Review

Translational Research in Alzheimer's and Prion Diseases

Giuseppe Di Fede[a], Giorgio Giaccone[a], Mario Salmona[b] and Fabrizio Tagliavini[a,*]
[a]IRCCS Foundation "Carlo Besta" Neurological Institute, Milan, Italy
[b]IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy

Abstract. Translational neuroscience integrates the knowledge derived by basic neuroscience with the development of new diagnostic and therapeutic tools that may be applied to clinical practice in neurological diseases. This information can be used to improve clinical trial designs and outcomes that will accelerate drug development, and to discover novel biomarkers which can be efficiently employed to early recognize neurological disorders and provide information regarding the effects of drugs on the underlying disease biology. Alzheimer's disease (AD) and prion disease are two classes of neurodegenerative disorders characterized by incomplete knowledge of the molecular mechanisms underlying their occurrence and the lack of valid biomarkers and effective treatments. For these reasons, the design of therapies that prevent or delay the onset, slow the progression, or improve the symptoms associated to these disorders is urgently needed. During the last few decades, translational research provided a framework for advancing development of new diagnostic devices and promising disease-modifying therapies for patients with prion encephalopathies and AD. In this review, we provide present evidence of how supportive can be the translational approach to the study of dementias and show some results of our preclinical studies which have been translated to the clinical application following the 'bed-to-bench-and-back' research model.

Keywords: Alzheimer's disease, amyloid, APP A673V, Creutzfeldt-Jakob disease, dementia, doxycycline, Gerstmann-Sträussler-Scheinker disease, prion, prion protein, recessive mutation

THE COMPLEXITY OF AD GENETICS AND THE PHENOTYPIC VARIABILITY OF FAMILIAL FORMS OF THE DISEASE

Alzheimer's disease (AD) is a genetically complex and heterogeneous disorder. Whereas most AD cases are sporadic with late onset by the age of 65 years and older, about 1% are early-onset familial AD (EOAD) cases [1]. Continued identification of variants contributing to EOAD is being spearheaded by multiple groups, including the Dominantly Inherited Alzheimer Network (DIAN) collaboration (http://www.dian-info.org/), among others. A comprehensive up-to-date list of mutations associated with EOAD is continuously reported by the following website: https://www.molgen.ua.ac.be/AD Mutations/. Fully penetrant causal mutations leading to predominantly EOAD have been identified in three genes (*APP*, *PSEN1*, *PSEN2*), while for the more common late-onset form of the disease (LOAD), novel partially penetrant genetic risk factors, mainly apolipoprotein E (*APOE*), have been established to date [2]. Several lines of evidence suggest that additional susceptibility genes exist for both EOAD and LOAD [3]. These genes may account only for a small fraction of the attributable AD risk and therefore rare variants and epistatic gene interactions should be taken into account to get the full picture of the genetic

*Correspondence to: Fabrizio Tagliavini, Scientific Director, IRCCS Foundation 'Carlo Besta' Neurological Institute, Via Celoria 11, 20133, Milan, Italy. Tel.: +39 0223942335; Fax: +39 022363973; E-mail: fabrizio.tagliavini@istituto-besta.it.

risks associated with AD. Indeed, the discovery of novel AD genes has great importance for the design of new prediction/prevention strategies for AD based on genetic risk profiling of patients [4, 5].

Over the past three decades, genetic research on AD have unveiled over 30 dominant mutations in the *APP* gene, accounting for about 15% of EOAD cases [6, 7]. These mutations have been shown to cause AD by altering *APP* processing, including enhancing C99 production (Swedish mutation KM670/671NL) [8], inhibiting non-amyloidogenic α-cleavage of *APP* (Arctic mutation E693G) [9], increasing $A\beta_{42}$ level or the $A\beta_{42}/A\beta_{40}$ ratio (London mutation V717I and Florida mutation I716V) [10, 11], or accelerating $A\beta_{40}$ fibril formation (Dutch mutation E693Q and Iowa mutation D694N) [12, 13]. In line with these advances in AD genetics, during the last twenty years, our research group has identified and/or characterized three new *APP* variants, four *PSEN1*, and three *PSEN2* causal mutations.

In 2004, our laboratory provided a detailed description of the clinical and neuropathological pictures associated with the *PSEN2* M239V mutation in a large Italian pedigree indicated as FLO10 [14]. The M239V mutation presented with some peculiarities involving not only Aβ deposition but also neuronal pathology such as the development of ectopic white matter neurons. The cognitive profiles of the affected members in FLO10 family also showed some distinctive features [15].

The A85V mutation in the *PSEN2* gene was studied neuropathologically by our group in 2008. The A85V carriers developed AD or Lewy body dementia and the neuropathological most relevant feature was the presence of diffuse Lewy bodies in the neocortex in addition to AD hallmark lesions [16].

In 2009, we described an Italian patient with a novel *PSEN2* mutation (Y231C) who showed behavioral abnormalities and language impairment as presenting symptoms, with only later involvement of other cognitive abilities [17]. This observation provided additional proofs of the variability of the phenotypes associated with *PSEN2* mutations.

Our study of the *PSEN1* S169L mutation in 2001 in collaboration with Bernardino Ghetti's group in Indianapolis revealed the presence of ectopic neurons in the white matter, which may represent the substrate for the early-onset seizures often associated with some *PSEN1* mutations [18]. The *PSEN1* I143V mutation was identified in a four-generation family with AD. The peculiarity of the age at onset (not very early), the long course, and the frontal

involvement, together with the rather complete absence of $A\beta_{40}$ and of amyloid angiopathy, widened the spectrum of *PSEN1*-linked phenotypes [19]. More recently, two novel *PSEN1* mutations (H214N and R220P) associated with familial AD were identified in our laboratory by targeted exome sequencing. The findings of this study confirmed the contribution of *PSEN1* genetic variants also to LOAD, underlying the need of extending the genetic screening of presenilin mutations to LOAD patients [20].

The results of these studies further supported the view that differences in the clinical/neuropathological features may be relevant even among family members with identical mutations of presenilins, further suggesting a phenotypic modulation by other genetic and/or environmental factors [21–23].

In 1987, van Duinen et al. [24] found that the cerebral amyloid angiopathy (CAA) with multiple hemorrhagic strokes previously described in several Dutch families [25, 26] is due to Aβ, and Levy et al. [27] showed that it is linked to a mutation at codon 693 of *APP* causing the E⟶Q substitution at position 22 of the Aβ sequence. The absence of neuritic plaques and tangles made it possible to distinguish the disease from familial AD with CAA and to call it hereditary cerebral hemorrhage with amyloidosis. The peculiar phenotype associated with the polymorphic 693 *APP* codon was confirmed by our observation about several members of four Italian families which had multiple strokes related to Aβ-CAA and a mutation localized to the same codon that leads to an E⟶K substitution [28]. Autosomal dominant dementia and multiple strokes due to a severe cerebral amyloid angiopathy were recognized also as distinctive features of the *APP* A713T mutation that differs from *APP* E693K mutation for the coexistence of severe tau pathology [29]. Our findings supported the view that the angiotoxic effects of Aβ may be independent of the neurotoxic effects that progresses to neuritic plaques and tangles, widening the spectrum of phenotypes linked to mutations in the *APP* gene.

A great number of studies have demonstrated that *APP* processing leads to the release of $A\beta_{1-40}$ and $A\beta_{1-42}$ which are the main responsible for the pathogenic events that, according to the amyloid cascade hypothesis, cause AD. However, a series of N- and C-terminal truncated Aβ species are also generated. The role of these additional Aβ peptides in the pathogenesis of AD is not yet clear. We analyzed $A\beta_{38}$ in the brains of patients with Aβ

deposition linked to sporadic and familial AD, hereditary cerebral hemorrhage with amyloidosis, and Down syndrome, and found that *APP* mutations localized in the Aβ coding region favor $A\beta_{38}$ accumulation in the brain. This observation suggested that the molecular mechanisms of Aβ deposition in carriers of mutations within the Aβ domain differ from those occurring in patients with FAD associated with other genetic defects and in sAD cases [30].

THE DISCOVERY OF AN AUTOSOMAL RECESSIVE MUTATION IN AD

In 2009, we discovered a novel mutation consisting of an Alanine-to-Valine substitution at codon 673 in *APP* gene, corresponding to the position 2 in the Aβ sequence (A673V or A2V) [31]. The mutation was found in the homozygous state in a patient with early-onset AD and in his younger sister who presented with multiple-domain mild cognitive impairment. Neuropathological examination of the proband revealed a peculiar profile characterized by large size Aβ deposits, mostly perivascular and showing a close correspondence between the pattern elicited by amyloid staining and the labeling obtained with immunoreagents specific for $A\beta_{40}$ or $A\beta_{42}$. This feature was in agreement with *in vitro* studies showing that the aggregation kinetic of the A2V mutant Aβ species is much faster than that of wild-type peptides suggesting that, once triggered, the nucleation of Aβ species proceeds very rapidly towards the formation of large amyloid assemblies [31]. Moreover, Aβ deposition spared neostriatum while deeply affecting cerebellum, and therefore was not in compliance with the hierarchical topographical sequence of involvement documented in sporadic AD cases [32].

Genetic screening of the proband's pedigree detected several family members from both parental lineages who had the A673V mutation in the heterozygous state. Neuropsychological assessment showed that none of these individuals had signs of cognitive decline even in advanced age. Furthermore, clinical information on the expected obligatory heterozygous carriers whose DNA was not available for testing ruled out any dementia in these individuals even in the ninth decade of life. These data were consistent with an autosomal recessive pattern of inheritance of the A673V genetic defect in this family, at variance with all the other previously reported mutations associated with AD [33].

These findings enlarged the scenario of AD genetics, suggesting that also autosomal recessive *APP* variants, although rarely, may be responsible for EOAD. In the same years, a further support to this view came from the discovery of the Osaka (E693Δ) intra-Aβ *APP* mutation. Either a possible recessive pattern of inheritance or a dominant pattern with incomplete penetrance was suggested for this mutation that leads to AD-like dementia despite low brain amyloid deposition [34].

To gain information on the mechanisms of the A2V recessive mutation in causing disease, we investigated the effects of the A673V variant on *APP* processing in cellular models and in brain tissue from the homozygous A673V carrier and found that this mutation promotes a shift of *APP* processing toward the amyloidogenic pathway, resulting in a significant increase in the sAβPPβ:sAβPPα and C99:C83 ratios, enhances Aβ production, increases particularly the brain levels of the A2V $A\beta_{1-40}$ ($A\beta_{1-40A2V}$) peptide, especially in the insoluble fraction of brain homogenates (i.e., formic acid extracts) where it is predominant over $A\beta_{1-42A2V}$), suggesting that this amino acid substitution strongly endorses the engagement of $A\beta_{1-40}$ in the aggregation pathway.

We then investigated the effects of the A673V mutation on the aggregation and amyloidogenic properties of Aβ and demonstrated that the A673V mutation increases the propensity of Aβ to adopt a β-sheet structure, modifies its aggregation kinetics strongly boosting Aβ's tendency to form oligomers and amyloid fibrils, enhances neurotoxicity of Aβ peptides bearing the A2V substitution, and produces specific toxic effects on neuronal cells by interfering with the cholinergic circuits [31, 35–37]. Similar conclusions were achieved by *in vivo* studies on transgenic *C. elegans* expressing human $A\beta_{A2V}$ showing that A2V mutation causes a pathologic behavioural phenotype with abnormalities in locomotor activity, pharyngeal contraction and a shortening of the lifespan [38]. Other groups showed additional pathogenic mechanisms for the A2V mutation, mainly involving effects on Cu2 + coordination [39].

The finding that the A673V mutation strongly boosts Aβ production and aggregation explained the presence of the early-onset dementia in homozygous carriers but raised the question why heterozygous carriers do not develop disease. Following the observation that humans with the mutation in the heterozygous state do not develop AD, we carried out *in vitro* and *in vivo* studies in order to simulate what happens in brains of A673V heterozygous carriers, where

$A\beta_{A2V}$ and $A\beta_{wt}$ are equally expressed [31]. These studies unveiled the extraordinary ability of $A\beta_{A2V}$ to interact with $A\beta_{wt}$, interfering with its nucleation and polymerization, leading to the formation of unstable aggregates, which can be easily removed by cell scavengers. In particular, circular dichroism (CD) spectroscopy, SDS-PAGE analysis, electron and atomic force microscopy (AFM) showed that the heterologous interaction between $A\beta_{A2V}$ and $A\beta_{wt}$ at equimolar concentration, resulted in a decrease of β-sheet secondary structure and inhibition of assembly into oligomers and fibrils [35]. Most interestingly, $A\beta_{A2V}$ was very efficient in counteracting Aβ-dependent neurotoxicity in human neuroblastoma cells [31], and rescuing normal phenotype and restoring dendritic spine density of hippocampal neurons from brainbow mice treated with $A\beta1\text{-}42_{wt}$ [40].

X-ray and neutron diffraction experiments combined with polarized light microscopy, AFM, and modeling provided a rational basis for the paradoxical effects of A2V-Aβ mutation in humans, explaining its aggressiveness in homozygous carriers and its protective effect in heterozygotes. Since the N-terminal Aβ residues are always located at the outside of the fibril, this makes high probable that alanine-alanine interaction in wt and valine-valine interaction in A2V are involved in inter-fibrillar interactions and dimerization. The higher degree of orientation in the latter suggests tighter packing or less sterically-hindered interference between laterally-adjoining protofilaments or fibrils, that favors the fibril polymerization. Conversely, an interaction of alanine2 in the wt with valine2 in the A2V peptide may interfere with the fibril interactions owing to a mismatch of the side chains and steric hindrance that disrupts the hydrogen-bonding and inter-sheet interactions, and thereby prevent fibrillogenesis [41].

Overall these data suggested that the interaction between mutant and wt Aβ is able to hinder amyloidogenesis and neurotoxicity, thus protecting the A673V heterozygous carriers. The results of our studies leaded also to a reconsideration of the relevance of the N-terminal sequence of Aβ in misfolding and disease, since it was underestimated by the previous scientific literature [42]. Our data reinforced the hypothesis that the N-terminal domain of Aβ is selectively perturbed in amyloidogenesis and that changes in its primary sequence my deeply affect peptide assembly and fibril formation [43]. The importance of this domain is further supported by the finding that antibodies against it are optimal for plaque clearance in animal models [44] and, most intriguingly, are

under evaluation in human clinical trial as disease-modifying drugs for AD [45].

Interestingly, evidence for a natural protection against AD was shown in human carriers of the A2T Aβ mutation—another human Aβ variant characterized by an alanine-to-threonine substitution at the same *APP* codon of the A2V-Aβ mutation (*APP*-A673T or $A\beta_{A2T}$ variant) [46, 47]. Additional studies tried to clarify the molecular basis of the A2T-induced protection for AD, suggesting a likely composite mechanism including effects on *APP* processing, with consequent decrease of Aβ production, and on Aβ structure, aggregation and neurotoxicity [48–50].

The discovery of protective genetic variants like $A\beta_{A2V}$ and $A\beta_{A2T}$, although rare, should prompt a novel vision of genetic studies, until now limited to the identification of pathogenic variants, expanding the genetic research into the detection of 'protective' DNA variations as useful grounds for the design of efficient disease-modifying therapies in medicine [51].

THE DEVELOPMENT OF A POTENTIAL DISEASE-MODIFYING THERAPY FOR AD

The finding that the interaction between $A\beta_{A2V}$ and $A\beta_{wt}$ hinders amyloidogenesis offers grounds for the development of a therapeutic approach based on the use of modified $A\beta_{A2V}$ peptides for AD.

In collaboration with the Mario Negri Pharmacological Institute in Milan, we envisaged an integrated A2V-based strategy for treatment of AD by designing a short peptide homologous to residues 1–6 of $A\beta_{A2V}$ ($A\beta1\text{-}6_{A2V}$) that retains *in vitro* the anti-amyloidogenic properties of the parental *full-length* mutated Aβ. The correspondent *D-isomer* [$A\beta1\text{-}6_{A2V}(D)$] was then designed because predicted to be resistant to degradation by endogenous proteases, and resulted even more effective than the *L-isomer* in hindering Aβ aggregation. Molecular dynamics simulations showed that the native peptide is characterized by a "closed" configuration in which the N- and C- termini are strongly interacting. Conversely, the structure of the mutated peptide is marked by higher flexibility, which facilitates the heterotypic interaction with Aβ and hinders Aβ assembly [35, 51].

CD spectroscopy, SPR, and AFM showed that $A\beta1\text{-}6_{A2V}$ inhibits acquisition of β-sheet sec-

ondary structure by *full-length* wt Aβ, elongation of wt Aβ amyloid fibrils and assembly of Aβ into amyloid fibrils, dramatically reducing the formation of protofibrils and filamentous structures [35, 51]. Moreover, toxicity studies on cellular models showed that Aβ1-6$_{A2V}$ is not toxic on SY-SH5Y neuroblastoma cells even at high concentrations (20 μM) and hampers the toxicity induced by Aβ1-42$_{wt}$ on these cells [51]. Finally, to improve the translocation across the blood-brain barrier (BBB), we linked an amino acid sequence highly rich in basic residues (TAT) to the six-mer peptide so generating the Aβ1-6$_{A2V}$-TAT(D) compound. The *in vitro* studies were largely replicated by our groups with Aβ1-6$_{A2V}$-TAT(D) confirming that this peptide hinders (1) production of fibrils and amyloid structures by Aβ1-42$_{wt}$, (2) toxicity induced by Aβ1-42$_{wt}$ peptide on SYSH-5Y cells, and (3) synaptopathy caused by Aβ$_{wt}$ in hippocampal neurons [40, 51]. All these findings elected Aβ1-6$_{A2V}$-TAT(D) as our lead compound for *in vivo* studies.

We first demonstrated that Aβ1-6$_{A2V}$-TAT(D) prevents Aβ oligomer formation and protects transgenic *C. elegans* from Aβ toxicity. The compound was indeed effective in protecting CL4176 worms (expressing human oligomeric Aβ$_{1-42}$ in the body wall muscles) from the paralysis induced by the Aβ1-42$_{wt}$ expression, was able to protect against the motility defect in a dose-dependent manner and was successful in reducing Aβ fibril formation and amyloid deposition in CL2120 transgenic worms [52].

Then, we used murine models of AD to assess the efficacy of our approach. We showed that Aβ1-6$_{A2V}$TAT(D) is able to cross the BBB after intraperitoneal administration in the double transgenic APPswexPS1dE9 mice. Short-term treatment (2.5 months) resulted in the exciting prevention of cognitive deterioration, Aβ production/aggregation and amyloid deposition in the brain. However, the final outcome (5 months after the beginning of treatment) consisted of an unexpected increase of amyloid burden and attenuation of the effects on Aβ production, while the prevention of cognitive impairment was maintained and even more evident [51]. Concomitant studies performed in our labs revealed a great propensity of TAT(D) to target amyloid deposits. These data – together with the results of previous reports showing that the co-expression of TAT and human *APP* carrying the Swedish mutation in mice results in an acceleration of amyloidogenesis [53] and that TAT increases Aβ levels by inhibiting neprilysin [54] or enhancing β-secretase cleavage of *APP* [55] – suggested that the anti-amyloidogenic effects of Aβ1-6$_{A2V}$(D) were undermined in the chronic treatment by the TAT intrinsic amyloidogenic activities.

So, regardless its optimal BBB delivery abilities and cell penetrating activity [56], the design of therapeutic strategies for AD [57] should, in our opinion, take in account some intrinsic properties of TAT sequence, which can promote or worsen amyloidogenesis in long treatment schedules [51].

New studies are ongoing in our laboratories to design new brain delivery strategies for Aβ1-6$_{A2V}$(D), such as the intranasal administration of the peptide, or to develop novel Aβ$_{A2V}$-containing compounds, incorporating different peptide "shuttles" to efficiently drive the drug to the brain, without interfering with its anti-amyloidogenic ability.

MECHANISTIC AND NEUROPATHOLOGICAL STUDIES IN PRION DISEASES

Prion diseases are fatal, rapidly progressive, neurodegenerative disorders of humans and animals. They are also called transmissible spongiform encephalopathies (TSEs), a term that underlines their infectious character and their main neuropathological hallmark, i.e., neuropil vacuolation [58, 59]. The transmissible agent, the prion, is devoid of informational nucleic acid and consists only of protein [60]. Several lines of evidence indicate that prions are composed of an abnormal isoform of the prion protein (PrP). The normal form of PrP (PrP^C) is widely expressed in the central nervous system but little is known about its function(s) [61]. The human cellular gene which encodes PrP^C has been called *PRNP*. The pathogenic isoform (PrP^{Sc}) results when the normal form undergoes a conformational change, converting α-helical regions to β-sheet motifs, and has abnormal physicochemical properties such as detergent insolubility and protease resistance (PrP^{res}) [62–65].

Human TSEs include four main groups of pathologic conditions: Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI), and kuru. CJD has diverse phenotypes and can be familial (fCJD), acquired [iatrogenic (iCJD) and variant CJD (vCJD)] or sporadic (sCJD) when it arises for no obvious reasons [58, 66]. Possible causes of sCJD include spontaneous formation of PrP^{Sc} as a rare stochastic event, somatic mutations of *PRNP*, or unrecognized prion

exposure [67]. Kuru is an acquired prion disease confined to Papua New Guinea and related to cannibalistic mourning rituals. GSS, fCJD, and FFI are genetically determined by mutations within the open reading frame of *PRNP* and inherited as autosomal dominant traits [68]. Several reports by our group provided evidence that the spectrum of phenotypes associated with *PRNP* mutations is wide [69–78].

Over the last decades, huge data consolidated the concept that phenotypic diversity of human prion diseases is mainly dependent on the existence of distinct PrP conformers, which, together with other genetic and unknown environmental factors, generate different clinic-pathological profiles of TSEs. Regarding sCJD, two main types of the unglycosilated isoforms of PrP^{res} were found in sCJD cases: type 1 (21 kDa) and type 2 (19 kDa). Our group was the first to report that the two different types of PrP^{res} may be present in the same sCJD brain [79, 80]. During the last few years, these observations led to a classification of sCJD cases based on the type of PrP^{res} and the Methione/Valine polymorphism at codon 129 of *PRNP* gene [66, 81].

Moreover, our group contributed actively to the definition of neuropathological diagnostic criteria for CJD and other human prion diseases [82–85], as well as to pathogenic studies on prion encephalopathies. In 1991, we demonstrated that the GSS amyloid is mainly composed of an 11 kd fragment of the human prion protein with an N-terminal glycine at codon 58 [86, 87]. These pivotal studies were performed in tight collaboration with Blas Frangione in New York and Bernardino Ghetti in Indianapolis who identified and deeply characterized brains derived from members of a large family (Indiana kindred) reported to be affected by GSS [88]. Interestingly, the GSS amyloid deposits in brain from carriers of the *PRNP* mutations associated to the disease contain only the fragments originating from mutant PrP alleles [89]. This implies that mutant PrP fragments are dominant factors for amyloidogenesis in GSS and that *full-length* PrP is deposited in the extracellular compartment, partially degraded by proteases and further digested by tissue endopeptidases. This processing leads to generation of an approximately 7-kDa protease-resistant core that is similar in patients with different mutations [90]. Moreover, we deeply analyzed the composition of amyloid deposits in GSS patients and found that the epsilon isoform of 14-3-3 protein is among the components of the prion protein amyloid deposits in GSS [91]. Definitely,

our data together with studies by other authors provided evidence that GSS amyloid is composed of differently sized PK-resistant PrP fragments (mainly ranging from 7- to 11-kDa) forming patterns not previously described in other prion diseases, which may in part explain the peculiar pathology of GSS [92–94].

Neuropathological studies on GSS patients carrying different *PRNP* mutations revealed the significant presence of tau-related pathology in association with the core features of the disease, i.e., PrP-amyloid deposits [95]. Indeed, neurofibrillary tangles (NFTs) are diversely represented in different GSS genotypes, being most evident in F198S, D202N and Q217R carriers, so it is possible to distinguish between GSS with or without NFTs. This conclusion is interesting because GSS patients with NFT have a distinct PrP^{res} profile, suggesting that tau pathology may be related to pathogenic properties of specific PrP^{res} isoforms [96].

Additional studies by our group, demonstrated that tau pathology is also a relevant feature of human and experimental vCJD, suggesting that the abnormal forms of PrP associated with vCJD may trigger a tauopathy, and providing a paradigm for the early stages of tau pathology associated with cerebral amyloidoses, including AD [97]. The involvement of microglia was also reported by our group as relevant in the progression of CJD [98, 99], confirming previous animal and cell studies showing more rapid neurodegeneration, pathology spreading, and prion infectivity following microglia ablation, depletion, or deficiency [100, 101].

In 1993, we showed that the main pathogenic features of PrP^{res} peptide is retained in its 106–147 peptide fragment [102]. In particular, synthetic 106–126 and 127–147 PrP fragments resulted to be prone to form amyloid-like fibrils *in vitro*. Moreover, 106–126 PrP peptide has neurotic properties [103], induces activation of glial cells [104, 105] and increases plasma membrane microviscosity [106].

FROM BED TO BENCH AND BACK IN PRION DISEASES

The progress in the knowledge of molecular mechanisms driving the pathology in human and animal prion diseases offered grounds to (1) the discovery of disease biomarkers and (2) the design of experimental treatments in this group of fatal neurodegenerative diseases.

Biomarkers

The implementation in clinical practice of novel diagnostic tools for prion diseases was recently accelerated by the employment of two innovative amplification assays named Protein Misfolding Cyclic Amplification (PMCA) and Real Time Quaking-Induced Conversion (RT-QuIC) generated to model the process of prion misfolding *in vitro* in a very short time [107, 108]. PMCA consists of cycles of incubation and sonication of samples containing small amount of PrP^{res} in the presence of an excess of PrP^{C}, so enabling the exponential amplification of minute amount of PrP^{res} [108]. Thus, this technology allows the detection of PrP^{res} even in presymptomatic stages of prion diseases. In 2005, our group, in collaboration with others, showed that by PMCA it is possible to detect PrP^{res} in the brain of pre-symptomatic hamsters, enabling a clear identification of infected animals as early as two weeks after inoculation. Furthermore, PMCA was able to amplify minute quantities of PrP^{res} from a variety of experimental and natural TSEs. These findings indicated PMCA as useful to recognize the illness in humans in early phases [109]. Following this approach, a recent study performed with the Claudio Soto's laboratory in Houston, showed that urine samples from patients with vCJD contain minute quantities of PrP^{res} that can be detect by using PMCA [110]. These results provided a powerful diagnostic tool for vCJD considering its high sensitivity (92.9%) and specificity (100.0%). RT-QuIC employs Thioflavin-T fluorescence to detect femtomolar amounts of PrP^{res} in biological samples, using soluble recombinant prion proteins as substrate in a reaction cyclically alternating incubation and shaking. This technology was shown to enable the detection of PrP seeds in the cerebrospinal fluid of patients with sporadic sCJD or genetic forms of prion diseases [111, 112].

Further evidence for the effectiveness of amplification technologies for the detection of prions in biological fluids came from the observation that PrP^{res} could be detected with 100% sensitivity and specificity in blood samples from vCJD patients [113] as well as from sheep and primates experimentally infected with vCJD in preclinical stages [111]. RT-QuIC has been further adapted to detect minimal amounts of PrP^{res} in other tissues, such as the olfactory mucosa of sCJD patients [114]. Moreover, we recently demonstrated that the olfactory mucosa of patients with FFI contains trace amounts of PrP^{res} which are detectable by PMCA and RT-QuIC [115].

Taken together, these findings suggest that RT-QuIC and PMCA have a huge potential to detect trace-amount of PrP^{res} (≥ 1 femtogram) in peripheral tissues, validating a possible implementation of these arrays into novel diagnostic criteria for human prion diseases [116–118].

Treatments

Initial steps on the road to the development of experimental strategies for the treatment of prion diseases [119–121] followed previous observations by the group led by Giampaolo Merlini in Pavia about the effectiveness of an approach based on the use of the anthracycline 4'-iodo-4'-deoxy-doxorubicin (IDX) in systemic amyloidosis [122, 123]. In 1997 we found that, after treatment with IDX, clinical signs of disease were delayed and survival time was prolonged in an experimental prion disease in Syrian hamsters [124]. Other studies in collaboration with Mario Negri Institute in Milan indicated that the IDX-structurally similar compounds tetracyclines bind to PrP amyloid, prevent aggregation of PrP peptides, disrupt amyloid fibrils generated by PrP peptides, abolish neurotoxicity of PrP peptides, and revert protease resistance of PrP peptides and generation of PrP^{res} from sCJD, vCJD, bovine spongiform encephalopathy, and scrapie [125, 126]. Moreover, tetracyclines reduce prion infectivity through a direct interaction with PrP^{Sc} and are potentially useful for inactivation of bovine spongiform encephalopathy- or vCJD-contaminated products and thus they are potentially useful to design prevention strategies for prion diseases [119, 120, 127–129], also considering that they have favorable kinetics, high ability to cross BBB, low toxic effects, and good tolerability even in long-lasting treatment schedules [130].

On these bases, we began a pilot study on a small series of patients with CJD diagnosed at Carlo Besta Neurological Institute in Milan, between 1996 and 2004. They received a compassionate treatment with doxycycline at a daily oral dose of 100 mg from the time of diagnosis to death. The retrospective analysis showed that the patients treated with doxycycline survived significantly longer than untreated patients [131]. Similar results were obtained in an independent observational study in Germany [132]. We therefore designed a randomized, double-blind study of doxycycline versus placebo in CJD with the primary objective of assessing the effectiveness of doxycycline in increasing survival time in CJD patients. The study provided class 1 evidence that

an oral dose of doxycycline at 100 mg per day does not prolong survival. Quantification of the brain concentrations of doxycycline suggested, however, that a higher daily dose of doxycycline should be recommended in future trials and that the enrolment of patients in early stages of the disease is a crucial factor to enhance the beneficial effects of tetracyclines [133]. The possibility that tetracyclines may be more effective in treatment schedules starting very early along the course of the disease is being tested in a preventive clinical trial with doxycycline in FFI, designed with the help of asymptomatic carriers, who have agreed to be exposed over a 10-year period to doxycycline. The results of this ongoing study are not available at present [134].

The care of patients with AD and prion diseases is challenging because of the complexity of these disorders. Past approaches to drug development were effective in developing symptomatic agents, but they failed in the attempt to develop disease-modifying compounds. New means of discovering agents and predicting human effects, better animal models, improved trial designs and outcomes, and more predictive biomarkers are needed. The approach based on translational research can assist in diagnosing, preventing or treating neurodegenerative disorders. Translational neuroscience may accelerate these achievements by providing more efficient biomarkers and promoting successful drug development programs for AD and prion diseases.

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