβ_{42}$ aggregates bound to neuronal membranes

SH-SY5Y cells were seeded on glass coverslips and then treated for 15 min with A+ oligomers or ADDLs at a concentration of 3.0 µM (monomer equivalents), in the absence or presence of a pre-incubation with increasing molar ratio (1:0.1, 1:0.25, 1:0.5, and 1:1, monomer equivalents) between Abs and $Aβ_{42}$ species. After incubation, the cells were washed with PBS and the plasma membranes were counterstained for 15 min using 5.0 µg mL^{-1} of Alexa Fluor 633-conjugated wheat germ agglutinin (Life Technologies) (Evangelisti *et al.* 2016). Cells were fixed in 2.0% (w/v) paraformaldehyde and $Aβ_{42}$ assemblies were detected using 1:800 diluted mouse monoclonal 6E10 Ab (Biolegend Way) and then with 1:1000 diluted Alexa Fluor 488-conjugated anti-mouse secondary Abs (Thermo Fisher Scientific). To detect only the oligomers bound to the cell surface, the cellular membrane was not permeabilized, thus preventing Ab internalisation. Fluorescence emission was detected after double excitation at 633 and 488 nm by a TCS SP8 scanning confocal microscopy system (Leica Microsystems), as previously described (Bigi *et al.* 2023a). The degree of colocalization of $Aβ_{42}$ aggregates and cell membranes was estimated for regions of interest in 30-32 cells, via the use of ImageJ (NIH, Bethesda, MD, USA) and JACOP plugin (http://rsb.info.nih.gov) software (Rasband WR).

2.9 MTT reduction inhibition assay

The cytotoxicity of the different Aβ_{42} aggregates was assessed in SH-SY5Y cells seeded in 96-well plates by the MTT assay (Mosmann 1983; Evangelisti *et al.* 2014). Briefly, Aβ_{42} species (monomer, A+ and A- oligomers, ADDLs, and two types of fibrils) at a concentration of 3.0 μM (monomer equivalents) were added to the culture medium of SH-SY5Y cells for 24 h following or not a pre-incubation with equimolar concentrations of DesAb-O, 19.3, A11, or OC Abs. After treatment, the culture medium was removed, cells were washed with PBS and the MTT assay was assessed as previously reported (Bigi *et al.* 2023a). Cell viability was expressed as the percentage of MTT reduction in treated cells as compared to those untreated, or to those treated with Aβ_{42} species in the absence of Abs.

In a set of experiments, CSF samples (n = 4 for AD as well as controls) were added to the culture medium of SH-SY5Y cells in presence or absence of DesAb-O at 3 μM for 24 h. Treatments with CSF samples and DesAb-O were pre-incubated for 1 h at 37 °C. ADDLs oligomers at 1 μM (monomer equivalent) were used as positive control. These solutions were then added to SH-SY5Y cells for 24 h and the analysis was performed as reported above in *section 1.11.*

2.10 Measurement of cytosolic free Ca^{2+} levels

The intracellular calcium levels were measured in SH-SY5Y cells as previously described (Cascella *et al.* 2017; Bigi *et al*. 2023a). SH-SY5Y cells were treated for 5 h with ADDLs at 1 μM (monomer equivalents), or with the CSF samples (n = 4 for AD as well for controls) following or not a 1 h pre-incubation in the absence or presence of DesAb- O at 3 μM. At the end of the treatment, the cells were washed in PBS and loaded with 10 μM fluo-4 AM (Thermo Fisher Scientific) for 10 min and cytosolic Ca^{2+} levels were detected as previously described in *section 1.10*.

2.11 Measurements of calcein leakage

The intracellular calcein levels were measured in SH-SY5Y cells. Briefly, the cells were washed in PBS and loaded with 0.5 μM Calcein-AM (Thermo Fisher Scientific) for 20 min at 37 °C. Then, they were washed two additional times and treated with CSF (n = 4 for both AD and control CSF samples) and ADDLs at 1 μM (monomer equivalents; n = 4), pre-incubated for 1 h in the absence or presence of DesAb-O at 3 μM for 5 h. After fixation in 2.0% buffered paraformaldehyde for 10 min, fluorescence emission was detected after excitation at 488 nm by the TCS SP8 scanning confocal microscopy system as previously reported (Cascella *et al.* 2021). Images were then analyzed as reported above.

2.12 Statistical analysis

Data were expressed as means ± standard deviation (S.D.), or as means ± standard error of mean (S.E.M). Comparisons between the different groups were performed by using Student t test or by one-way ANOVA followed by Bonferroni's multiple-comparison test (GraphPad Prism 5.0 software). P values lower than 0.05, 0.01 and 0.001 were considered to be statistically significant (*), highly statistically significant (**) and very highly statistically significant (***), respectively.

3.Third section

3.1 Dimeric-DesAb-O design

To design the Dimeric-DesAb-O, a flexible linker composed by a repetition of glycine (Gly) and Serine (Ser), was attached to the C-terminus of a first DesAb-O monomer and the N-terminus of a second monomer of DesAb-O, depleted of the His-tag region. Gene sequence design was carried out through the use of SnapGene software (www.snapgene.com).

3.2 Protein Expression and Purification

DesAb-O was expressed and purified as previously reported in *section 2.3*. The Dimeric-DesAb-O construct was expressed using pET28a (+) vector in *E. coli* Origami™ 2(DE3) Competent Cells (Merck Millipore), as previously described (Sormanni, Aprile and Vendruscolo 2015). Cells were grown at 37 °C in Luria–Bertani (LB) medium (MerckMillipore) supplemented with Kanamycin (50 µg /ml) under shaking at 200 rpm in a New Brunswick Innova 44R incubator shaker (Eppendorf) until reaching an OD_{600} of 0.6. Cells were then harvested by centrifugation; resuspended 20 mM phosphate buffer, pH 8.0, with the addition of one EDTA-Free Complete Protease Inhibitor Cocktail Tablet (Roche) per 500 ml of cell growth; and lysed using sonication (15s on and 45s off pulses, 40% amplitude). Cell debris was removed using centrifugation at 20,000 x *g* (JA-20 rotor, Beckman Coulter) for 45 mins. The cleared lysate was loaded onto a Ni^{2+}-NTA Superflow column (Qiagen), previously equilibrated with 20 mM phosphate buffer containing 15 mM imidazole. After washing with 20 mM phosphate buffer containing 30 mM imidazole, the His-tagged Dimeric- DesAb-O was eluted with 20 mM phosphate buffer with 300 mM imidazole and dialyzed extensively against 20 mM phosphate buffer. Dimeric-DesAb-O was finally purified using size-exclusion chromatography with a HiLoad 16/600 Superdex 75 pg column (GE Healthcare), previously equilibrated in 20 mM phosphate buffer. Protein concentration was determined by absorbance measurement at 280 nm using theoretical extinction coefficients calculated with ExPASy ProtParam (Gill and Von Hippel 1989). Both the flow through and peak fractions were then loaded on 4–12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific) to verify the sample purity.

Purification of $A\beta_{42}$ was carried out as previously described (Abelein *et al.* 2020; Vadukul *et al.* 2023). Briefly, the spider silk domain-conjugated $A\beta_{42}$ peptide, known as fusion protein (20 kDa) was expressed by heat-shock transformation in BL21 *E. coli*. Cells were grown at 37 °C in LB broth supplemented with kanamycin (50 µg/mL) with shaking at 200 rpm in a New Brunswick Innova 44R incubator shaker (Eppendorf) until an OD_{600} of 0.8 was reached. Protein expression was then induced with 1 mM IPTG, and cultures were incubated overnight at 20 °C with shaking at 200 rpm. Then, cells were collected, centrifuged, and resuspended in 20 mM Tris −HCl and 8 M urea, pH 8.0 to be sonicated on ice for 20 min (15 s on and 45 s off pulses, 20% amplitude). Following another centrifugation, the supernatant was passed through a 0.22 µm filter and loaded onto two tandem 5ml HisTrap HP columns (Cytiva), pre-equilibrated with a binding buffer (20 mM Tris−HCl and 8 M urea, pH 8, supplemented with 15 mM imidazole). After washing the columns with the same buffer to remove nonspecific binding, the fusion protein was eluted using an elution buffer 20 mM Tris-HCl, 8 M urea, pH 8.0 supplemented with 300 mM imidazole (elution buffer). 8.0), followed by concentration measurements via Nanodrop. To release the target peptide, the fusion protein was incubated with TEV protease (1:15 molar ratio) overnight at 4 °C. To ensure complete cleavage and denaturation of the spider silk domain, the sample was treated with 7 M guanidine-HCl on ice for at lease 2h. The sample was then applied on to a Superdex 75 Increase pg 10/300 column (Cytiva), previously equilibrated with 20 mM phosphate buffer, pH 8, for size-exclusion chromatography.

The monomeric peak was collected manually, and the concentration of monomeric $A\beta_{42}$ (in µM) was determined from the chromatogram using the following equation:

$$A\beta_{42}(\mu M) = (\frac{A_{280}/2}{0.2 * 1490}) * 10^6 \qquad (4)$$

Precisely, A_{280} is the absorbance at 280 nm of the elution peak of $A\beta_{42}$, 0.2 is the path length (cm) of the ATKA Pure (Cytiva) and $1490 M^{-1}\ cm^{-1}$ is the molecular coefficient of $A\beta_{42}$.

For all cellular biology experiments and ADDLs oligomers (Lambert et al., 1998) preparation, we used $A\beta_{42}$ lyophilised peptide (Bachem) dissolved in 100% hexafluoro- 2-isopropanol (HFIP), as previously reported in *section 2.1.*

3.3 Electrospray Ionization Mass Spectrometry

Purified Dimeric-DesAb-O (~20 μM) was analysed by electrospray ionization mass spectrometry (ESI-MS) to confirm molecular weight and sample purity. The plot shows a visible a major peak at 34.270 Da, corresponding y to the molecular weight of Dimeric-DesAb-O predicted by bio-informatic softwares such as ExPASy ProtParam and Peptide Nexus (34.270 Da).

3.4 CD

Far-ultraviolet (UV) CD spectra of Dimeric-DesAb-O were acquired using a Chiroscan spectropolarimeter (Applied Photophysics) equipped with a Peltrier temperature control unit. Measurements were performed in a quartz cuvette with 1 mm path length. Samples contained 6 μM proteins in phosphate-buffered saline (PBS), pH 7.4. The far-UV CD spectra of the dimeric structure of DesAb-O were recorded from 200 to 240 nm at 20°C, and the spectrum of the buffer was subtracted from the spectra of Dimeric-DesAb-O, scanning 10 accumulations. In another set of experiments, temperature-wavelength CD spectra of 6 μM DesAb-O and Dimeric-DesAb-O were recorded between 20°C and 90°C at 5 °C intervals. A background spectrum of the sample buffer was subtracted from all sample spectra. Raw data of θ (units of mdeg) were converted to mean residue ellipticity ($[\theta]_{res}$, units deg cm^2 $dmol^{-1}$) using (Greenfield 2006):

$$[\theta]_{res} = \theta/[(n-1)*l*c] \qquad (5)$$

where θ is the raw data, n the number of amino acids, l the cuvette pathlength (cm), and c the protein concentration (M). The denaturation curves were plotted θ against temperature, and fitted using Santoro and Bolen equation (Santoro and Bolen 1988) and normalised to fraction folded (%) values:

$$[\theta]_{res} = \frac{([\theta]_{res}(F) + m(F) * T) + ([\theta]_{res}(U) + m(U) * T) * e^{\left(\frac{-\Delta G}{RT}\right)}}{\left[1 + e^{\left(\frac{-\Delta G}{RT}\right)}\right]} \qquad (6)$$

where $[\theta]_{res}$ is the measured molar residue ellipticity at temperature T (°C), $[\theta]_{res}(F)$ and $[\theta]_{res}(U)$ are the [θ]res values for the folded and unfolded states at 20 °C, respectively, m(F) and m(U) are the slopes of the folded and unfolded baselines, respectively, ΔG is the Gibbs free energy change upon unfolding and R is the universal gas constant. By fitting the data obtained with the above equation, it was possible to determine the ΔG with the temperature increase. In order to calculate the temperature of half-denaturation (Tm), we firstly determined the fraction folded with the following equation:

$$Fraction\ folded\ (\%) = \frac{([\theta]res - [\theta]res(U))}{([\theta]res(F) - [\theta]res(U))} \qquad (7)$$

The Eq. (7) works by normalizing the observed signal [θ]res against the known spectral endpoints. The denominator ([θ]res(F)-[θ]res(U)) establishes the maximum possible signal change between the fully folded ([θ]res(F)) and fully unfolded ([θ]res(U)) states. The numerator measures how far the observed signal is from the completely unfolded state, allowing the calculation of the fraction that has successfully attained the native conformation. The Tm values represent the temperature at which the protein is 50% folded and 50% unfolded (fraction folded = 50%).

3.5 Thioflavin T Fluorescence Assays

Monomeric recombinant Aβ_{42} (1 µM) was incubated alone or in presence of Dimeric-DesAb-O at decreasing molar ratios (1:1, 1:0,5, 1:0,25, 1:0,125 corresponding to 1 µM, 0,5 µM, 0,25 µM, 0,125 µM Dimeric-DesAb-O concentrations) in 20 mM phosphate buffer pH 8. Samples were prepared with a final concentration of 10 µM thioflavin T (ThT) dye, gently vortexed, and pipetted into nonbinding surface black 96-well plates (Greiner Bio-One) in triplicates. The plate was read in a ClarioStar Plus microplate reader (BMG LabTech) at 37 °C. The excitation and emission wavelengths were set to 440 and 480 nm, respectively, and fluorescence intensity measurements were taken using spiral averaging (3 mm diameter). Buffer-only values were not subtracted from the sample readings but shown in the not normalized graph. Readings were taken every 2 min. To test the capability of the dimeric structure of DesAb- O compared to the monomeric sdAb, in another set of experiments monomeric Aβ_{42} (1 µM) was incubated alone or in presence of 1 µM Dimeric-DesAb-O or increasing DesAb- O molar ratios (1:1, 1:2 corresponding to 1 and 2 µM DesAb-O concentrations. The data were plotted using GraphPad Prism version 9.3.1 for Windows (GraphPad Software).

To obtain Aβ_{42} oligomers and perform experiments on SH-SY5Y cells, the lyophilized peptide (Bachem) was dissolved in 100% HFIP to obtain a monomeric form, followed by evaporation under nitrogen flux. Subsequently, the peptide was resuspended in PBS, resulting in a final concentration of 10 µM. For visualization of the emerging β-sheets in Aβ_{42}, samples were added with a final concentration of 25 µM ThT, gently vortexed and pipetted into no binding surface black 96-well plates (Grenier Bio-One) in quadruplets. The plate was read in a BioTekSynergyTM H1 Hybrid Multi-mode reader (Agilent, Santa Clara) at 37 °C. The excitation and emission wavelengths were set to 440 and 485 nm, respectively. Buffer-only values were not subtracted from the sample readings but shown in the final graph. Readings were taken every 2 min. The data were plotted using GraphPad Prism version 9.3.1 for Windows (GraphPad Software). To characterize the different type of aggregates formed during the Aβ_{42} aggregation process, we collected Aβ_{42} samples at various timepoints (0, 2, 4, 8, and 24 h) to conduct further experiments (see details below).

3.6 Real-Time based ELISA assay

Real-time based ELISA experiment was performed aggregating 1 μM monomeric Aβ_{42} peptides in 20 mM sodium phosphate buffer (8 pH) in quiescent conditions. Twenty- microliter aliquots were taken at precise timepoints (0, 0.5, 1, 2 and 20 h) from aggregation reactions and immobilized on a 96- or 384-well Maxisorp ELISA plate (Nunc) with no shaking overnight at 4°C. Aβ_{42} fibrils obtained after 4 days of incubation at 37 °C were used as a control. At the end of the incubation, the plate was then washed three times with TBS (20 mM Tris, pH 7.4, and 100 mM NaCl) and incubated in TBS supplemented with 5% bovine serum albumin (BSA) under constant shaking for 1h at RT. The plate was then washed six times with TBS and then incubated with 30 μL solutions 1 μM DesAb-O or 1 μM Dimeric-DesAb- O under constant shaking either for 1 h at RT or overnight at 4 °C. At the end of this incubation, additional six washes with TBS were performed and the plate was incubated with 30 μL solutions of rabbit polyclonal 6x His tag horseradish peroxidase (HRP) conjugated (Abcam) at a dilution of 1:4.000 in 20 mM Tris, pH 7.4, 100 mM NaCl, and 5% BSA under constant shaking for 1 h at RT. Finally, the plate was washed two times with 20 mM Tris, pH 7.4, and 100 mM NaCl, then three times with 20 mM Tris, pH 7.4, 100 mM NaCl, and 0.02% Tween- 20 and again three times with 20 mM Tris, pH 7.4, and 100 mM NaCl. Finally, the amount of bound sdAbs was quantified by using 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific), according to the manufacturer's instructions, and the absorbance was measured at 450 nm by means of a CLARIOstar plate reader (BMG Labtech) as previously reported (Aprile *et al*. 2020).

3.7 Transmission Electron Microscopy (TEM)

5 μM Aβ_{42} samples incubated in the presence or absence of 5 μM sdAbs were aggregated in a microplate without ThT and collected after 24h of aggregation at 37°C. Aβ_{42} aggregation was monitored by other replicates with ThT, allowing real-time monitoring of the reaction. Samples for TEM were then prepared spotting 4 μL onto Formvar/carbon- coated 300 mesh copper grids for 1 min. By blot dying the grid with Whatman filter, we removed excess sample were removed, allowing the grid drying for 2 min. Samples were then washed with 4 μL of water and stained with 4 μL of 2% w/v uranyl acetate (Vadukul *et al*. 2023). Grids were imaged on a T12 Spirit electron microscope (Thermo Fisher Scientific). The fibril diameter was measured using ImageJ software and all data were plotted using Excel (Version 16.89.1 (24091630)).

3.8 Dot Blot analysis

Dot blots were carried out on samples that were aggregated in the microplates without ThT at the endpoint of aggregation as previously reported (Vadukul *et al*. 2023). Samples were collected and centrifuged at max speed (~16,000g) for

30 min on a benchtop centrifuge to separate the soluble and insoluble aggregates. Prior to centrifugation, an aliquote of each sample was stored and considered as the total protein amount. At the end of the centrifuge, the supernatant was collected and the pellet was resuspended in 20 mM phosphate buffer. To analyze the proportion of soluble and insoluble Aβ_{42} species and Ab fragments, three repeats of each samples were spotted onto a nitrocellulose membrane and blocked in 5% nonfat milk in 0.1% PBS-Tween for 45 min at RT (Vadukul *et al*. 2023). The membranes were then incubated overnight at 4 °C with either 1:1000 6E10 Ab for Aβ_{42} detection or 1:1000 anti-6X His-tag Ab for DesAb-O and DiDesAb-O detection. The following day, the membrane were washed three times for 10 min each in 0.1% PBS-Tween. Membranes were then incubated for 1 h at RT protected from light with 1:2000 anti-mouse Alexa fluor 647 (Thermo Fisher Scientific) for 6E10 Ab and 1:1000 anti-goat anti-6X His Tag (Abcam) for the anti- 6X His Tag Ab, respectively. After three additional 10-minute washes in 0.1% PBS-Tween, the membranes were imaged with a Typhoon scanner (GE Healthcare) using the appropriate laser settings (Vadukul *et al.* 2023). The signal intensity of the supernatant was normalized to that of the total protein of the corresponding sample.

In another set of experiments, to characterize the different types of Aβ_{42} aggregates formed during the Aβ_{42} lyophilised peptide (Bachem) aggregation process, 2.0 µl (equivalent to 0.1 µg) of the samples at five different timepoints (0, 2, 4, 8, and 24 h) were spotted onto a nitrocellulose membrane. Following a 45-min blocking step (1.0% bovine serum albumin, BSA, in TBS-Tween 0.1%), the membrane was incubated with 1:15.000 diluted human monoclonal anti-ADDLs (19.3) Ab (Creative Biolabs), 1:1000 diluted rabbit polyclonal anti-amyloid fibrils (OC) Ab (Sigma-Aldrich) and 1:800 diluted mouse monoclonal anti-Aβ (6E10) Ab (Biolegend Way) for 1 h and 30 mins. Subsequently, the membrane was washed three times in TBS-Tween 0,1% for 10 mins each and incubated with 1:3000 diluted goat anti-6X His tag (Abcam), goat anti-human (Sigma-Aldrich), or goat anti-rabbit (Abcam) or rabbit anti-mouse (Abcam), all conjugated with horseradish peroxidase (HRP) for 1 h. After three additional washes in TBS-Tween 0,1%, the immunolabelled dots were detected using a Super-SignalWest Dura (Pierce) ImageQuant™ TL software (GE Healthcare UK Limited version 8.2) as previously reported (Bigi *et al*. 2024b).

3.9 PK Digestion

Fibrils obtained after 4 days at 37 °C under constant conditions in the presence or in the absence of sdAbs were centrifuged at max speed (~16,000g) for 1 h. The supernatant was discarded. The pellet was resuspended in 20 mM phosphate buffer and treated with increasing PK concentrations (0, 10, 25, 50 µg/ml) for 30 min at room temperature. Samples were then incubated at 95 °C for 5 min to stop the enzymatic reaction, and samples were prepared for SDS-PAGE and Western blotting analysis. 6E10 antibody diluted 1:1000 in 0.1% PBS-Tween was used as primary antibody. Data analysis was performed assuming the band intensity at 0 μg/ml of PK of each sample as the 100%. Intensity of each 0 μg/ml of PK band was compared as well.

3.10 SDS-PAGE and Western Blotting

Fibrils from the coincubation sample were collected by centrifugation at max speed for 1h as previously reported in *section 3.9.* Samples were prepared in 4× LDS sample buffer and 10× reducing agent after which they were boiled at 95 °C for 5 min. Samples were then run on 4−12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific) and transferred onto a 0.45 μm nitrocellulose membrane for 7 min at 20 V with the iBlot 2 (Thermo Fisher, Waltham). Blocking, incubation with antibodies, and detections were carried out as described above (Vadukul *et al*. 2023).

3.11 CSF samples

CSFs from human aged controls (n = 4) or AD patients (n = 4) were obtained from BioIVT, stored and processed as previously reported in *section 2.2*.

3.12 STED microscopy

Aβ_{42} aggregates collected at different timepoints (0, 2, 4, 8, and 24 h) of a ThT aggregation assay were spotted and visualized by STED microscopy as described in *section 2.7*.

3.13 Confocal microscopy

To detect Aβ_{42} oligomers bound to the cellular membranes and internalized into the cytosol without pre-incubating with sdAbs, we performed the experiment adding 0,5 μM Aβ_{42} oligomers for 1 h to the culture medium of SH-SY5Y. The experiment was conducted as previously described in *section 2.8*. Aβ_{42} species were then detected with 3 μM and 1 μM DesAb-O or Dimeric-DesAb-O and 1:800 6E10 Ab as a control. As a secondary Ab, we used diluted FITC anti-6X His-tag secondary Abs (Abcam) or Alexa Fluor 488-conjugated anti-mouse secondary Abs (Thermo Fisher Scientific). In another set of experiments, we calculate the number of Aβ_{42} oligomers bound to neuronal membranes of SH-SY5Y. To perform this experiment, 0.5 μM Aβ_{42} oligomers were pre- incubated or not with increasing sdAbs molar ratios (1:0.1, 1:0.25, 1:0.5, 1:1, 1:2, 1:3) corresponding to 0.25, 0.63, 1.25, 2.5, 5 and 7.5μM of sdAbs concentrations) for 1 h at 37 °C under soft shaking, and the binding experiment was then conducted as reported in *section 2.8.*

3.14 Measurement of cytosolic free Ca^{2+} levels

The intracellular calcium levels were measured in SHSY-5Y cells as previously described (Bigi *et al.* 2023; Cascella *et al.* 2017). SHSY-5Y cells were treated for 15 mins with 0,5 µM $A\beta_{42}$ oligomers previously incubated or not for 1 h at 37 °C under gentle shaking with increasing sdAbs molar ratios (1:0.1, 1:0.25, 1:0.5, 1:1, 1:2, 1:3 corresponding to 0.25, 0.63, 1.25, 2.5, 5 and 7.5µM of sdAbs concentrations). Cells were then washed in PBS and loaded with 4.5 µM Fluo-4 AM (Thermo Fisher Scientific) for 10 min and cytosolic Ca^{2+} levels were detected after excitation at 488 nm by the TCS SP8 scanning confocal microscopy system as previously reported (Bigi *et al.* 2023a; Cascella *et al.* 2021). In another set of experiments, SH-SY5Y cells were treated for 5 h with the CSFs (n = 4 for AD as well for controls) pre-incubated or not with DesAb-O or Dimeric-DesAb- O at 3 µM and 1 µM for 1 h at 37 °C under shaking. Experiments were conducted as previously described in *section 1.10*.

3.15 MTT reduction assay

To assess the cytotoxicity of $A\beta_{42}$ aggregates formed during the $A\beta_{42}$ aggregation process, a MTT reduction assay was conducted. Briefly, 1 µM $A\beta_{42}$ species collected from a ThT aggregation assay at different timepoints (0, 2, 4, 8 and 24 h) were added for 24 h to culture medium of SH-SY5Y cells. ADDLs were used as positive control. For our experiments, we decided to use $A\beta_{42}$ oligomers obtained after 8 h of incubation at 37 °C. In another set of experiments, we performed an MTT test with decreasing concentrations $A\beta_{42}$ oligomers (1 µM, 0.5 µM, 0.25 µM, 1 nM, 0.5 nM, 0.25 nM and 1 pM) in order to optimize our experimental conditions, deciding to use 0.5 µM $A\beta_{42}$ oligomers concentration for the following cellular biology experiments. In another set of experiments, $A\beta_{42}$ oligomers were pre-incubated for 1 h at 37 °C with increasing sdAbs molar ratios (1:0.1, 1:0.25, 1:0.5, 1:1, 1:2, 1:3 corresponding to 0.25, 0.63, 1.25, 2.5, 5 and 7.5 µM of sdAbs concentrations). The solutions were then added to the extracellular medium of SH-SY5Y cells. Then experiments were conducted and analyzed as reported in *section 1.11*.

3.16 Statistical analysis

Data were expressed as means ± standard error of mean (S.E.M). Comparisons between the different groups were performed by using unpaired and one-tailed Student t test or by one-way or two-way ANOVA followed by Bonferroni's multiple-comparison test (GraphPad Prism 10.3.1 software). P values lower than 0.05, 0.01, 0.001 and 0.0001 were considered to be statistically significant (*), highly statistically significant (**) and very highly statistically significant (***), and extremely statistically significant (****) respectively.