Oligomeropathies and inflammation: new and old concepts in neurodegenerative disorders

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We coined the term *oligomeropathies* to define the protein misfolding disorders where the oligomers exert a central pathogenetic role. The contribution of inflammation in the neuronal dysfunction that characterize the Alzheimer's disease (AD) and in general the neurodegenerative disorders, is well established, although the approaches based on the control of inflammatory factors show poor or none therapeutic efficacy. Recent evidence indicate that the inflammatory mechanisms proposed in the last two decades based on the activation of glial cells as consequence of neurodegeneration, are juxtaposed with more direct contribution of inflammatory factors in the initial neuronal dysfunction. Our results in experimental models demonstrate the efficacy of anti-inflammatory treatments to prevent the cognitive deficits induced by β amyloid oligomers directly applied into the brain. The application of α-synuclein oligomers potentially involved in Parkinson's disease (PD) and Lewy bodies Dementia (LBD), induced similar cogntive decline but different inflammation reaction. Other studies support the concept that the glial activation might contribute to the primum movens responsible of the neuronal damage and the cognitive decline. The presence of inflammatory factors in the biological fluids has been proposed as possible markers of the neuroinflammation associated to AD and other neurological disorders. In the same cases the presence of altered levels of cytokines or other inflammatory markers in CSF correlate with the severity of the disease. In cognitively impaired elderly an association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers has been found, indicating the possible influence of peripheral inflammatory state on cognitive decline. Genetic variants of several genes encoding inflammatory factors, including the triggering receptor expressed on myeloid cells 2 (TREM2), have been linked to AD and PD. The recent results on inflammation and neurodegeneration might indicate, according to the precision medicine principles, innovative therapeutic approaches to AD and other neurodegenerative disorders. This would be based on the inflammatory state of the patients determined by the combination of genotype/phenotype information.

In vitro amplification of protein misfolding: diagnostic and therapeutic applications

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Neurodegenerative disorders are characterized by the accumulation of misfolded proteins in the Central Nervous System (CNS). In particular, abnormal prion proteins (PrP^{Sc}) are found in the brain of patients with prion diseases, amyloid-β (Aβ) and tau proteins accumulate in the brain of patients with Alzheimer's disease (AD), α-synuclein is found in the CNS of patients with Parkinson's disease (PD) and Lewy body dementia (DLB), while tau is found in patients with frontotemporal lobar degeneration (FTLD-tau). Therefore, these misfolded proteins are considered disease-specific biomarkers. Two innovative techniques named Protein Misfolding Cyclic Amplification (PMCA) and Real Time Quaking Induced Conversion (RT-QuIC) were generated to mimic the process of protein misfolding *in vitro* in an accelerated manner. Firstly optimized for the amplification of prion proteins, both techniques have been recently extended for the amplification of abnormal forms of tau, Aβ and α-synuclein. Considering their ability to reproduce the pathological process *in vitro,* PMCA and RT-QuIC have been used to study (i) the molecular mechanisms which lead to protein misfolding, (ii) the efficacy of specific therapeutic compounds to interfere with these processes and (iii) to detect trace amount of misfolded proteins eventually circulating in peripheral tissues of patients with different forms of neurodegenerative diseases. Here we show how PMCA and RT-QuIC were able to successfully detect trace-amount of disease specific biomarkers circulating in blood, urine, cerebrospinal fluid and olfactory mucosa of patients with different types of dementia, including Alzheimer's disease, Parkinson's disease and prion diseases. These findings demonstrate that disease-specific biomarkers are not only confined to the CNS and that the analysis of peripheral tissues might potentially lead to an early, not invasive and definitive diagnosis of dementia when patients are alive.

HIV-1 matrix protein p17 misfolding forms toxic amyloidogenic assemblies that induce neurocognitive disorders

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The advent of combined antiretroviral therapy (cART) has converted the course of human immunodeficiency virus type-1 (HIV-1) from an incurable disease to a treatable chronic disease. HIV-1-associated neurocognitive disorder (HAND) is one of the most frequent complications in HIV-1-seropositive patients and may result in the development of neurological deficiencies which adversely affects the quality of life, treatment adherence and lifespan of these patients. The pathogenesis of HAND is unknown, even though many mechanisms have been proposed. A role of viral proteins has been also suggested but the direct infection of neuronal cells by HIV-1 is limited indicating that the virus is unlikely to cause a direct effect and that the mechanism of HAND may rely on indirect actions of the molecules released into the microenvironment by HIV-1 infected cells. The role of the HIV-1 matrix protein p17 (p17) in the onset and development of HIV-1-associated neurocognitive disorder (HAND) was considered here, with the hypothesis that the formation of toxic soluble oligomers secondary to the partial misfolding of the protein, might underlie this pathology.

For the first time we have produced a morphological and dimensional characterisation of the assemblies produced by p17 in cell-free conditions. Starting from a monomeric form, p17 changes its conformation to form small oligomers with hydrophobic residues on the external surface that grew rapidly, forming annular or rosary-like structures that preceded the prefibrillar soluble structures. Applying the well-established *Caenorhabditis elegans* approach for the specific recognition of toxic assemblies of amyloidogenic proteins, we prove that the soluble assemblies of p17, at concentrations that fall within those detected in the blood of HIV^+ patients, were toxic. We have also identified new epitopes of the protein, unrelated to the region interacting with the p17 receptors, responsible for this biological effect.To further substantiate the observations in *C. elegans*, we did selected experiments in mice proving that intrahippocampal injection of p17 reduced their cognitive function and induced behavioral deficiencies. To explore pharmacological approaches aimed at neutralising the toxic activity of p17, we again used *C. elegans* and showed that tetracyclines, antibiotics with a peculiar pleiotropic action, counteract the pharyngeal impairment caused by p17.

These findings offer a new way of thinking about the possible cause of neurodegeneration in HIV-1-seropositive patients, involving the ability of p17 to form soluble toxic assemblies and put forward innovative hints for the development of novel pharmacological strategies.

Aggregation and dis-aggregation process of α-synuclein in the presence of Oleuropein aglycone

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The presence of proteinaceous intracellular aggregates, Lewy bodies and Lewy neurites, is one of the histopathological features of Parkinson’s disease (PD). These aggregates are mainly constituted by α-synuclein (Syn) in fibrillar form. Syn is a presynaptic protein, highly expressed in the central nervous system, natively unfolded. Its aggregation from monomer into amyloid fibrils through oligomeric intermediates is supposed to be the toxic disease-causative mechanism in PD. These intermediates, soluble oligomers, are generally considered as the principal toxic species that mediate cellular homeostasis disruption and neuronal death.

Current pharmacological treatment for PD involves the use of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa), dopamine receptor agonists (such as bromocriptine), and monoamine oxidase B inhibitors (such as deprenyl). They alleviate only some symptoms (bradykinesia, tremor), by restoring dopamine levels in the basal ganglia, but none of the currently available treatments have been proved to slow the progression of PD. A promising strategy would involve the use of small molecules with the potential to alter the conformation of oligomeric forms of Syn and render them non-pathogenic.

Here, we show that oleuropein aglycone (OleA), an olive-derived polyphenol, exhibit anti-amyloidogenic activity, interacting with Syn monomers and interfering with Syn oligomers formation, *in vitro*. This capacity was determined by following the aggregation process by Thioflavine T and ANS binding assay and electron microscopy (TEM) examination of the amyloid fibrils spontaneously formed. The interaction between OleA and Syn was analyzed by several chemical-physical techniques (gel filtration, CD, limited proteolysis and cross-linking experiments). Moreover, the presence of OleA reduces the cytotoxicity of Syn aggregated species hindering their binding to cell membrane components and preventing the resulting cellular damages as an increased ROS production.

The main finding is that OleA stabilizes monomeric form of Syn and induces the formation of soluble non-toxic Syn aggregates that do not evolve into other type of structures. OleA was shown to exert similar effect *in vitro* and *in vivo* also with amyloid fibril formation by other proteins/peptides, avoiding the growth of toxic oligomers and displaying protection against cognitive deterioration. Taken together, these data suggest a possible therapeutic use of this molecule to treat and to stem amyloid-related pathologies.

Oleuropein Aglycone and its metabolite Hydroxytyrosol: two different ways against toxic amyloid formation in Alzheimer’s disease

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The pathological features of systemic amyloidosis are based on the concurrent presence of amyloid deposits and circulating amyloidogenic precursors. Aβ1-42 aggregation is a multistep step, where monomers assemble to form oligomers, protofibrils, and eventually mature fibrils. Drug discovery efforts are focused on preventing the formation of toxic oligomers or fibrils and/or at favoring their disaggregation. However, there are limited studies exploring at the molecular level how drugs bind to Aβ1-42 and prevent fibril formation and toxicity. Dietary polyphenols such as resveratrol, curcumin, and myricetin have shown anti- Aβ aggregation properties. Attention has been paid to the main phenolic compounds found in extra-virgin olive oil, 3,4-dihydroxyphenylethanol (hydroxytyrosol, HyT) and oleuropein aglycone (OleA) as new tools to combat Alzheimer’s disease (AD). Recent data show that OleA interferes with the aggregation path of some proteins including amylin. APP, tau and transthyretin skipping the growth of toxic oligomers both in vitro, in C. elegans and in TgCRND8 mice, a model of Ab deposition. In particular, OleA administered to Tg mice with diet was found in mouse brain as its main metabolite, HyT. However, the few mechanistic data presently available on the HyT-AD relation, concern only its strong antioxidant power. In order to elucidate the molecular and cellular determinants of the protection by, OleA and its main metabolite, HyT, against protein aggregation and/or aggregate cytotoxicity a set of in vitro experiments were performed using biophysical analysis and cell biology techniques. Our preliminary results highlight a modulation by OleA and HyT of the molecular mechanism of Aβ aggregation; in particular, HyT was found to be able to accelerate Aβ aggregation skipping the appearance of toxic oligomers. Our data will offer the possibility to validate and optimize the use of OleA and/or HyT to rationally design novel and promising drugs for AD prevention and therapy.

Understanding the frustration arising from the competition between function, misfolding and aggregation in a globular protein

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Folding and function impose different requirements on the amino acid sequences of proteins, thus potentially giving rise to conflict. Such conflict, or frustration, could potentially result in the formation of partially misfolded intermediates that can compromise folding and promote aggregation. We investigated this phenomenon by studying frataxin, a protein whose normal function is to facilitate the formation of iron-sulfur clusters, but whose mutations are associated with Friedreich’s ataxia. I will present the folding pathway of this protein as probed by Φ–value analysis and restrained molecular dynamics simulations. The analysis of the transition state structure reveals that the regions that are critical for folding are highly aggregation prone. By contrast, the regions that are functionally important are partially misfolded in the transition state but highly resistant to aggregation. Taken together, these results indicate that in frataxin the competition between folding and function creates the possibility of misfolding, and that in order to prevent aggregation the amino acid sequence of this protein has evolved to be highly resistant to aggregation from the partially misfolded states populated along its folding pathway.

Inhibition of mechano-enzymatic transthyretin amyloidogenesis by monovalent and bivalent ligands: just a matter of binding affinity and cooperativity?

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Dissociation of the native transthyretin (TTR) tetramer is widely accepted as the critical step in TTR amyloid fibrillogenesis. It is modelled by exposure of the protein to non-physiological low pH *in vitro* and is inhibited by small molecule compounds, such as the drug tafamidis. We have recently identified a new mechano-enzymatic pathway of TTR fibrillogenesis *in vitro*, catalysed by selective proteolytic cleavage, which produces a high yield of genuine amyloid fibrils. This pathway is efficiently inhibited only by ligands that occupy both binding sites in TTR. Tolcapone, which is bound with similar high affinity in both TTR binding sites without the usual negative cooperativity, is therefore of interest. Here we show that TTR fibrillogenesis by the mechano-enzymatic pathway is indeed more potently inhibited by tolcapone than by tafamidis but neither, even in large molar excess, completely prevents amyloid fibril formation. In contrast, mds84, the prototype of our previously reported bivalent ligand TTR ‘superstabiliser’ family, is notably more potent than the monovalent ligands and we show here that this apparently reflects the critical additional interactions of its linker within the TTR central channel. Our findings have major implications for therapeutic approaches in TTR amyloidosis.

Structural determination of toxic and nontoxic HypF-N oligomers

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Oligomers formed by the N-terminal domain of *E. coli* HypF (HypF-N) are considered an important model for investigating the structure of misfolded protein oligomers involved in neurodegenerative disorders, particularly Alzheimer’s disease. Indeed, such oligomers have morphological, structural and tinctorial features similar to those formed by proteins involved in diseases and impair cell viability in neuronal cells and neurons both *in vitro* and *in vivo*. Two types of stable HypF-N oligomers were previously formed *in vitro*, showing similar morphological and tinctorial features, but structurally disclosing different degrees of packing within their cores and different toxicities.

In this work we attempted to characterise the toxic type A and nontoxic type B oligomers of HypF-N in detail using solid-state NMR and site-directed fluorophore-labelling coupled to fluorescence resonance energy transfer (FRET) to report on intermolecular distances between 12 different positions within the two oligomer types. The aim of the work is to obtain information on the structure and dynamics of the two oligomeric forms at the level of individual residues and interactions and identify the specific structural elements and sites responsible for their difference of toxicity.

Concurrent structural and biophysical traits link with immunoglobulin light chains amyloid propensity

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Light chain amyloidosis (AL), the most common systemic amyloidosis, is caused by the overproduction and the aggregation of monoclonal immunoglobulin light chains (LC) in target organs. Due to genetic rearrangement and somatic hypermutation, virtually, each AL patient presents a different amyloidogenic LC. Because of this complexity the fine molecular determinants of LC aggregation propensity and proteotoxicity are, to date, unclear; significantly, their decoding requires investigating large sets of cases. Aiming to achieve generalizable observations, we systematically characterised a pool of thirteen sequence-diverse full length LCs. Eight amyloidogenic LCs were selected as responsible for severe cardiac symptoms in patients; five non-amyloidogenic LCs were isolated from patients affected by multiple myeloma. The comprehensive approach (consisting of spectroscopic techniques, limited proteolysis, and X-ray crystallography) showed that low fold stability and high protein dynamics correlate with amyloidogenic LCs, while hydrophobicity, structural rearrangements and LC dimeric association interface (as observed in seven reported crystal structures here presented) do not appear to play a major role in defining amyloid propensity. Based on the structural data and on the biophysical characterizations, our results highlight the fundamental properties driving LC amyloid propensity, thus setting principles and building the bases for the design of synthetic inhibitors of LC aggregation.

Direct observation of calcium-dependent misfolding in single neuronal calcium sensor-1 molecules

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Neurodegenerative disorders are strongly linked to protein misfolding, and crucial to their explication is a detailed understanding of the underlying structural rearrangements and pathways that govern the formation of misfolded states. Here we use single-molecule optical tweezers to monitor the structural rearrangements leading to misfolded conformations of the human neuronal calcium sensor-1 (NCS-1), a protein linked to serious neurological disorders. By manipulating one NCS-1 molecule at a time, we directly observed competing pathways leading to either the native state or two distinct misfolded conformations. The relative probability of each pathway could be affected by modulating the relaxation rate of the applied force, demonstrating unprecedented real-time control over the energy landscape of a protein. Constant-force measurements in combination with hidden Markov model analysis reveal a complex kinetic network where the two distinctly misfolded conformations can be accessed only from an on-pathway intermediate state through either a downhill or barrier-limited transition, in a calcium dependent manner. Strikingly for a calcium sensor, higher Ca^{2+} concentrations increase the occupation probabilities of the two misfolded conformations and slow productive folding to the native state. We propose a rugged, multidimensional energy landscape for neuronal calcium sensor-1 and speculate on a direct link between protein misfolding and calcium dysregulation that could play a role in neurodegeneration.

Protein dynamics and aggregation

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Proteins aggregation is a ubiquitous phenomenon associated with the intrinsic properties of proteins polypeptide chains, nonetheless while in general all proteins can undergo a transition to the amylodogenic state, this transition is often associated with specific mutations.

To investigate the possible roles of mutations in monomers behaviour and their links with aggregation, we used a combination of NMR spectroscopy and molecular modelling to compare the native state dynamics of Beta-2 microglobulin (β2m), whose aggregation is associated with dialysis-related amyloidosis, its aggregation-resistant mutant W60G and a rare mutation D76N. The study suggests that together with the stability of the protein, the dynamic of the monomer plays a critical role in determining protein aggregation.

A maltose terminated dendrimer to prevent amyloid fibril formation induced by Copper ion: a computational study.

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Dendrimers are unique tree-like branched polymers with biomolecules-like properties, low polydispersity and high degree of versatility which emerged as a new class of structure with outstanding features for nanomedicine [1]. In recent years, dendrimers have been extensively studied for their potential application, including their ability to in vitro impair the aggregation of amyloid peptides related to Alzheimer disease [2,3]. The present work is focused on the computational characterization of the interaction between G4-maltose-terminated PPI dendrimers and the amyloid peptide $A\beta_{1-40}$. In detail, the effect of dendrimer structure on the peptide-peptide interaction triggered by copper ion was investigated by molecular dynamics techniques (Figure 1). Advanced computational simulations have been applied to estimate the free energy landscape of peptide-peptide interaction. Our results [4] have demonstrated that $A\beta_{1-40}$ binding to Copper ion promotes a notable increase of β sheet components, suggesting that Cu-binding to Aβ accelerates the protein aggregation. Moreover, results from enhanced sampling have revealed how PPI dendrimer is able to reduce the protein-protein interaction free energy, affecting the overall conformation of the Aβ dimer.

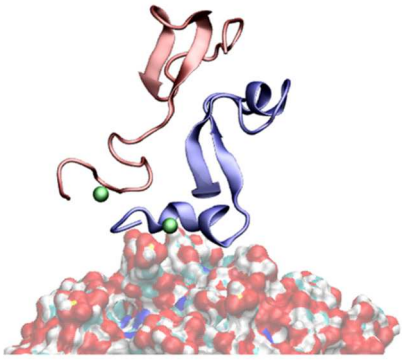

Figure 1. $A\beta_{1-40}$ peptides in presence of Copper ion interacting with G4 maltose-terminated PPI dendrimer.

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Autophagic removal of aggregating dipeptides produced in C9ORF72 related neurodegenerative diseases

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two neurodegenerative diseases characterized by partial clinical overlap. Similar pathogenic mechanisms are involved with the contribution of TAR DNA binding protein (TARDBP/TDP-43) and the fused in sarcoma gene (FUS). Recently, the discovery of the causative gene *C9ORF72* has clarified the ALS and FTD as a spectrum disorder. The 50% of ALS/FTD familial cases are linked to an expansion of the repeated G4C2 hexanucleotide sequence in *C9ORF72* gene. A loss-of-function mechanism in C9ORF72 ALS/FTD is suggested by the reduction of all three *C9ORF72* mRNA variants in patient tissue samples (haploinsufficiency). In parallel the gain of toxic function may be well described by repeated RNA mediated toxicity that sequesters RNA and protein in RNA foci and impairs nucleocytoplasmic trafficking. Moreover, although the G4C2 expansion is localized in an untranslated region of the *C9ORF72* transcript, it drives an unconventional ATG independent translation, known as RAN translation. That leads to the synthesis of five different di-peptide repeat proteins (DPRs), which are not "classical" misfolded proteins. DPRs as misfolded protein may be processed by protein quality control (PQC) system to prevent their aggregation and toxicity by enhancing their degradation via proteasome and/or autophagy.

We have developed a stable transfected and inducible SH-SY5Y cell line for each DPR. We compare the biochemical behaviour of the five DPRs. We observe that low expression level of stable transfection is insufficient to reach a detectable protein level in western blot or in filter retardation assay. Nevertheless, 5 days after induction we observe an increase of 4-8% of dead cells in each line. In order to enhance the DPRs protein level, we deeply evaluated the biochemical behaviour of the five DPRs in immortalized motoneurons transiently transfected with DPRs. In NSC34 cells we found that although the DPRs are mainly processed via autophagy, this system is unable to fully clear their aggregated forms, which tend to form PBS insoluble aggregates in basal condition. We analysed the role of the small heat shock protein HSPB8, a protective protein that reduces a large variety of classical misfolded aggregation-prone proteins. We observed that HSPB8 overexpression significantly decreased the accumulation of most DPRs insoluble species while HSPB8 silencing increase the level of PBS insoluble DPRs. Thus, the induction of HSPB8 might represent a valid approach to prevent DPR-mediated toxicity and improve motoneuron viability.

Control of microglial activation and autophagic response as possible strategy to counteract the neurotoxicity induced by amyloidogenic prion protein fragment PrP90-231

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Causal role of prion protein scrapie (PrP^{Sc}) in brain spongiosis is a crucial and debated question to control neurodegeneration associated with prion diseases. We focused our study to characterize cellular signalling that mediates the neurotoxicity of amyloidogenic prion protein fragment PrP90-231 in neurons. In particular, we examined the possibility to unravel the heterogeneous nature of PrP90-231 neurotoxicity, dissecting direct from glia-mediated mechanisms. As neuronal models, we employed primary cultures of cerebellar granule cells (CGCs), containing different amounts of glial cells, and the immortalized mesencephalic cell line A1 whose sensitivity to PrP90-231 can be modulated by stimulating autophagic process. We observed that PrP90-231 elicited CGCs death and produces sustained increase of [Ca++]i in a glia-dependent manner, suggesting that type I astrocytes and microglia, could increase the sensitivity of granule neurons to PrP90-231 or be stimulated to produce a oxidant neurotoxic *milieu.* Noteworthy, glia-enriched cultures showed upregulation of phagocytic NADPH oxidase (PHOX) and inducible nitric oxide synthase (iNOS); moreover, the inhibition of iNOS and PHOX prevented PrP90-231 neurotoxicity in glia-enriched cerebellar granule cultures only, whereas NMDA blockade by AP-V proved protective only in “pure” cerebellar granule cultures. These data indicate that PrP90-231 toxicity, *in vitro*, could involve a direct excitotoxic-like mechanisms on neurons and an indirect pathway mediated by microglial release of free radical species that predominates when glial contamination overcomes a threshold.

In addition, we have observed that PrP90-231 neurotoxicity proceeds through its intracellular accumulation as protease-resistant aggregates that destabilize lysosomal impermeability and lead to the cytosolic release of lysosomal proteases. Pharmacological enhancement of autophagy increases A1 resistance to PrP90-231 internalization suggesting that autophagic defensive response in A1 cells could be mediated by increased degradation of PrP90-231 or by removal of damaged cytoplasmic structures.

Application of a new method of amyloid fibrils extraction and SDS-PAGE/Western Blot proteomic analysis for the diagnosis of cardiac amyloidosis

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Aim of this study was to evaluate the role of SDS-Page/Western Blot technique for amyloid proteins detection and characterization on cardiac tissues. We first set up a new protocol of amyloid fibrils extraction from cardiac tissue, which not involved protein purification and buffer changing before SDS-PAGE and mass spectrometry procedures. We considered 20 frozen, 20 FFPE endomyocardial biopsies amyloid positive and 20 cardiac samples amyloid negative as control group. Different conditions of temperature, pH, buffer composition and additives were tested in all samples during extraction procedure. SDS-PAGE/Western Blot (WB) analysis was performed for the following proteins: Transthyretin (TTR), lambda and kappa-light chain (λ or k), amyloid-A, Apolipoprotein AIV(APO-AIV) and APO-E. SDS-PAGE/WB results were compared to those of mass spectrometry (MS) technique and immune-electron microscopy (IEM). To validate the method in a diagnostic setting, we examined from August 2014 to April 2016, 37 consecutive samples (29 endomyocardial biopsies (BEM) and 8-autopsy heart) with suspected amyloidosis.

We found that the proteomic extraction of 34/37 (91.9%) samples showed agreement data of IEM. We identified AL- λ fibrils in 13/34 (38.23%), AL-k fibrils in 8/34 (23.52%) and TTR fibrils in 8/34 (23.52%). Five out of 34 (14.7%) samples were negative. Proteomic analysis showed discordant results compared to IEM in three cases: one case identified positivity for TTR protein while EM showed AL-k positivity; in the other two cases a double component (TTR+/light chain+ and TTR+/APO-AIV+) were identified. Data were confirmed by MS. Moreover, in three samples, WB showed TTR-electrophoresis double band, in keeping with the presence of mutation or post-translational TTR-protein modification. MS confirmed the presence of post-translational TTR/Cys10-Sulfonate modification. Time required for proteomic analysis ranged from 5 to 7 days.

The new microextraction methods combined with SDS-PAGE/WB proteomic analysis is a reliable tool for amyloid proteins detection and characterization, and should be applied in clinical setting with cost and time-effective improvement. It is the only technique that could identify the presence of amyloid proteins post-translational modification and of the double protein components.

Metal ions drive oxidative stress and mitochondrial damage in cardiac light chain amyloidosis

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The knowledge of the mechanism underlying the cardiac damage in immunoglobulin light chain (LC) amyloidosis (AL) is essential to develop novel therapies and improve patients’ outcome. Although an active role of radical oxygen species (ROS) in LC-induced cardiotoxicity has already been envisaged, the actual mechanisms behind their generation remain elusive.

This study was aimed at further dissecting the action of ROS generated by cardiotoxic LC *in vivo* and investigating whether transition metal ions are involved in this process. In absence of reliable vertebrate model of AL we employed the nematode *Caenorhabdistis elegans,* whose pharynx is an “ancestral heart”.

LC purified from patients with severe cardiac involvement intrinsically generated high levels of ROS and, when administered to *C. elegans* induced ROS production, activation of the DAF-16/FOXO pathway and expression of proteins involved in stress resistance and survival. Profound functional and structural ROS-mediated mitochondrial damage, similar to that observed in amyloid-affected hearts from AL patients, was observed. All these effects were entirely dependent on the presence of metal ions since addition of metal chelator or metal-binding 8-hydroxyquinoline compounds (Chelex, PBT2 and clioquinol) permanently blocked the ROS production and prevented the cardiotoxic effects of amyloid LC.

Our findings identify the key role of metal ions in driving the ROS-mediated toxic effects of LC. This is a novel conceptual advance which paves the way for new pharmacological strategies aimed at not only counteracting but totally inhibiting the vicious cycle of redox damage.

Drug repositioning for the therapy of transthyretin amyloidoses: anti-amylodogenic potential of CSP-1103

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Wild type transthyretin (TTR) and its pathologically more aggressive amyloidogenic mutant forms represent relevant examples of amyloidogenic proteins. A large number of synthetic ligands possess the ability to kinetically stabilize the native state of TTR, inhibiting its in vitro, and presumably in vivo, pathological aggregation. CSP-1103 (formerly, CHF5074), a chlorinated derivative of the NSAID (nonsteroidal anti-inflammatory drug) flurbiprofen, is a brain penetrating compound developed by Chiesi Farmaceutici and currently studied by Cerespir as a drug effective for the therapy of Alzheimer’s Disease, owing to its protective role on neurons. We report on an in vitro and ex vivo comparative study to evaluate the antiamyloidogenic potential of CSP-1103, in prospect of its use as a drug effective in TTR amyloidoses. We have found that CSP-1103 is also a potent TTR kinetic stabilizer in comparison with other well established inhibitors of TTR amyloidogenesis, such as tafamidis and diflunisal, suggesting that it is a promising candidate for the therapy of TTR amyloidoses.

Methacycline displays a strong efficacy in reducing *in vitro* ataxin-3 aggregation and toxicity in a SCA3 *Caenorhabditis elegans* model

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Ataxin-3 (ATX3) is a protein endowed with deubiquitylating activity that triggers the inherited neurodegenerative disorder spinocerebellar ataxia type 3 (SCA3) when its polyglutamine (polyQ) stretch close to the C-terminus exceeds a critical length [1]. The expansion results in misfolding and other structural rearrangements in the protein product, which leads to aberrant interactions of the expanded protein and to the consequent formation of fibrillar amyloid-like aggregates [2]. To date, no effective treatment has been developed for SCA3. We have recently demonstrated that tetracycline and epigallocatechin-3-gallate (EGCG), two well-known inhibitors of amyloid aggregation, differently affect AT3 fibrillogenesis [3]. Here, we have screened a small library of tetracyclines using Thioflavin T and solubility assays, which led us to identify methacycline as the most effective compound of the family against ATX3 aggregation. We performed the assays on the N-terminal Josephin Domain (JD) that is directly involved in the onset ATX3 fibrillation [4]. As previously observed for tetracycline, methacycline did not change the aggregation kinetics nor did it affect the secondary structures of the final aggregates, as supported by Fourier Transform Infrared spectroscopy, but increased the solubility of the aggregated species. Furthermore, STD NMR spectroscopy analyses clearly demonstrated the capability of methacycline to bind only the oligomeric species of JD. Competition assays performed by incubating JD with either methacycline or tetracycline showed that the former binds to JD more tightly than the latter, thus providing a possible rationale for its stronger effect. The pharmacological efficacy of methacycline was evaluated on a SCA3 *C. elegans* model, whereby the treatment induced a significant increase in mobility and improvement in locomotion in the diseased worms without extending the lifespan. Methacycline was also proven to be more effective than tetracycline in the animal model. Noteworthy, unlike tetracycline methacycline was able to ameliorate the motility of even the healthy nematodes, probably contrasting the aging effect. Finally, the absence of toxic effects by methacycline makes it a possible candidate for a chronical treatment of the disease.

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Characterization of amyloid aggregate surface charge by Zeta potential measurements

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Determining the charge of amyloid aggregates can be relevant to elucidate their interactions with other molecules in the surroundings, including amyloid markers. The state of charge of a biological surface in solution depends both on the degree of dissociation of anionic and cationic surface groups and on the adsorption of counterions, and it is related to the surface electric potential. The latter can be determined experimentally as Zeta potential from measurements of the aggregate electrophoretic mobility by phase analysis light scattering.

We focused our attention on the aggregates formed by three amyloidogenic proteins with different isoelectric points: the S52P variant of transthyretin (S52P TTR), the D76N variant of β_2-microglobulin and hen egg white lysozyme. Amyloid fibrils were obtained under conditions mimicking the physio-pathological ones; aggregate morphology was checked by atomic force microscopy. We measured the Zeta potential of the aggregates and we monitored the changes induced by the addition of different concentrations of salts in solution. In particular, we studied the interaction of S52P TTR aggregates with calcium ions and we found that the addition of $CaCl_2$ promotes a progressive increase of Zeta potential values.

Our results demonstrate that this technique is able to discriminate the difference of charge between aggregates obtained from different proteins. Moreover, Zeta potential values can be modulated by the addition of electrolytes in solution.