Chapter 5
Discussion

AD accounts for 60-70% of cases of dementia worldwide (https://www.alz.org). The NIA-AA have proposed a research framework based on a biomarker-grounded biological, rather than syndromal, definition of AD, where the disease has to be regarded as a continuum (Jack *et al*. 2018). In this spectrum, seven biomarkers have attained widely recognized diagnostic relevance. These include the low levels of the $A\beta_{42}$ peptide and the high concentration of t-tau and p-tau in the CSF. They also include the high cortical amyloid deposition and cortical tau deposition measured with PET, poor brain glucose metabolism measured with FDG PET, and significant brain atrophy imaged with magnetic resonance imaging. The availability of this group of CSF-based and imaging-based biomarkers has led to the introduction of the AT(N) system for a biological characterization and staging of the disease (Jack *et al*. 2018).

The study and identification of novel biomarkers are important to enrich the aforementioned research framework, but also as diagnostic tools for supporting the existing biomarkers that often produce uncertain diagnoses in early AD, to address the pathological complexity and heterogeneity of the disease, and to enrich our biomarker list with others with more prognostic value (Hampel *et al*. 2021a).
All the classical protein-based biomarkers reveal the soluble and aggregation states of specific proteins, such as $A\beta_{42}$, t-tau and p-tau. However, it is recognized that protein misfolding diseases, including AD, are characterized by a generic failure of the PN, which physiologically maintains proteins in a soluble non-aggregated state (Labbadia and Morimoto 2015). In a compromised PN status a great number of proteins lose solubility and gain a propensity to misfold and aggregate (Hipp, Park and Hartl 2014). Accumulation of protein aggregates is both an effect and a cause of PN decline, driving a vicious cycle that ultimately leads to its collapse (Hipp, Park and Hartl 2014; Labbadia and Morimoto 2015).

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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, *Discussion*, © Author(s), CC BY 4.0, DOI 10.36253/979-12-215-0993-9.07, in Liliana Napolitano, *A multidisciplinary approach for the early diagnosis of Alzheimer's disease and potential therapeutic applications*, pp. 117-125, 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

Consistently, in all neurodegenerative disease the main characterizing protein deposits are often associated with those of other proteins.

Building on this idea, in this work we compared CSFs extracted from AD and non-AD cases in a novel Italian study named PRAMA and we sought the presence of aggregated protein species, detectable with biophysical methods, and proteotoxicity, in the form of misfolded protein oligomers able to cause cell dysfunction to cultured cells using cell viability assays. This idea is based on the detection of misfolded proteins not just of the $A\beta_{42}$ and tau proteins, that represent a very small fraction of the protein population composing the CSF, but of the overall CSF proteome.

29 patients with fin al clinical diagnosis of AD (AD cases) and 20 patients with final clinical diagnosis of other diseases affecting the CNS (non-AD cases) were recruited. Their CSF samples were collected and treated as reported in *section 1.1* in *Chapter 1*. At the time of CSF collection and analysis, the diagnosis was uncertain and was ascertained only after following clinical examination. None of the final diagnoses were post-mortem. In this work, we reported the mean and individual demographic characteristics of both groups, values of the classical CSF biomarkers ($A\beta_{42}/A\beta_{40}$ ratio and levels of t-tau and p-tau), percentages of patients with the ε4 allele of the *APOE* gene and scores of MMSE tests.

The total protein concentrations of the CSFs, measured using the Bradford assay, ranged from *ca.* 0.2 to 1.0 mg/ml in both groups, indicating similar distributions in the two groups in agreement with previous analyses (Dufour-Rainfray *et al.* 2013). Scatter plots of $A\beta_{42}/A\beta_{40}$ *versus* t-tau and $A\beta_{42}/A\beta_{40}$ *versus* p-tau, with the thresholds (t^*) derived from optimization of the Youden's indexes of the two parameters, show a good separation between non-AD and AD cases. This analysis validates our cohort as it indicates that the two groups are good representatives of non-AD and AD cases, respectively.

Once observed that our cohort was representative of non-AD and AD cases, CSFs were first compared by recording the size distributions of their particles with DLS, for five representative non-AD and five representative AD CSFs. In both cases, a peak of small species having an apparent hydrodynamic diameter (D_h) of *ca.* 10 nm is evident, which arises from the dominant largest CSF proteins, such as human serum albumin. This occurs because the all-α human serum albumin (HSA) is by far the most abundant protein in CSF and only one of the first ten most abundant proteins of the CSF is all-β, with the other nine being either mixed α/β or all-α proteins, as previously reported (Lardinois *et al*. 2014). However, large species arising from protein aggregates are also present in both groups, all having D_h values around or higher than 100 nm. The *LSI* arising from large species (D_h> 30 nm) is higher in AD cases, indicating a larger proportion of protein aggregates in this group. In 2019, De and co-workers analysed the size and morphology of aggregates present in CSF samples using a newly developed super-resolution technique called AD-PAINT (AD-PAINT) and measured the size of individual aggregates, which ranged from 20 nm to 300 nm (De *et al.*

2019).To examine the aggregate morphologies, they compared the size distribution of protein aggregates in the CSF of control, MCI, and AD subjects. Analysis of the aggregate size histograms revealed a statistically significant (99% confidence) increase in smaller aggregates (< 50 nm) in MCI CSF compared to controls. In line with our results, they demonstrated that AD CSF showed a ten-fold increase in the proportion of larger, mature aggregates (~ 40 to 200 nm) relative to controls. Furthermore, their finding suggests a potential link between smaller aggregates and increased membrane permeability observed in MCI (Kremer *et al.* 2000, De *et al.* 2019). Indeed, since protein aggregates added to the extracellular medium of cultured cells have the ability to bind and destabilize biological membranes and cause an influx of Ca^{2+} ions into the cytosol (Bigi *et al.* 2020; Cascella *et al.* 2021; Fani *et al*. 2021; Fani *et al*. 2022; Bigi *et al*. 2023a), the levels of intracellular Ca^{2+} ions of cultured cells exposed to CSF samples are a good indicator of CSF proteotoxicity (Fani *et al*. 2022). We therefore added the CSFs to the extracellular medium of SH-SY5Y neuroblastoma cells (1:1) and measured the intracellular Ca^{2+} levels after 5 h, using the Fluo-4 AM probe and confocal fluorescence microscopy. From our study, cells treated with AD CSFs showed higher Ca^{2+} levels compared to cells treated with non-AD CSFs (except one outlier sample) in agreement with others findings (Yerbury and Wilson 2010; Bigi *et al*. 2024b). To sum up, AD CSFs are characterized by higher values of *LSI* from large protein species in the DLS distributions and higher ability to induce high cytosolic Ca^{2+} levels when added to the medium of cultured cells. These findings have a rationale in the presence of higher amounts of large protein particles and misfolded protein oligomers inducing Ca^{2+} dyshomeostasis in cells, respectively.

We then combined the three classical CSF biomarkers ($A\beta_{42}/A\beta_{40}$, t-tau, p-tau) with the two novel putative biomarkers identified here (*LSI*, Ca^{2+} levels). The comparison between scatter plots built with pairs of classical biomarkers and mixed classical and novel biomarkers indicate good separations with $p<0.0001$ in all cases, using both the Fisher's exact and Chi-square tests. This observation legitimates the use of the two novel CSF parameters analyzed here for AD diagnosis. In this context, many efforts have been made to find new biomarkers and combine them with traditional biomarkers to study their application in the early and differential diagnosis of AD (Westin *et al*. 2012, Sutphen *et al*. 2015, Lusardi *et al*. 2017, Wiedrick *et al*. 2019). In line with our results, in 2023, Liu and coworkers performed an analysis of CSF and blood serum revealing distinct protein profiles in individuals with AD compared to controls. AD patients exhibited altered levels of multiple proteins, with 8 increased and 60 decreased in CSF, and 55 increased and 10 decreased in serum. These findings highlight a systemic dysregulation of protein expression associated with AD, affecting both the central nervous system and peripheral blood. Among the 8 proteins over-expressed in the CSF, 3 of them, the sodium-/potassium-transporting ATPase subunit beta-1 (AT1B1), serglycin (SRGN) and thioredoxin-dependent peroxide reductase mitochondrial (PRDX3), were found to highly differentiate AD subjects from controls subjects with an AUC lower but close to those of CSF $A\beta_{42}$, t-tau,

and p-tau (Liu *et al*. 2023).

These results showed that although the AD/non-AD segregation using novel biomarkers is not as high as that obtained with classical biomarkers, it is very highly significant. In the broader context of AD, the possibility to distinguish AD and non-AD cases based on the novel biomarkers identified here reinforces the view that PN is compromised in AD leading to an aggregated and proteotoxic status of Aβ and tau, and many other proteins of the entire proteome. With the aim of recognizing aggregated toxic species for the development of possible treatment for AD or research purposes, major efforts have been made over the years to design conformational Abs raised by different investigators against independently generated Aβ oligomers, detected in AD brains unlike age-matched healthy individuals (Kayed *et al*. 2003; Gong *et al*. 2003; Kayed *et al*. 2007; Hillen *et al*. 2010). Indeed, considerable attention was invested in the development of robust assays to characterize and quantify oligomers, discriminating them from the monomeric form (Hefti *et al*. 2013; Savage *et al.* 2014; De *et al*. 2019). In all the ELISA-based methods, a significant overlap in the total mass of Aβ oligomers between patients and controls has been observed, although a small increase in the oligomeric mass has been reported for some cohorts (Jekel *et al*. 2015; Savage *et al*. 2014). However, due to the lack of suitable sensitive methods, the detection, quantification and isolation of these soluble neurotoxic species from biological fluids remain difficult, because of their heterogeneity, transient nature and very low concentration.

Nanobodies or sdAbs have been recently proposed as promising tools for basic research and potential candidates for diagnostic and therapeutic applications in a range of pathological conditions, thanks to their high target specificity and affinity, as well as low immunogenic potential (Muyldermans 2013, Zheng *et al*. 2022). In particular, the rational design of sdAbs that selectively target specific Aβ conformers neutralising their neurotoxicity has a great potential of diagnostic and therapeutic value for AD (Zameer *et al*. 2008; Lafaye *et al*. 2009; Kasturirangan *et al*. 2012; David, Jones and Tayebi 2014; Aprile *et al*. 2020; Zheng *et al*. 2022). A dozen of sdAbs have shown their therapeutic or diagnostic potential value for AD *in vitro* (Bélanger *et al*. 2019), and two of them, namely R3VQ and A2, have reached *in vivo* imaging as they bind brain Aβ deposits and tau inclusions, respectively (Li *et al*. 2016).

In this work, we examined the potential role of a sdAb, named DesAb-O, targeting a conformational epitope formed by residues 29-36 of $A\beta_{42}$ and exposed by the oligomeric species (Aprile *et al.* 2020), to selectively detect and neutralise $A\beta_{42}$ oligomers both from synthetic origin and present in AD CSFs. We first characterised its ability to detect a range of pathologically relevant, highly stable and well-characterised $A\beta_{42}$ aggregates (A+ and A- oligomers, ADDLs, and two type of fibrils) (Lambert *et al*. 1998; Dahlgren *et al*. 2002; Ladiwala *et al*. 2012), taking advantage of a panel of commercially available conformation-sensitive Abs, as controls. The immunoassays analysis revealed a high affinity and selectivity of DesAb-O for $A\beta_{42}$ oligomers, at least equal to that of the A11 and 19.3 Abs,

raised against prefibrillar oligomers and ADDLs, respectively (Kayed *et al*. 2003; Savage *et al*. 2014), with only a minor cross-reaction with the monomeric protein, nontoxic oligomers, and fibrillar conformers. We also evaluated the ability of DesAb-O to selectively detect $A\beta_{42}$ oligomers in cultured cells, demonstrating a great performance also in a more physiological condition.

We then revealed that the pre-incubation of DesAb-O with $A\beta_{42}$ oligomers strongly reduces their interaction with neuronal membranes in a dose-dependent manner, and this protective effect appears more evident for DesAb-O than the A11 Ab, at least at low $A\beta_{42}$:Abs molar ratios. This suggests that DesAb-O can detect the key epitopes normally exposed on the surface of toxic oligomers and responsible for the interaction with the membrane more effectively than the A11 Ab, presumably because of its smaller size, that enables a more precise targeting of critical epitopes. Our results are consistent with those obtained with conformation-sensitive Abs, such as ACU-954 and A-887755, specifically raised against Aβ oligomers, namely ADDLs and globulomers, respectively, that were found to prevent their binding to neurons (Shughrue *et al.* 2010; Hillen *et al.* 2010), rescuing the impaired synaptic transmission (Hillen *et al.* 2010). The capture of $A\beta_{42}$ oligomers by DesAb-O was also found to prevent their induced mitochondrial dysfunction, in agreement with large body of evidence supporting the protective effects of Abs, such as A11, OC, AUC-954, A-887755, and PMN310, against neuronal dysfunction (Kayed *et al.* 2003; Kayed *et al.* 2007; Hillen *et al*. 2010; Shughrue *et al*. 2010; Gibbs *et al*. 2019; Bigi *et al*. 2020). Notably, DesAb-O showed no inherent toxicity when added alone to the cell culture medium, thus making it an excellent tool for future tentative therapeutic applications.

Other sdAbs have been reported to target pathologically relevant $A\beta_{42}$ oligomers. For example, the V31-1 was able to recognize intraneuronal oligomers in human brain slices and to inhibit fibril formation preventing their induced neurotoxicity (Lafaye *et al.* 2009), suggesting a great potential for the detection and diagnosis of Aβ oligomers in AD patients (Pain, Dumont and Dumoulin 2015). In addition, two sdAbs, namely PrioAD12 against anti-$A\beta_{40}$ and PrioAD13 against $A\beta_{42}$ (David, Jones and Tayebi 2014) can detect Aβ oligomers simultaneously in the blood and retina of APP/PS1 mice before their appearance in the brain, with respect to age-matched wild-type mice controls (Habiba *et al*. 2021).

When we moved our approach to CSFs, we revealed a remarkable ability of DesAb-O to selectively identify $A\beta_{42}$ oligomers also in a biological fluid from AD patients compared to age-matched control subjects. Previous studies detected $A\beta_{42}$ oligomers in AD CSFs by using two-site ELISA assay and the couple 19.3/82E1 Abs (Savage *et al.* 2014), or homotypic ELISA using 82E1 Ab (Hölttä *et al*. 2013) or BAN50 Ab (Herskovits *et al*. 2013). Even if these reported assays used different oligomer standards, a trend has emerged suggesting that a sub-pg/ml sensitivity is required to detect CSF oligomers (Georganopoulou *et al.* 2005; Hölttä *et al*. 2013; Savage *et al*. 2014). Indeed, in our assay we have been

able to measure an average oligomer concentration of c.a. 4.5 pg/ml, in good agreement with previous findings (Hölttä *et al.* 2013; Savage *et al.* 2014;Yang *et al*. 2015). It is important to clarify that some studies reported a lack of significant change in total aggregates between AD and controls, using both a sdAbs, named Nb3, and a mAb, named Bapineuzimab (Yang *et al.* 2015; Drews *et al.* 2017). This suggests that the total amount of the aggregates is not a critical factor in AD CSFs, but it is rather the nature of these aggregates and their effects to be important.

Further analyses were conducted in this work on neuroblastoma cells to test the possible therapeutic potential of DesAb-O against the neurotoxic $A\beta_{42}$ oligomers present in the CSFs of AD patients. We first demonstrated that the CSFs of AD patients caused significant Ca^{2+} influx, membrane permeabilization and mitochondrial dysfunction, in agreement with previous findings (Walsh *et al.* 2002; Klyubin *et al*. 2008; Yerbury and Wilson 2017) and suggesting that the AD CSF contains neurotoxic species. Notably, DesAb-O was found to prevent neuronal dysfunction caused by Aβ oligomers present in the CSFs of AD patients, in agreement with other studies using Nb3 and other Abs against Aβ (Walsh *et al.* 2002; Klyubin *et al*. 2008), or extracellular chaperones (Yerbury and Wilson 2010). It is well known that the shielding of toxic hydrophobic regions exposed on the oligomer surface by either polyclonal or monoclonal Abs, extracellular chaperones and even other proteins present in biological fluids such as transthyretin, appears to be an effective method to suppress the toxicity of misfolded $A\beta_{42}$ oligomers (Keyed *et al.* 2003; Savage *et al*. 2014; Cascella *et al.* 2013a; Cascella *et al*. 2013b), but the high sensitivity and selectivity of DesAb-O and possibly other sdAbs relative to other Abs and chaperones could offer a remarkable potential of these biotechnological tools against AD particularly in its early stages.

In this context and with the aim of improving the DesAb-O specificity and affinity for $A\beta_{42}$ oligomers, we design the Dimeric-DesAb-O. The dimeric structure was obtained connecting two monomers with a flexible $(GGGGS)_3$ linker and removing the His-tag region from the N-terminus of the second monomer. Together, the loop region of DesAb- O and the flexible GS linker compose a *partial natural linker* of 39 aa length. We then characterize the structure of Dimeric-DesAb-O investigating the secondary structure, molecular weight and thermal stability by CD and ESI-MS. We found that the dimeric sdAb shared the same structure and, surprisingly, a close temperature of half-denaturation with its monomer (Klement *et al*. 2015).

Thereafter, we tested the ability of the Dimeric-DesAb-O to interfere with the $A\beta_{42}$ aggregation process performing ThT assays and whether it showed an increased specificity and affinity for $A\beta_{42}$oligomers with respect to the monomeric sdAb, performing a Real-Time Based ELISA assay. Interestingly, the Dimeric-DesAb-O showed a massive interference in the $A\beta_{42}$ aggregation at an equimolar concentration with the $A\beta_{42}$ monomer and in a dose dependence manner decreasing the sdAb concentration. We also tested DesAb-O as a control

observing that it can interfere with $A\beta_{42}$ aggregation at a lower extent compared to the dimeric sdAb, which demonstrates a substantially enhanced potency. Furthermore, our results showed a higher ability of the Dimeric-DesAb-O in recognizing $A\beta_{42}$ oligomers with respect to the monomeric sdAb. Indeed, the highest absorbance values, as determined by ThT assay, was observed approximately close to the half-time of aggregation, time in which oligomeric species reach their maximum amount.

A significant difference was obtained compared to DesAb-O at the same time of aggregation, suggesting that the engineering of DesAb-O sharply improved the previous outstanding affinity and specificity for $A\beta_{42}$ oligomers.

In literature, the engineering of sdAbs through the application of a flexible linker was extensively used over the past years with the aim of increase the binding avidity and specificity for their antigen. In 2011, Hultberg and coworkers obtained multivalent constructs against the Respiratory Syncytial Virus fusion protein (RSV F), the highly pathogenic avian influenza virus A (H5N1 HA) and Rabies G protein. Surprisingly, a bivalent protein composed of two identical anti-F VHHs extremely increased the RSV neutralizing ability by 4000-fold. Similarly, multivalent constructs against influenza H5N1 and vesicular stomatitis virus (VSV) also showed significantly increased potency. They observed that the optimal length of the linker connecting the VHHs, ranging between 9 and 35 aa, varied depending on the virus and specific antibody combination, highlighting the need for case-by-case optimization. For instance, a shorter linker worked better for trivalent anti-influenza constructs, while linker length did not significantly impact on bivalent anti-RSV constructs (Hultberg *et al*. 2011). Later in 2014, Cardoso and colleagues fused two identical anti-influenza neuraminidases (NA) VHHs using the llama IgG2c hinge as a flexible linker. Similar to the study mentioned above, the antiviral potency of the bivalent format was found to be significantly enhanced (Cardoso *et al*. 2014). These studies support the possibility that the addition of a flexible linker is a successful method for the optimization of the binding avidity and affinity of sdAbs for their antigen and that the linker length optimized case-by-case ensures wide range of movement of both domains and the ability to bind antigenic sites spaced between them. We further investigated the impact of the Dimeric-DesAb-O on the $A\beta_{42}$ aggregation, by using TEM. Our data revealed that the co-incubation of $A\beta_{42}$ monomer with sdAbs induced morphological and structural changes in $A\beta_{42}$ fibrils. In particular, in the presence of the dimeric DesAb-O we observed a reduction in width fibrils dimeter, a jagged appearance and a highly increased fragility. Similar results have been reported with other nanobodies, also known as Nbs, such as, V31-1 (Lafaye *et al.* 2009), A4 and E1 (Kasturirangan *et al*. 2012), able to selectively recognize low-molecular-weight Aβ42oligomers and inhibit fibrils formation (Lafaye *et al.* 2009, Kasturirangan *et al*. 2012). In 2022, another Nbs was developed to specifically binds the PHF6 sequence, located in the core structure of au aggregation, inhibiting this process (Danis *et al*. 2022).

Further evidences of the inhibition of $A\beta_{42}$aggregation have been observed with anti-Aβ single chain fragment variable antibodies (scFvs), that are composed by the heavy and light variable chain variable regions of an entire immunoglobulin (Bird *et al*. 1989), sharing a similar structure with the dimeric form of DesAb-O that differs from an scFv only for the presence of two identical variable heavy domains as recognition sites. In 2011, Marín-Argany and colleagues tested the conformational changes induced in $A\beta_{42}$oligomers by scFv-h3D6 binding. This scFv was able to induce nontoxic conformational changes in $A\beta_{42}$ oligomeric species, abolishing their detrimental effects (Marín-Argany *et al.* 2011). Our results agreed with a large body of evidence supporting the morphological and structural changes in $A\beta_{42}$ fibrils by scFv binding, such as the scFv59 (Fukuchi *et al*. 2006a; Fukuchi *et al*. 2006b), scFv B6 (Yoshihara *et al.* 2008), and scFv-1E8 (Nisbet *et al*. 2013).

We then evaluate the ability of the Dimeric-DesAb-O to selectively detect $A\beta_{42}$ in cultured cells, as previously reported (Bigi *et al*. 2024b). The pre-incubation of the dimeric sdAb with $A\beta_{42}$ oligomers caused a massive reduction of their interaction with neuronal membranes in a dose-dependent manner, in agreement with another scFv, called NUsc1, able to prevent the interaction of $A\beta_{42}$ oligomers in mature rat hippocampal cultures (Selles *et al*. 2022). Furthermore, we observed a decreased alteration of intracellular Ca^{2+}free levels and an increased prevention of the mitochondrial dysfunction at lower $A\beta_{42}$ oligomers:sdAb molar ratios compared to DesAb-O, suggesting that the presence of two cooperative binding sites for $A\beta_{42}$ oligomers enhances the detection of key epitopes exposed by toxic oligomers, thus preventing the interaction with neuronal membranes more efficiently than DesAb-O.

These results appear to be consistent with those obtained with other scFv, such as A4- scFv (Zameer *et al*. 2008), scFv-1E8 (Nisbet *et al*. 2013), and HCDR3 (Manoutcharian *et al*. 2004) able to capture $A\beta_{42}$ oligomers and prevent detrimental effects, such as mitochondrial dysfunction. Indeed, Zameer and coworkers performed a lactate dehydrogenase (LDH) release assay on human SH-SY5Y neuroblastoma cells, observing that the treatment with $A\beta_{42}$ monomer in the presence of scFv for 96 h determined no signs of toxicity. Taking advance of the AFM, they observed morphological changes in $A\beta_{42}$ aggregates formed in the presence of scFv (Zameer *et al*. 2008). In line with our results, this data was further confirmed using an MTT assay and evaluating the mitochondrial dysfunction induced by $A\beta_{42}$ oligomers (Zameer *et al*. 2008). In agreement, Nisbet and colleagues performed a cell counting Kit-8 assay (CCK-8) and LDH assay on primary cortical neuronal cultures treated with $A\beta_{42}$ oligomers in the absence or in the presence of scFv-1E8. In both analyses, the co-incubation with the scFv significantly restored the cell viability, suggesting the ability of this scFv to prevent the detrimental effects induced by $A\beta_{42}$ oligomers on cultured cells (Nisbet *et al*. 2013). With a different approach, Manoutcharian and coworkers obtained two new anti-$A\beta_{42}$ phage- displayed scFvs Abs. They focused on a specific part of the Ab called the heavy chain complementarity-determining

region 3 (HCDR3), a crucial part that directly interacts with the antigen and created two small synthetic peptides, N44 and C44, that mimicked the HCDR3 region of the scFv with the highest $A\beta_{42}$ oligomers affinity. They studied the effect of HCDR3-derived synthetic peptides on hippocampal cultures that were exposed to $A\beta_{42}$ peptide in the absence or in the presence of the synthetic peptides N44 and C44, by the MTT assay. Their results showed that the co-incubation with N44 did not prevent the mitochondrial dysfunction induced by the $A\beta_{42}$ peptide, whereas C44, in its linear form, significantly prevented the $A\beta_{42}$ toxicity (Manoutcharian *et al.* 2004), appearing perfectly in line with our results. Further evidence was obtained in mice models where a scFv, called W20, was found to prevent the neuropathology induced by the accumulation of αSyn and mutant huntingtin protein aggregates (Zha *et al*. 2016).

This work also included a proof-of-concept analysis on the therapeutic potential of Dimeric-DesAb-O against neurotoxic $A\beta_{42}$ oligomers present in the CSF samples of AD patients. Firstly, we demonstrated that the administration of AD CSFs to human SH- SY5Y neuroblastoma cells caused significant Ca^{2+} influx, in agreement with previous findings (Walsh *et al*. 2002, Klyubin *et al.* 2008, Yerbury and Wilson 2017, Bigi *et al*. 2024b) and suggesting that the AD CSF contains neurotoxic species. Notably, the Dimeric-DesAb-O was found to prevent neuronal dysfunction caused by $A\beta_{42}$ oligomers present in the CSFs of AD patients at lower concentrations than DesAb- O, demonstrating again the increased specificity for $A\beta_{42}$ oligomers and its ability to detect these toxic aggregates in complex biological fluid, such as the CSF extracted from AD patients.

While further studies in larger cohorts of AD patients and controls are needed, our data support the idea that engineering sdAbs directed against $A\beta_{42}$ oligomers greatly improves the performance of nanobodies. Furthermore, they may represent a new biotechnological tool for the diagnosis of AD at a very early stage of pathology due to their high specificity for $A\beta_{42}$ oligomers and their ability to detect them in complex biological fluids.