Potential biomarkers and novel pharmacological targets in protein aggregation-related neurodegenerative diseases

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Abstract

The aggregation of specific proteins plays a pivotal role in the etiopathogenesis of several neurodegenerative diseases (NDs). β-Amyloid (Aβ) peptide-containing plaques and intraneuronal neurofibrillary tangles composed of hyperphosphorylated protein tau are the two main neuropathological lesions in Alzheimer's disease. Meanwhile, Parkinson's disease is defined by the presence of intraneuronal inclusions (Lewy bodies), in which α-synuclein (α-syn) has been identified as a major protein component.

The current literature provides considerable insights into the mechanisms underlying oligomeric-related neurodegeneration, as well as the relationship between protein aggregation and ND, thus facilitating the development of novel putative biomarkers and/or pharmacological targets.

Recently, α-syn, tau and Aβ have been shown to interact each other or with other "pathological proteins" to form toxic heteroaggregates. These latest findings are overcoming the concept that each neurodegenerative disease is related to the misfolding of a single specific protein.

In this review, potential opportunities and pharmacological approaches targeting α-syn, tau and Aβ and their oligomeric forms are highlighted with examples from recent studies. Protein aggregation as a biomarker of NDs, in both the brain and peripheral fluids, is deeply explored. Finally, the relationship between biomarker establishment and assessment and their use as diagnostics or therapeutic targets are discussed.

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1. New insights into protein aggregation-related neurodegenerative diseases

Neurodegenerative diseases (NDs) are characterized by the dysfunction and loss of neurons in specific areas and are associated with pathologically misfolded proteins that accumulate in the human brain and in peripheral organs [1-3]. Although these diseases are distinguished by specific clinical signs and symptoms, NDs share conspicuous similarities and have been defined as "proteinopathies", which include Alzheimer's disease (AD); Parkinson's disease (PD); all the synucleinopathies; transmissible spongiform encephalopathies (TSEs; also known as prion diseases); amyotrophic lateral sclerosis (ALS); Dementia with Lewy body (DLB) and frontotemporal dementia (FTD).

The molecular classification of NDs relies upon meticulous morphological evaluation of protein aggregates, their distribution within the brain, genetic alterations and clinical symptoms. Each of these diseases has been generally related to specific type of protein aggregates. For example, β-amyloid$_{1-42}$ (Aβ) and tau protein represent the principal constituent of AD pathological hallmarks. Aβ aggregates to form extracellular deposits called senile plaques (SP) (Fig. 1); simultaneously, tau produces intraneuronal neurofibrillary tangles (NFTs), composed of abnormal filaments of hyperphosphorylated protein (Fig. 1) [4].

Conversely, α-syn has been generally associated with PD, DLB, and glial cytoplasmic inclusions in Multiple System Atrophy (MSA). The common hallmarks of these pathologies are the abnormal accumulation of misfolded α-syn forming amyloid fibrils which subsequently accumulate in Lewy Bodies (LB) and Lewy neurites (LN) [5].

Post-mortem analyses commonly identify a mixed pattern of proteinopathies, frequently accompanied by signs of chronic cerebrovascular disease pathology [6]. Moreover, in addition to homoaggregates, monomers of a single protein could also aggregate with other misfolded proteins leading to the formation of heteroaggregates [7, 8]. This evidence has provided a link between the different NDs.

In this review, the most current studies on protein aggregation in NDs will be summarized, with a specific focus on linking protein oligomerization to the molecular classification of NDs. Because the combination of proteinopathies is an emerging topic in the recent literature, our commentary will place an emphasis on the roles of mixed oligomers and heteroaggregation as biomarkers and innovative targets of NDs with a focus on AD and PD.

1.1 Protein aggregation in neurodegenerative diseases: oligomers and heteromers

1.1.1. Aβ, tau and α-syn misfolding in oligomeropathies

Several efforts have been directed towards establishing an understanding of prion protein folding and the structural features of prion proteins after oligomerization and aggregation [9]. The detailed

mechanisms of aggregation have been extensively described in recent reviews [10-13]. Briefly, the pathological mechanisms responsible for the gradual transition from a highly soluble, functional conformation to an insoluble, filamentous pathological aggregate containing characteristic cross-β-sheet structures are not completely understood [14]. The accumulation of protein aggregates increases with age as the ubiquitin/proteasome system [15] and autophagy processes [16] lose their ability to control and degrade misfolded monomers (Fig. 1).

Although there is considerable information regarding oligomers and fibril structures [17], the toxic types of prion proteins and the mechanisms underlying their cytotoxicity are still being evaluated.

The oligomeric structures of **Aβ** are soluble in nature and considerably the most toxic of all the aggregate types [11].

Conversely, **α-syn** generates oligomeric species, protofibrils and fibrils similar to Aβ but with slower kinetics [18], and its toxic forms consist of oligomers rather than fibrils. In particular, the non-amyloid component (NAC) of the central α-syn fibrillogenic fragment has been established to directly induce toxic effects (Fig. 1) [19, 20].

Tau phosphorylation in specific sites has been shown to direct proteins to aggregate [21] by decreasing their affinity for microtubules [22]. For this reason, tau hyperphosphorylation is the hallmark of all tauopathies, even though hyperphosphorylation states differ among and within different pathologies [23]. The identity of the toxic form of tau is still actively debated, but the most toxic form appears to be the oligomeric form [24-26].

Despite the aforementioned differences between Aβ, α-syn and tau, they share a common hypothesis: smaller diffusible oligomers rather than the insoluble cross β-sheet amyloid fibrils drive the degenerative process [27]. The current hypothesis states that oligomeric forms are produced by monomeric aggregation but could result from either fragmentation of preformed fibrils or unsuccessful degradation of fibrils by lysosomes or the proteasome.

Several microenvironmental factors and intracellular mechanisms have been proposed as triggers for spreading the aggregation process; however, it is difficult to determine whether the presence of these protein aggregates is a consequence or a cause. The misfolding of disease-specific proteins has been correlated with neuroinflammatory processes [28], increased levels of oxidative stress [29], vascular degeneration [30], and neuronal cell death [31]. However, even if the involved proteins differ among the NDs, the common feature remains oligomer toxicity. This evidence led to the generation of the term “oligomeropathies”, which is a better description of all the protein misfolding-related diseases [32, 33].

1.1.2 Aβ, tau and α-syn heteroaggregation

In addition to the co-existence of plaques, tangles and LBs, the recent literature has indicated that Aβ, tau, and α-syn promote the accumulation of one another [34-36], which creates a vicious cycle in ND pathogenesis and supports the hypothesis that structural and functional cooperation occur between misfolded proteins. For example, α-syn has been demonstrated to promote tau polymerization or its *in vivo* accumulation [34, 36].

Aβ has been shown to influence α-syn and tau aggregation as well. For example, double-transgenic α-syn/amyloid precursor protein (APP) mice exhibit enhanced α-syn deposition compared to single-transgenic mice [37]. Moreover, using a genetic approach to combine the pathologies of AD and dementia with Lewy bodies, Clinton and co-workers have confirmed that Aβ, tau, and α-syn interact *in vivo* to promote the aggregation and accumulation of one another and accelerate cognitive dysfunction [36].

The hypothesis of the structural and functional cooperation between misfolded proteins has been confirmed by several clinical observations demonstrating a high comorbidity and overlapping between pure synucleinopathies and tauopathies. Indeed, the co-occurrence of tau and α-syn inclusions is frequent in several NDs, such as PD, DLB, a Lewy body variant of AD [38, 39], and even Down's syndrome [38, 40]. Furthermore, α-syn seems to contribute to AD pathogenesis as well [6, 41], with 30 – 40% of AD cases presenting with LB and LN [42].

In addition to oligomers of the same protein, the role of heterocomplexes in NDs has also been emerging. Aβ and α-syn have been shown to form complexes and co-immunoprecipitate from patient brain samples and transgenic mouse models, providing clear evidence for their direct interaction (Fig. 1) [43, 44]. Aβ accumulates primarily in the extracellular regions, but it has also been found in different subcellular areas, including mitochondria and the Golgi apparatus [45]. This abnormal localization allows Aβ to interact with a variety of intracellular proteins, including α-syn [43, 44]. In physiological condition, α-syn, in a helical conformation, is associated with dopamine containing vesicles and is involved in the vesicle transport process. When the vesicles are fused to the membrane, α-syn is completely released into the cytosol and is absent in the synaptic membrane [46]. In pathological conditions with neuronal damage, α-syn tends to increase and to from cytosolic aggregates, which interact with membrane-associated $A\beta_{40}$ and $A\beta_{42}$ peptides [47]. Membrane-bound α-syn associates with Aβ peptides at multiple locations. Mandal and co-workers [43] have demonstrated with NMR experiments that both 15N-labelled $A\beta_{40}$ and $A\beta_{42}$ interact with membrane-associated α-syn, particularly with the latter (81–95) residues, as confirmed by a solid phase binding assay [46]. α-Syn-induced structural alteration is more substantial in combination with $A\beta_{42}$ compared to $A\beta_{40}$, as demonstrated in transgenic mice [37] and indicates a greater pathogenic role

for $A\beta_{42}$. Of note, the three residues involved in α-syn-$A\beta_{42}$ interaction (residues G67, G73 and V74) [43] belong to the NAC component of α -syn. Considering that the NAC contributes up to 10% of the SDS-insoluble protein in amyloid plaques [43] and that α-syn is expressed in regions of the brain characterized by abundant AD lesions, the interaction between the two proteins might play a key role in both DLB and AD pathogenesis.

α-Syn has been demonstrated to promote tau polymerisation *in vitro* and to co-localize with the same protein in neurons [34, 48]. α-Syn is known as a preterminal protein and is not expected to co-localize with tau in the axon. However, Jakes and co-workers [49] have provided evidence of axonal α-syn transport in the rat optic system, suggesting that the two proteins have wide opportunities for interactions within the axonal compartment. In particular, in the same study the authors identified the microtubule binding (MT)-binding protein tau as a ligand for the C-terminus of α-syn in human brain cytosol and established the direct protein interaction (Fig. 1) [49]. Affinity chromatography experiments have confirmed that α-syn directly binds to tau and induces fibrillation [49]. In different cellular systems, the physical interaction between the two proteins has been demonstrated to be abolished by the most common tau mutation (P301L) associated with frontotemporal dementia [50]. Interestingly, high tubulin concentrations, present in microtubules, has been shown to inhibit α-syn binding to tau, indicating that α-syn is a ligand for the soluble tau pool in contrast to the protein phosphatases 1 and 2A [51, 52].

In conclusion, there is mounting evidence of protein heteromers playing a role in ND pathogenesis. The stoichiometry of the interaction among the misfolded proteins and the correlation between the content of heteroaggregates and ND progression are currently unknown.

2. Biomarkers of protein aggregation-related neurodegenerative diseases

The pathological processes that characterize NDs begin decades before the first symptoms of cognitive dysfunction, thus making it difficult to identify pathology based on the clinical phenotype alone. For this reason, ND management would strongly benefit from the availability of biomarkers (BMs) for *early* diagnosis [53]. A biomarker is defined as an indicator of normal biological processes, pathological processes or of pharmacological responses to a therapeutic intervention [54].

The most attention has been focused on identifying genes that may be causative or associated with specific diseases and on unravelling the functional mechanisms induced by products of those genes as BMs for early diagnosis. In regards to AD, the identification of Aβ precursor protein (APP) and presenilin 1 and 2 mutations has supported the amyloid hypothesis and identified potential targets for pharmacological interventions. In particular, the genetic association with enhanced AD risk in families that carry the apolipoprotein E ε4 allele remains the primary issue [55]. However, it should

be emphasized that mutations in these genes identify at-risk family members but are not applicable to sporadic forms of AD [56, 57].

In contrast, five pathogenic mutations in genes linked to familial PD have been identified: autosomal dominant LRRK2 and SNCA, autosomal recessive Parkin, PINK1 and DJ1 [58, 59]. In addition, mutations in α-syn, parkin, ubiquitin c-terminal hydrolase (UCH)-L1 and DJ-1 have been linked to juvenile forms of PD [53].

2.1 Neuroimaging and central biomarkers

Potential central and peripheral biomarkers and the respective methodology of identification are summarized in Table 1.

During the past decade, the phenotype of NDs has been defined extensively by neuroimaging techniques, both in preclinical and early clinical disease stages. Aβ plaques in post-mortem studies were first detected using coloured dyes, such as Congo red [60], and fluorescent dye, such as Thioflavin-T (ThT) [61], that are capable of binding the β-sheet structure of Aβ. Such compounds have been utilized in the development of the first radiolabelled molecules for positron emission tomography (PET), including 2-(1-(6-[(2-[^{18}F]-fluoroethyl)(methyl)amino]-2-naphthyl)ethylidene)malononitrile ([^{18}F]-FDDNP), that can detect SP in AD patients [62]. Novel *in vivo* imaging techniques have been used to detect pathogenic Aβ accumulation, providing novel AD diagnostics and offering a new instrument to track changes in amyloid plaques in response to amyloid-lowering therapeutics. In particular, researchers have identified a Thioflavin-T analogue that can bind β-sheet–rich fibrils [63], which allows PET visualization of amyloid burden. Another example is the benzothiazole derived [^{11}C]-Pittsburgh compound B PET ([^{11}C]-PiB), which crosses the blood–brain barrier and binds amyloid deposits in the brain parenchyma where binding of carbon-11-PIB can be detected by PET imaging. Promisingly, an inverse correlation has been demonstrated between [^{18}F]-2-fluoro-deoxy-D-glucose PET imaging of glucose metabolism and PiB binding in the parietal cortex [64]. Conversely, no clinical studies on Aβ imaging using magnetic resonance imaging (MRI) have been reported, even though fluorine-19 ([^{19}F]) probes capable of detecting Aβ deposition have been in development in mouse models of AD [65-69].

Among all the efforts in the development of tracers to detect Aβ, only three probes have been approved by the European Medicines Agency and the Food and Drug Administration in the USA:[^{18}F]-florbetapir (Amyvid®) [70], [^{18}F]-flutemetamol (Vizamyl®) [71], and [^{18}F]-florbetaben (Neuraceq®) [72].

Finally, [^{18}F]-FDG PET has been used to trace and visualize the ‘typical’ metabolic deficiencies of AD, which allows it to be distinguished from other ND [73, 74]. In addition, AD and forms of vascular

dementia can be distinguished in regional cerebral perfusion studies by single photon emission computed tomography (SPECT) [75].

Beyond AD, the aforementioned PET tracers allow for exceptional *in vivo* research of other pathological conditions characterized by Aβ deposition, including cerebral amyloid angiopathy, brain trauma, Down's syndrome, and even cardiac amyloidosis [76-78].

Tau is a more complex target than Aβ, because of its intracellular localization, the existence of six dissimilar isoforms in different diseases, and its lower concentration. For these reasons, the requirements for a tau PET tracer are more stringent [79]. However, due to the unique role of this protein as a marker of neurodegeneration, the imaging of tau will allow for topographic distribution of tau proteins across diseases and *in vivo* assessment of tau pathology evolution. Furthermore, PET-tau could allow for precocious and precise diagnosis and monitoring of disease progression in tauopathies, cognitive impairment, movement disorders, and trauma [79]. Finally, the combination of Aβ and tau-PET will expand knowledge of the interactions between the two proteins in humans. To date, none of the tau imaging compounds described in preclinical and clinical studies have been approved. Among the more selective binders, [^{11}C]-PBB3 has been shown to allow tau imaging in AD and non-AD tauopathies [80]. Moreover, the ^{18}F-labelled tracers [^{18}F]-THK-5351 and [^{18}F]-AV1451 have been demonstrated to align with the known distribution of tau neurofibrillary tangles in live patients [81-83]. Additionally, [^{125}I]-phenylethenyl benzimidazole is currently under development for the detection of NFTs with SPECT [84].

Similarly, PD imaging includes either detecting alterations in brain structure or examining functional changes in brain metabolic patterns. Recently, a significant increase over controls in soluble α-syn levels has been found in AD brains, which correlates with cognitive impairment better than soluble Aβ or tau levels [41]. Based on these data, researchers are trying to develop α-syn imaging agents that could be useful for PD diagnosis as well. Furthermore, α-syn has advantages over dopamine as a biomarker for PD, because changes in α-syn often occur earlier than those in dopamine and are not affected by symptomatic treatment. Agents that bind brain α-syn would allow tracking of disease severity and localization, as well as monitoring of the therapeutic effects of compounds targeting α-syn. Thus, the Michael J. Fox Foundation has assembled a consortium of researchers to develop an α-syn PET radiotracer [85].

Studies focusing on the development of PET or SPECT tracers for α-syn are ongoing but are still in their infancy [86]. For example, [^{18}F] 3-(2-fluoroethoxy)-7-nitro-10H-phenothiazine and [^{11}C]-3-methoxy-7-nitro-10H-phenothiazine have been identified as more specific binders for α-syn and can cross the blood–brain barrier in animals [87]. However, these compounds have not been applied to human imaging yet.

Nevertheless the great efforts, the establishment of routinely biomarkers remains a strenuous objective, due to the dynamic anatomic localization and content of the different protein aggregates.

2.2 Peripheral biomarkers

Several studies have demonstrated that ND pathology and biomarkers are not restricted to the brain and that substantial biological changes appear in peripheral tissues as well. In particular, the typical brain alterations of NDs have been found, at the molecular level, in cerebrospinal fluid (CSF). For this reason, this fluid has been heavily studied in the search for ND biomarkers. CSF biomarkers should also reproduce brain pathological processes, such as synaptic/axonal degeneration, Aβ oligomerization, tau hyperphosphorylation, and α-syn accumulation [53]. Among CSF biomarkers, $A\beta_{42}$, total tau and α-syn are the most studied (Table 1).

In AD, a decrease in CSF $A\beta_{42}$ has been found [88, 89]. This reduction could be due to the deposition of the monomers in brain plaques that limit their diffusion to the CSF. Additional reasons include the detection limits of ELISA, which can be caused by $A\beta_{42}$ binding to other proteins that block antibody recognition or lack of recognition of the Aβ oligomer by the ELISA that generates false negatives [53]. $A\beta_{1\text{-}40}$ ($A\beta_{40}$), which is the most abundant amyloid form, has been found to be unaffected or marginally augmented in AD [90], consistent with the decreased $A\beta_{42}/A\beta_{40}$ ratio in the CSF of AD patients [91]. Aβ reduction has also been demonstrated in patients with FTD and vascular dementia, even if current measurements are insufficient to distinguish them from AD [92].

Contradictory data have been reported regarding the correlation between Aβ CSF concentrations and cognitive performance in AD: non-significant [93], inversely correlation [94] or even directed relationships [95] have been found. Based on these findings, the putative value of Aβ in AD progression should be further evaluated and consolidated.

In parallel, the quantification of Aβ oligomers in CSF has been carried out; even if debating data have been noted an overlap between the aged population and the low level of oligomers in CSF that make a reliable quantification challenging. Several studies have found increased Aβ oligomer levels in the CSF of AD patients [96-98], while other studies have reported no change [99, 100] or lower levels [101]. A possible explanation could reside in the selected method of detection (i.e., ELISA, Western Blot, ultrasensitive bead-based immunoassays) or the overlap of AD with other forms of dementia. Overall, these data suggest that Aβ42 detection in CSF allows for screening of early cases of AD and monitoring disease progression. However, this measurement constitutes only an additional test to confirm the disease diagnosis and provides little information on the disease progression.

The levels of tau protein in CSF have also been considered as possible biomarkers. A significant enhancement in CSF tau and phospho-tau have been identified in AD patients [66, 102-104].

Interestingly, the measurement of total and phospho-tau has not only been reported to identify AD pathology, but in combination with the Aβ measurement, it allows for the discrimination of AD from MCI with high accuracy [91, 105]. Moreover, autopsy studies have confirmed the association between high levels of CSF tau and AD severity [89], thus validating CSF tau as a biomarker of neuronal damage and neuronal and axonal degeneration.

α-Syn has been widely investigated as a potential peripheral biomarker to detect the NDs and to discriminate between different pathologies. Decreased CSF levels of α-syn have been shown in primary synucleinopathies, such as DLB and PD with respect to controls [106-108]. Conversely, oligomeric [109] and phosphorylated α-syn [110] have been reported to be significantly increased in PD patients. Regarding AD, CSF α-syn levels have been demonstrated to be higher with respect to controls [111]. However, conflicting data regarding the use of CSF α-syn levels to discriminate between different NDs have been reported [112]. One of the main issues could be the difficulty in the detection of α-syn oligomers and phosphorylated protein. Identification of innovative methodology to detect toxic species could help to validate the CSF α-syn levels as a biomarker for different NDs [113, 114].

Crucial disadvantages limit the clinical diagnostic and prognostic use of CSF biomarkers, such as cut-off values, the absence of assay standardization, and overlap between different types of dementia. Importantly, CSF sample collection requires a lumbar puncture, an invasive procedure which limits follow-up clinical trials [115]. Thus, there is a crucial necessity for peripheral biomarkers, in particular for AD, that could correlate with brain lesions and establish the efficacy of therapeutic drugs. In particular, more appropriate and manageable tissues need to be exploited by the introduction of specific and sensitive diagnostic tests at lower costs.

Great efforts have been devoted in exploiting biochemical markers in tissues other than CSF [103, 104, 116-118]. In this respect, the use of blood cells and plasma as a source of dementia biomarkers has been emerging because of its availability, lower cost and time effectiveness with respect to CSF. Pathological changes in blood proteins have been suggested to reflect the changes in CSF due to barrier impairment in dementia or merely by diffusion [119]. A CSF biomarker that can be potentially translated to blood is Aβ. Even if plasma Aβ species have been extensively investigated, literature results are inconsistent. Plasma $A\beta_{42}$ has been shown to increase in familial AD, while an opposite trend has been demonstrated during the disease-associated cognitive decline prior to the development of dementia [92]. Opposite results have been reported also by Hulstaert and co-workers [93]. The discrepancy in the quantification of Aβ could be ascribed to several reasons: i)the traffic across the blood-brain barrier; ii) the inability to measure Aβ oligomeric form; or iii) the use of inappropriate antibodies in ELISA protocols. The debate on the effective use of plasma Aβ as a biomarker is still

ongoing. In fact, Wood [120] stated that Aβ concentration in plasma is inappropriate to use as a biomarker due to the issues in detection and the interference of variables such as sex, age and other forms of dementia.

Tau is a brain-specific protein that can be converted to a relevant blood biomarker. To date, little is known about tau levels in blood due to its low abundance. Several efforts have been made in the detection of tau levels in the plasma of AD patients. However, these studies have shown contradictory data, including increased levels [121], mild increases [122], no differences [123] or reduced levels in AD compared to control [124]. Recently, Mattsson and co-workers [125] have demonstrated that higher levels of plasma tau partially reflect AD pathology, but the overlap between normal ageing and AD is large, thus highlighting the need of further investigation to clarify the potential of tau as a biomarker in AD and other NDs.

Interestingly, a few studies have reported the expression of high molecular tau in the platelets of AD patients [126, 127], but such changes do not correlate with cognitive decline [128]. Moreover, increased total tau levels in older AD patients compared to younger AD patients and healthy controls have been found, suggesting platelet tau as a diagnostic marker for the detection of disease onset [129].

The data regarding plasma α-syn levels in PD patients remain controversial. Plasma α-syn has been shown to increase early in PD [130]; such differences are particularly relevant following elimination of heterophilic antibody interference [131]. Conversely, other authors report no differences between PD patients and controls in total plasma α-syn [132, 133]. Foulds and co-workers [134] showed no difference in the total and oligomeric α-syn plasma levels, but they also noted a significant increase in phospho-α-syn (Ser129). Peripheral α-syn will probably not be used a diagnostic marker because it is produced not only in the brain but also in blood cells and skin [135, 136]. Another problem is the sensitivity of the detection methods, similar to the issues of CSF detection. To overcome this problem, Yang and co-workers [137] recently presented a new method of detection with improved sensitivity in immunomagnetic reduction (IMR). They used magnetic nanoparticles with antibodies against α-syn and were able to detect an increase in the protein in the plasma of PD patients.

Very recently, researchers have detected total and oligomeric α-syn in saliva: the authors showed that total α-syn decreases in PD patients, whereas its oligomeric form increases in the same cohort [138]. Such modifications correlate with several patients' clinical features, suggesting that the combined detection of total and oligomeric α-syn might aid in the early diagnosis of PD [138].

Although plasma Aβ, tau and α-syn have been investigated thoroughly in previous studies, little attention has been paid to the red blood cell (RBC) concentration of the proteins. Despite the widely accepted effects of Aβ on RBC function [139], few studies have reported Aβ quantification in RBCs,

which shows an increase in the protein with ageing and a decrease with antioxidant supplementation [140]. Similarly, to the best of our knowledge, no data have been reported on the quantification of tau in RBCs.

Some studies have been conducted in RBCs to assess levels of α-syn oligomer and total α-syn in PD. It has been shown that the ratio of RBC α-syn oligomeric/total protein is higher in PD patients than in control subjects; however, there is no correlation between this measurement and age of onset, disease duration, age, motor scale score or progression of motor degeneration in PD patients [141]. The results are consistent with previous findings showing an elevation of α-syn oligomers in CSF and plasma of PD patients. Because RBCs contain high levels of α-syn and detection of RBC α-syn can avoid contamination arising from haemolysis, this method should be more stable and reproducible compared with those detecting α-syn oligomers in plasma and CSF [141].

Other innovative approaches to detect oligomers/aggregates in peripheral fluid (CSF, plasma and RBC) have been recently reported by Horrocks and co-workers [142]. They developed a method of single aggregate visualization by enhancement imaging (SAVE) for the ultrasensitive detection of β-sheet rich fibrils and oligomers using single-molecule fluorescence microscopy. The methods do not allow for the discrimination of Aβ, tau and α-syn oligomers. However, the level of total oligomers are higher in CSF fluid of PD patients with respect to the aged control group.

Despite the efforts in the research of peripheral biomarkers able to detect the insurgence and the progression of NDs, conflicting data have been reported, which highlight the need to improve the accuracy and efficiency of the detection methods and the knowledge of the misfolded protein onset and fate.

3. New insights into protein aggregation inhibition as novel targets in neurodegenerative diseases

The issue of ND management is the development of therapeutic tools not only to delay worsening of symptoms but also to ameliorate the pathological signs. To date, there are no effective treatments that can prevent ND progression. Among the ND therapeutic strategies, the development of compounds able to prevent protein aggregation and remove diffusible toxic oligomers has been emerging (Table 2) [143].

3.1 Aβ aggregation inhibitor

In the last two decades, several disease-modifying strategies have been developed with the aim to decrease Aβ monomer production or to remove deposited Aβ [144, 145], such as the use of potent, highly selective inhibitors of β- and γ-secretases that can readily enter the brain and lower Aβ production. Similarly, efforts are also ongoing to develop small molecules that can up-regulate the enzymes that control Aβ degradation and thus lower Aβ levels by increasing

Aβ catabolism. The latest advances in the pharmacological inhibition of the Aβ formation or degradation enzymes have been extensively reviewed [144, 145].

Recently, innovative strategies to reduce Aβ toxicity have emerged (Table 2). Particularly, small derivatives have been developed that are able to interfere with Aβ aggregation (i) by reducing the oligomerization process and/or (ii) by inducing a conformational change in β-sheet assembly and/or (iii) by inducing quick conversion of soluble aggregates into less toxic fibrils [145].

In the literature, several small-molecules have been reported to interfere with Aβ aggregation, and most of these molecules share polyphenolic structures [146], such as 4-aminophenol [147], resveratrol [148], myricetin [149], curcumin [150], caffeine [151].

Another therapeutic approach targets the nucleation site of aggregation. This region is known as the KLVFFA and is the hexapeptide sequence that is believed to facilitate monomer-monomer interaction, leading to dimer and oligomer formation [152, 153]. An Aβ-steric zipper has been established as a useful model to investigate the binding interactions of small molecules with putative anti-Aβ activity [154]. Additionally, a few compounds have been identified by a high-throughput approach and demonstrated to interact with the KLVFFA region [155].

Among the Aβ-anti-aggregating strategies, an anti-Aβ immunotherapy approach has been emerging. In particular, antibodies that recognize the different toxic species of Aβ can act: i) directly by neutralizing them and blocking their toxic effects; ii) by stimulating microglial clearance; and/or iii) by promoting Aβ exit from the brain to the systemic circulation. This therapeutic approach has been demonstrated to decrease brain Aβ levels, reduce gliosis and neuritic dystrophy, and counteract memory impairment in AD transgenic mice [156]. More importantly, Alzheimer's disease patients who were immunized with aggregated Aβ showed diminished cognitive decline and slowed disease progression compared with patients who received the placebo [157]. Unfortunately, the phase IIa trial employing the AN1792 Aβ vaccine was stopped when ~6% of the immunized patients developed meningoencephalitis [158]. Great efforts are ongoing to avoid such problems and develop an effective immunization protocol.

Therapies directed at blocking Aβ oligomerization into toxic oligomers and aggregates have entered clinical trials. However, numerous phase II/III clinical trials for ADwith drugs targeting Aβ aggregation have failed [159, 160]. There are several reasons that can explain the high rate of clinical trial failure. Since Aβ plaque deposition may begin 10 years or more prior to the onset of cognitive symptoms [161, 162], one critical issue is the stage of disease generally targeted (mild-to-moderate dementia stages). This hypothesis is supported by the results of the solanezumab phase III trial in which a subgroup analysis showed a significant slowing of cognitive decline in subjects with mild AD dementia at baseline but not moderate AD [163]. Thus, ND drug discovery research has recently

shifted towards tau [164], because, in contrast to Aβ, tau pathology correlates with the degree of cognitive impairment and neuronal loss [165].

3.2 Tau aggregation inhibitor

Several therapeutic approaches targeting tau aggregation have been proposed (Table 2), such as inhibition of tau phosphorylation (kinase inhibitors), a microtubule stabilizer, a tau aggregation inhibitor (TAI), immunotherapy and chaperone-based drugs targeting disease-specific tau species [166, 167]. Thus, the new potential natural or synthetic molecules that are able to inhibit tau aggregation are reviewed and discussed.

Several small molecules developed to inhibit tau oligomer formation have already been tested in humans [166, 168, 169]. However, a discrepancy has been highlighted between the cell-based and/or *in vitro* data and the *in vivo* efficacy of TAI. In the last decade, different classes of agents able to interfere with tau aggregation have been reported, including but not limited to polyphenols such as green tea-derived (−)- epigallocatechin gallate (EGCG) [170], porphyrins such as hemin chloride [171], phenothiazines such as Methylene blue [171], benzothiazoles/cyanines such as N744 and Riluzole [172], thioxothiazolidinones (rhodanines), phenylthiazole-hydrazides, anthraquinones, and aminothienopyridazines (ATPZs) [167, 172] (Table 2). These compounds present two distinct mechanisms of action: the first includes the covalent TAIs, and the second includes the non-covalent inhibitors, which cause less secondary effects but also have less efficacy. Based on these scenarios, several clinical trials have begun. However, the most promising tau oligomer inhibitor is leucomethylthioninium (LMT, leucomethylene blue (MB), LMTX, TRx0237), developed by TauRxTherapeutics Ltd., Republic of Singapore, which is a second-generation TAI for AD treatment. TRx0237 shares the same active ingredient and mode of action of another first-generation TAI, methylthioninium (MT, Rember, TRx-0014) developed by the same company. LMTX is the reduced form of MT and is designed to have improved bioavailability and tolerability. They are both derived from methylthioninium chloride (MTC), a tricyclic phenothiazine, and they represent the most advanced TAIs in clinical development for the treatment of AD. Several clinical trials are currently ongoing for this class of compounds (ClinicalTrials.gov Identifier NCT01626391, NCT01689233, NCT01689246, NCT01626378, NCT02245568) for AD treatment and Behavioural Variant Frontotemporal Dementia (bvFTD). Overall, the trial failed to meet its primary efficacy outcome. However, the investigators found clinically meaningful and statistically significant reductions in the rate of disease progression in key study measures but only in the 15% of patients treated with LMTX monotherapy. These inconsistent results emphasize the issues with the use of TAI in regards to the stage of the pathology, similar to the amyloid inhibitor.

However, several other chemical entities and compounds have been reported [173-175]. Okuda and co-workers [173] reported a new compound, PE859 (3-[(1E)-2-(1H-indol-6-yl)ethenyl]-5-[(1E)-2-[2-methoxy-4-(2-pyridylmethoxy)phenyl]ethenyl]-1H-pyrazole), that inhibits tau aggregation *in vitro* and delays the onset and progression of motor dysfunction in an *in vivo* experiment with a reduction of tau aggregates in the central nervous system. In parallel, Saeda and co-workers [174] demonstrated that 1,2-dihydroxybenzene-containing compounds (i.e., isoproterenol, dopamine, epinephrine) can inhibit tau oligomerization. Of note, among these compounds, isoproterenol is able to decrease tau formation *in vivo*. As an innovative therapeutic strategy, Kim and co-workers [175] reported the use of specific tau-binding RNA aptamers that effectively delayed tau oligomerization *in vitro* and in tauopathy model cells.

The efforts to develop safe and efficacious anti-Aβ immunotherapy as active or passive vaccination have been translated to the development of immunotherapies targeting tau. Several active vaccines have entered clinical trials [167], such as the AADvac1 that is a synthetic peptide derived from tau amino acids 294 to 305 coupled to keyhole limpet hemocyanin (KLH) through an N-terminal cysteine, which has entered a Phase II clinical trial (ClinicalTrials.gov Identifier NCT02579252). Likewise, in active immunization, several antibodies have been developed directed to the phosphorylated sites, pS396/pS404/pS422 [176, 177]. RG7345 (RO6926496, MAb86) is a human monoclonal antibody specifically targeting pS422 that is able to counteract the signs of AD in a mouse model [177] and recently entered a phase I clinical study (ClinicalTrials.gov Identifier NCT02281786).

3.3 α-Syn aggregation inhibitor

In the discovery of effective inhibitors for the prevention and cure of NDs, amyloid and tau have received great attention with more than 4800 and 1500 papers, respectively, in PubMed. Recently, the development of molecules able to prevent the deposition of the toxic protein, α-syn, have arisen as an attractive therapeutic approach (Table 2) [178]. The reported inhibitors could be grouped into three categories: 1) small synthetic molecules and natural polyphenols, 2) peptides, and 3) aryl-residue-rich β-hairpins with no sequence homology to α-syn.

Polyphenols are a large group of aromatic compounds containing one or more phenolic hydroxyl groups. Among this class of compounds, baicalin [179], EGCG [180, 181], tannic acid (TA) [182], resveratrol [183] and curcumin [184] have been found to potently inhibit the assembly of α-syn into multimeric oligomers. Almost all these compounds reveal their effects only in preclinical studies. However, a phase III clinical study on the effect of EGCG as a neuroprotective agent is currently ongoing but not recruiting participants (ClinicalTrials.gov Identifier NCT02008721).

Among the synthetic molecules, NPT200-11 [185] and ANLE138b [186, 187] are emerging as promising candidates for PD treatment. Both compounds are able to pass the blood–brain barrier and have been reported to modify and thus reduce the aggregation of α-syn in preclinical studies [185]. Furthermore, NPT200-11 recently completed a phase I clinical trial (ClinicalTrials.gov Identifier NCT02606682) and exhibited low toxicity in healthy volunteers, thus opening the way for the experimentation on PD patients.

In the panel of disease-modifying therapies, the use of small peptides to control oligomerization has been on the rise. It has been widely accepted that the 71-82 region of α-syn is the site of aggregation. In light of this finding, several efforts have been made in the design and synthesis of β-sheet breakers based on this region. These efforts led to the discovery of unmodified peptides [188] and N-methylated peptides [189]. Nevertheless, the effects of the synthetic peptides *in vitro* have no data available in clinical trials. However, the research of effective peptides that target not only the aggregation site but also other pivotal sites for the correct folding of the protein is still ongoing [190, 191].

Another approach is active or passive immunization therapy that is based on the use of α-syn antibodies, as described for AD therapy [192]. Recently, a vaccine composed of short immunogenic peptides that mimic the C-terminus of α-syn (PD01A, AFFITOPE®, AFFiRiS) has been developed [193]. The vaccine results showed that it was well tolerated, and the next step (AFF008AA) is focusing on the long-term safety and the assessment of the immunological and clinical effects of a second vaccination ("reboost"). The results are expected in 2017.

Similarly, PRX002 is a monoclonal antibody able to bind the oligomeric form of α-syn. It is safe and well tolerated, as demonstrated by the positive results of a phase I, double-blind placebo clinical trial in healthy subjects conducted in 2014 (ClinicalTrials.gov Identifier NCT02095171). Based on these encouraging findings, another phase I study of multiple ascending doses has been initiated in patients with recent onset PD (ClinicalTrials.gov Identifier: NCT02157714).

A new frontier in immunotherapy is represented by the discovery and development of antibodies against the phosphorylated site of α-syn [194]. The phosphorylation of Ser129 seems to be a critical event in the accumulation of α-syn in the brain [195]. Thus, the development of a specific pSer129 antibody could represent an innovative target not only for imaging but also for the reduction of α-syn oligomerization.

Despite the efforts in the discovery and development of inhibitors of the transition to insoluble deposits of Aβ, α-syn and tau protein, several clinical trials have failed. One explanation could be the inadequacy of the disease-modifying strategy, however, in some cases, the use of the compounds was able to stabilize the non-toxic species preventing further polymerization. An example is Diflunisal, a

drug that can stabilize the mutant transthyretin (TTR) tetramer, which is one of the causes of familial amyloid polyneuropathy [196, 197]. Another possible explanation of the aggregation inhibitor failure is the stage of the disease during the drug administration. In fact, several clinical trials demonstrated positive outcomes in a subgroup of subjects. This evidence suggests that ND treatment should be initiated prior to the onset of clinical symptoms [198]. In accordance, the aggregate inhibitor should be used depending on the ND stage.

3.4 Broad-spectrum inhibitor

Recently, the synergy between Aβ, tau and α-syn in the acceleration of NDs has been highlighted [36, 41, 199]. Moreover, the discovery of heteromonomers and heteroaggregates supports the strategy to use broad-spectrum compounds that can interfere with the aggregation of more than one protein. Umeda and co-workers [199] reported that rifampicin, a well-known antibiotic, is able to prevent the aggregation of Aβ, tau and α-syn in a cell-free model. Moreover, they demonstrated that the drug can reduce Aβ and tau deposition in a mouse model of AD and reduces memory impairment. These findings are leading the way for the use of broad-spectrum compounds in the prevention of NDs and highlight the need to develop new effective anti-aggregation agents.

4. Future directions

In this review, the link between protein misfolding/aggregation and neurodegeneration was summarized, and the main pharmacological and clinical evidence at the basis of such a hypothesis were reported. Nevertheless, in the intensive research of aggregate-based biomarkers, most of the biomarkers demonstrate group differences but cannot reliably diagnose AD or PD in their early stages in an individual subject. This issue can be ascribed to an imprecise diagnosis that depends on clinical or pathological features, as well as phenotypic convergence, indicating the limit of the current understanding of NDs. Further efforts are needed to investigate and validate predictive biomarkers, in particular for the preclinical phase of neurodegeneration. In this scenario a greater attention have been directed to the heteroaggregates. The level of hetereoaggregates seem to be related to the progression of NDs thus highlighting their use a possible central and peripheral biomarker. Even if, further studies are mandatory to clarify their role in the unset and progression of NDs.

Of the therapeutic strategies targeting protein aggregates, the most appropriate and effective strategies will be selected depending on the nature of the target protein (e.g., intrinsically or natively disordered, whether it forms extracellular or intracellular deposits), as well as the disease stage. Unfortunately, the same lack of biomarkers for early diagnosis impedes the monitoring of the pharmacological

response to therapies. We expect that new biomarkers will be identified through basic research focused on quantifying all the aggregates present before and after clinical symptom presentation. Alternatively, new insights into the pathological role of heteromonomers and heteroaggregates could pave the way for the discovery and development of an aggregation inhibitor able to decrease neurodegeneration and possibly overcome the current barriers in disease-modification strategies.

Figure Legend

Fig. 1. Aβ, α-syn and tau production and aggregation. The monomers originate from different pathways. The Aβ monomer originates from the proteolytic activity of a secretase that leads to the production of the amyloidogenic fragment Aβ. The α-syn structure is characterized by three distinct regions: the N-terminal region (residues 1–60), the central region (residues 61–95) known as the non-amyloid component (NAC), and the C-terminal hydrophilic region that represents a different phosphorylation site (Ser129). Tau is a protein that stabilizes microtubules, and its hyperphosphorylation leads to the production of tau filaments that can form different types of aggregates. The pivotal mechanisms of monomer and oligomer degradation are mediated by the lysosomal/phagosomal machinery and proteasomal degradation. The monomers interact with each other to create oligomers and aggregates. The monomers of different proteins could also interact, producing heteromonomers and heteroaggregates.

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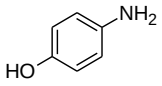
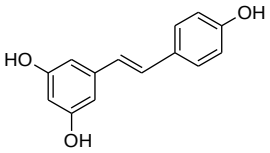
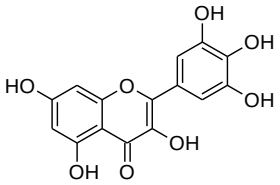
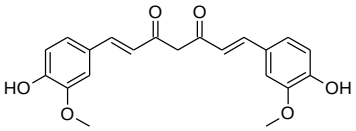
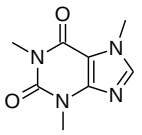
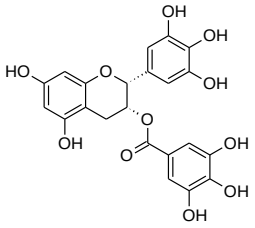
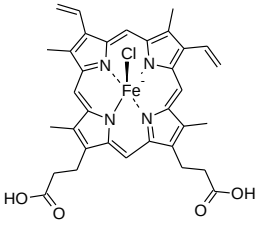
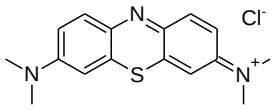
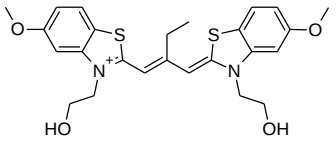
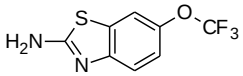
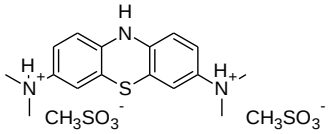
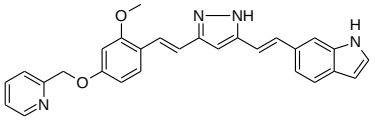
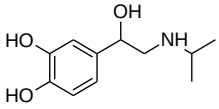
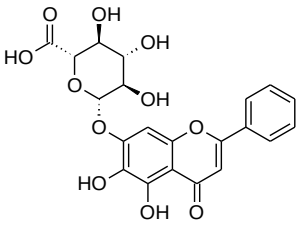
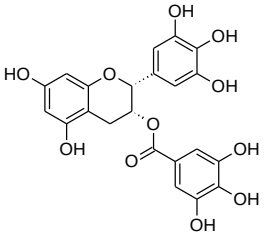
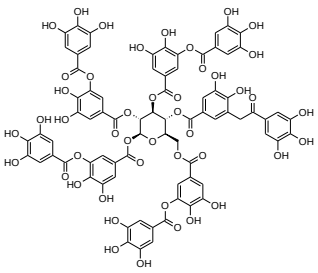
Table 1. Aβ, tau and α-syn as potential biomarkers in central and pheripheral fluids.

	Diagnostic site	Diagnostic sign	Metodology of detection	References
		Central		
Aβ	Post-mortem human brain	SP	Immunohistochemical, Congo red	[66]
	Post-mortem human brain	Aβ-deposit	Fluorescent microscopy, Thioflavin-T (ThT)	[67]
	Human brain	β-sheet structure, SP	PET, [^{18}F]-FDDNP	[68]
	Human brain	β-sheet structure, SP	PET, [^{11}C]-PiB	[69]
	Human brain	SP	PET, [^{18}F]-florbetapir, [^{18}F]-flutemetamol, [^{18}F]-florbetaben	[76-78]
	AD mice	Aβ-deposit	MRI, [^{19}F]-XP7, [^{19}F]-FMeC1, [^{19}F]-FSB	[71-75]
	Metabolic deficiencies of AD	Neuronal function	PET, [^{18}F]-FDG	[79-80]
tau	Human brain	NFT	PET, [^{11}C]-PBB3	[86]
	Human brain	NFT	PET ,[^{18}F]-THK-5351, [^{18}F]-AV1451	[87-89]
	Human brain section	NFT	SPECT [^{125}I]-phenylethenyl benzimidazole	[90]
α-syn	Macaque brain	LB, LN	PET, [^{18}F] 3-(2-fluoroethoxy)-7-nitro-10H-phenothiazine	[93]
		Peripheral		
Aβ	CSF	Total $A\beta_{42}$	ELISA	[94,95]
	CSF	$A\beta_{40}/A\beta_{42}$	ELISA	[96,97]
	CSF	Aβ oligomers	ELISA, Western-Blot	[102-107]
	Plasma	Total $A\beta_{42}$	ELISA	[98,99]
	RBC	$A\beta_{40}/A\beta_{42}$	ELISA	[152]
tau	CSF	tau, phospho-tau	ELISA	[108-111]
	Plasma	Total tau	ELISA, Western-Blot	[130-136]
	Platelet	tau oligomers	ELISA	[139,140]
α-syn	CSF	Total α-syn	ELISA, Western-Blot	[112-114, 119,120]
	CSF	α-syn oligomers	ELISA	[115,116]

CSF	phospho-α-syn	ELISA	[117,118]
Plasma	Total α-syn	ELISA	[141-144]
Plasma	phospho-α-syn	ELISA	[145]
Plasma	Total α-syn	IMR	[148]
RBC	Total/oligomeric α-syn	ELISA	[150]

Table 2. Aβ, tau and α-syn aggregate inhibitors.

Protein	Compound	Stage of development	Structure	References/ Clinical Trials
Aβ	*Smal molecules*			
	4-aminophenol	pre-clinical		[158]
	Resveratrol	pre-clinical		[159,160]
	Myrecetyn	pre-clinical		[161]
	Curcumine	pre-clinical		[162,163]
	Caffeine	pre-clinical		[164,165]
	Immunization therapy			
	AN1792	phase IIa (Failed)		[172]
tau	*Smal molecules*			
	Epigallocatechin gallate, EGCG	pre-clinical		[184]
	Hemin chloride	pre-clinical		[185]
	Methylene blue	pre-clinical		[185]

	N744	pre-clinical		[186]
	Riluzole	pre-clinical		[186]
	TRx0237 (LMT, leucomethylene blue, LMTX)	Phase II/III		NCT01626391NCT01689233NCT01689246NCT01626378NCT02245568
	PE859	pre-clinical		[187]
	Isoproterenol	pre-clinical		[188]
	Immunization therapy			
	AADvac1	Phase II		NCT02579252
	RG7345 (RO6926496, MAb86)	Phase I		NCT02281786 [191]
α-syn	*Smal molecules*			
	Baicalin	pre-clinical		[193]
	Epigallocatechin gallate (EGCG)	pre-clinical Phase III		[194,195]
	Tannic acid (TA)	pre-clinical		[196]

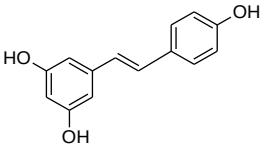
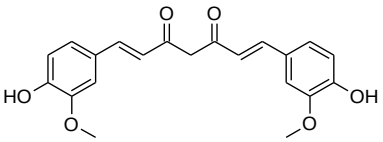
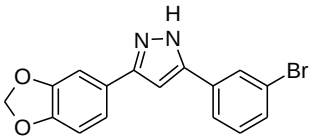
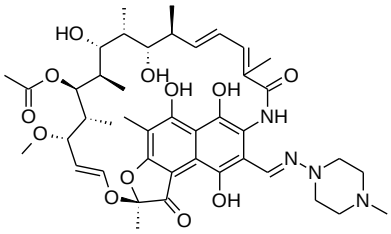
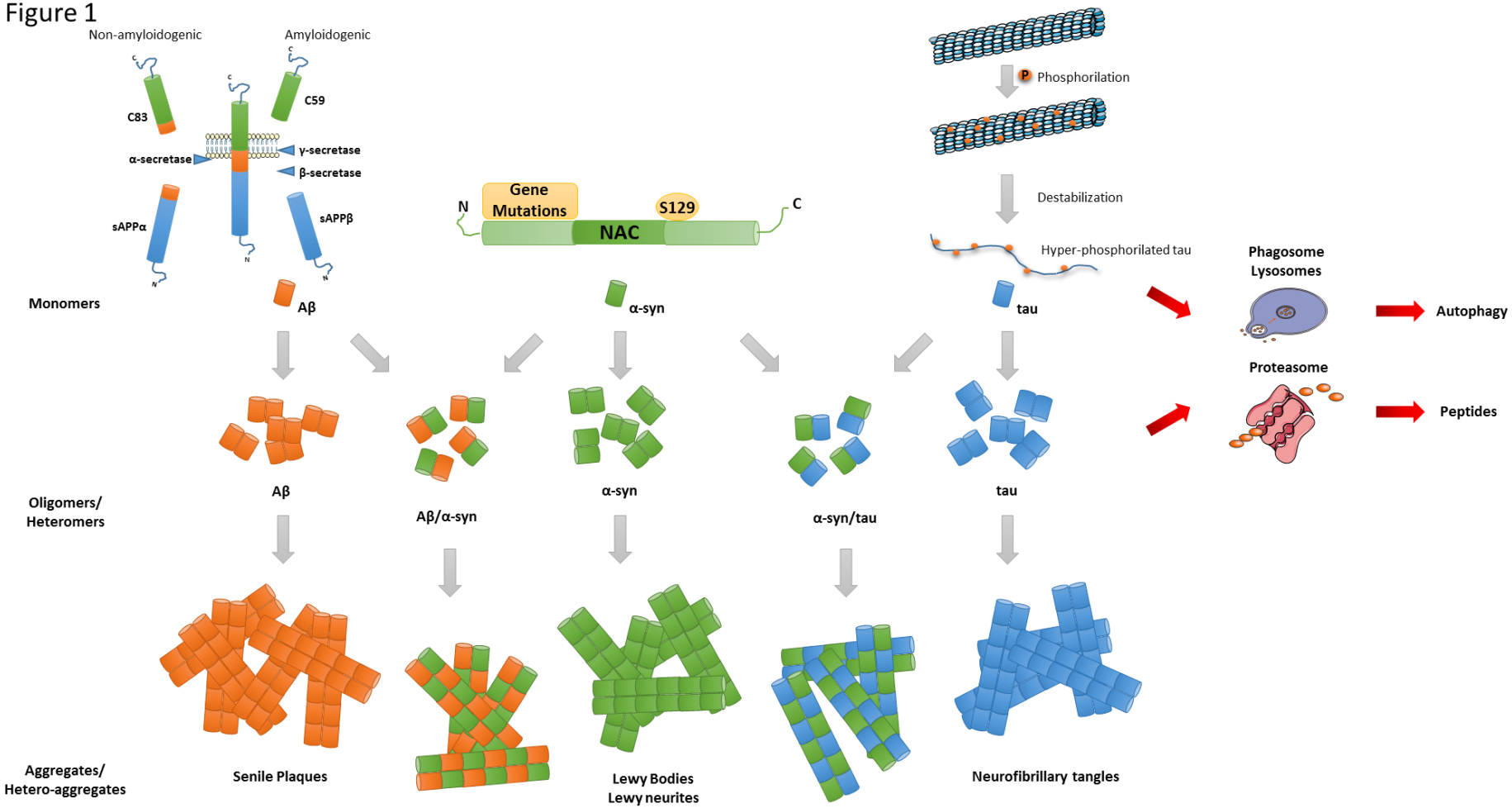
	Resveratrol	pre-clinical		[197]
	Curcumine	pre-clinical		[198]
	NPT200-11	Phase I		NCT02606682 [199]
	ANLE138b	pre-clinical		[200,201]
	Immunotherapy therapy			
	PD01A (AFFITOPE®, AFFiRiS)	Phase I		[207]
	PRX002	Phase I		NCT02095171 NCT02157714
Broad inhibitor	*Smal molecules*			
	Rifampicin	Pre-clinical		[213]

Figure 1


Non-amyloidogenic
Amyloidogenic
C83
C59
α-secretase
γ-secretase
β-secretase
sAPPα
sAPPβ
Gene Mutations
S129
N
C
NAC
Phosphorilation
Destabilization
Hyper-phosphorilated tau
Phagosome
Lysosomes
Autophagy
Proteasome
Peptides
Monomers
Aβ
α-syn
tau
Oligomers/
Heteromers
Aβ
Aβ/α-syn
α-syn
α-syn/tau
tau
Aggregates/
Hetero-aggregates
Senile Plaques
Lewy Bodies
Lewy neurites
Neurofibrillary tangles