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# Crossing research borders to combat protein aggregation disorders

**S. J. Roeters[1], A. Sapmaz[2], R. A. M. Buijsen[3], Patrick C. A. van der Wel[4] and V. Raz[3,*]**

[1]Department of Anatomy and Neurosciences, Vrije Universiteit, De Boelelaan 1117 1081 HV, Amsterdam, The Netherlands; [2]Department of Cell and Chemical Biology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands; [3]Department of Human Genetics, Leiden University Medical Center, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands; [4]Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands.

## ABSTRACT

Protein aggregation disorders (PADs) are characterized by accumulation of misfolded proteins leading to cell dysfunction and tissue degeneration. A network of cellular processes impacts cellular proteostasis, including protein synthesis, folding, and breakdown. Pathogenic protein aggregates, the hallmark of a variety of adult-onset diseases, can be caused by genetic mutations that increase a protein's aggregation propensity, in combination with proteostasis-associated cellular mechanisms. Protein aggregates can be found across all tissues and cell types, but pathogenic protein aggregates are most often found in post-mitotic cells of the neuromuscular system. Except for treatment strategies that aid in improving the quality of life, currently, no curative treatments are available for many protein aggregation disorders. Our as-yet insufficient understanding of the processes leading to protein aggregation impedes the development of innovative treatment strategies. A big challenge in such research is that a full understanding requires in-depth studies from the organismal, cellular, molecular, to atomic level. Thus, we need multidisciplinary research of protein aggregation in humans and in disease-relevant models, to open novel avenues for therapeutical development and to accelerate translational research. Here, we advocate for multidisciplinary networks that cross disease-specific borders and discuss the requirements for collaboration and communication across traditional research niches. Ultimately, this will advance our understanding, diagnosis, and treatment of these debilitating protein aggregation diseases.

**KEYWORDS:** multidisciplinary research networks, disease models, protein aggregation structure, disease mechanisms, therapy development.

## INTRODUCTION

The class of protein-aggregation disorders (PADs), which encompasses more than 50 disorders, can be divided into heritable disorders, driven by known genetics, and sporadic disorders, without known genetics. In both subclasses the cellular mechanisms leading to protein aggregate formation are controlled by (dys)regulation of protein homeostasis (proteostasis). Aggregates formed by misfolded proteins are associated with these diseases as a hallmark of PAD pathology. The aggregated proteins can themselves be toxic to cell function (gain-of-function) or contribute to disease mechanisms by depleting the levels of vital proteins to cell function (loss-of-function). Either way, pathogenic protein aggregates lead to cell dysfunction and tissue degeneration.

*Corresponding author: v.raz@lumc.nl

Protein aggregation is predominantly implicated in neurodegenerative and neuromuscular diseases (NDDs and NMDs, respectively), which emerge with age and often progress over years. Many neurodegenerative disorders are accompanied by pathological accumulation of abnormal protein aggregates in the brain. Examples are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), spinocerebellar ataxias (SCA), dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and frontotemporal dementia (FTD) [1, 2]. Other PADs affect the neuromuscular system, where protein aggregates are found in neuromotors, such as amyotrophic lateral sclerosis (ALS) or X-linked spinobulbar muscular atrophy (SBMA; Kennedy's disease) [3]. PADs also affect skeletal muscles, with examples such as hereditary and sporadic inclusion body myopathy (hIBM and sIBM) and oculopharyngeal muscular dystrophy (OPMD) [4]. Intracellular aggregates can accumulate in the cytoplasm, as in AD, PD, ALS, and h/sIBM, in the cell nucleus as in OPMD, or in both subcellular compartments (HD). The incident percentage of NDDs and NMDs ranges from common such as AD and PD (>10% or 1% in the age group over 60 years-old, respectively), to rare such as ALS (prevalence 4-6:100,000) or highly rare such as OPMD (prevalence 1:100,000).

Despite the diversity in disease prevalence, the tissue-specific accumulation of aggregated proteins, and the degenerated cell and tissue types, in most PADs the onset of symptoms is age-associated. The hereditable PADs are characterized by two stages: at the pre-symptomatic stage carries of the mutated gene do not show clinical symptoms, and at the later stage (symptomatic stage) clinical symptoms are presented and symptom severity progresses with age. The age-association of disease onset and progression emphasize a crucial impact of ageing-related regulators in disease mechanisms [5, 6]. Some of the PADs are fatal and lead to premature death, while others affect quality of life and limit daily functioning [7]. Currently, there are limited medical options to treat PADs. Therapeutic developments are concomitant with biomarkers.

Additionally, natural history studies in patients are essential for understanding disease progression and tailoring intervention strategies. Without safe therapies there is no prospect for this group of patients for normal life and full participation in society. As such, there is an urgent need to engage in fundamental and translational research that will enable novel therapeutical developments [8].

## Models – overexpression in contrast with physiological conditions

A crucial challenge in protein aggregation research is the establishment and development of proper cellular and animal models. Models generated by high overexpression of the mutant gene or part of the pathogenic genes were instrumental to demonstrate the formation of aggregates and an association with cell or tissue phenotype. However, aggregation due to high overexpression may not represent physiological expression levels and may not mimic the disease condition. Modeling PADs with physiologically relevant expression levels and expression of the full-length gene is desirable. Incorporating only the disease-specific mutation is crucial for pinpointing its direct impact on the phenotype. This method isolates the mutation's effects, providing clearer insights into its role in the disease process.

Overexpression of genes encoding for proteins prone to aggregation is not limited to cell-type, but *in vivo* pathogenic protein aggregates are mostly manifested in post-mitotic cells, such as neuronal cells or fused muscle cells. Recent studies demonstrated that the landscape of RNAs and proteins are impacted during cell differentiation. In the context of protein aggregates, protein-protein interactions and protein homeostasis networks are influenced by differentiation [9, 10]. Although there are common molecular regulators of proteostasis [11], cell-specific and differentiation-associated molecular regulators could trigger or suppress the aggregation of proteins in a cell-specific context. Such considerations highlight the importance of studying protein aggregation in disease-relevant cells models that appropriately mimic disease conditions. Since modelling aggregation in cells often requires overexpression, comparing overexpression of the wild-type gene to the mutant gene in a disease-relevant cell type can be a valuable approach to shed light on molecular

and functional differences between pathogenic and non-pathogenic aggregates [12, 13].

Patient-derived induced pluripotent stem cells (iPSCs) are another valuable tool for disease modeling. They enable the exploration of disease mechanisms, discovery of new drugs, and development of personalized-medicine approaches. Conflicting data on protein aggregation in iPSC-derived models highlight the complexity of accurately replicating disease pathologies, underscoring challenges in standardization and interpretation [14-18].

Finally, studies in invertebrate models often assist in demonstrating the effect of the aggregated protein in an organism context, but differences in tissue architecture and cellular complexity between simple organisms and human must be considered. Lower animals may not represent the differentiation complexity as in human brain or may not have skeletal muscles, lacking multinucleated cells as in vertebrates [19]. Additional challenges include the choice of a tissue-specific promoter, codon optimalization, transcript stabilization and more; all could affect expression level, aggregation, and symptoms.

Researchers should be mindful of the advantages and limitations of their models. Disease-relevant and cell-type-specific cell and animal models can illuminate cell-type-specific aggregation processes and mechanisms, thereby facilitating the development of targeted therapeutics.

**Protein aggregation associated molecular mechanisms**

Proteostasis is strongly impaired in PADs, leading to exacerbated protein deposition and the formation of insoluble aggregates. Conversely, age-associated proteostasis decline is accelerated in neuromuscular disorders that are associated with protein aggregation. Proteostasis aims to maintain the delicate balance between protein synthesis and protein degradation.

Protein synthesis efficiency declines with age [20], and is closely related to the availability of transcription factors and RNA to the translational machinery [21]. Evidently, translation efficiency is impacted by the aggregation-prone RNA binding proteins, such as PABPN1 [22]. In general, a functional protein has a defined three-dimensional structure, the so-called “native” structure, which requires proper folding of the newly synthesized polypeptide. Other proteins (known as intrinsically disordered proteins, or IDPs) lack such a folded state, but are nonetheless able to function in their IDP state. The first line in protein folding control is mediated by chaperones that effectively correct protein folding and thus reduce or prevent protein aggregation.

Under environmental stress conditions, proteins may partially unfold or misfold to generate aggregation-prone conformers [5], which chaperones can detect and try to disaggregate. Over the years, the neuroprotective properties of chaperones have been well-established and modulation of molecular chaperones has been indicated to eliminate the proteotoxicity of protein aggregates related to AD, PD, ALS, HD, and more [23]. A complex meshwork of refolding machineries and clearance processes is key to maintaining protein homeostasis. The ubiquitin-proteasome and endolysosomal systems are key components of the clearance process, the degradative functions of which are highly regulated by (de)ubiquitinating enzymes [24]. For example, the proteosome-associated de-ubiquitinating enzyme USP14 is linked to the accumulation of multiple disease-associated protein aggregations, including Tau, Huntingtin, and TDP43 [25], and its inhibition has been shown to facilitate the degradation of Tau protein in murine embryonic fibroblasts and primary neuronal cultures [26, 27], while the membrane-associated Nedd4 E3 ligase plays a significant role in the clearance of α-Synuclein *via* endolysosomal system [28]. Autophagy is a dynamic process that degrades large protein aggregates and damaged organelles by directing them to lysosomal degradation. Loss-of-function mutations in genes regulating the autophagy-lysosomal degradation pathway can cause a decrease in the degradation capacity of autophagy and are linked to many neurological disorders [29, 30]. Due to the complexity of the PAD-associated pathway network and challenges in therapeutic approaches, as highlighted below, current medical options to arrest or slow-down neurological or neuromuscular disorders are limited.

Therefore, in addition to targeting genetic factors, targeting disease-modifiers involved in common

molecular mechanisms in PADs for therapeutic approach will likely be an important therapeutic route against PADs in the future [31, 32]. Those urges for collaborative and communicative networks between multidisciplinary fields of expertise.

**Understanding protein aggregation from a structural perspective**

Unraveling the structure of protein aggregates is critical to understand aggregation and its pathogenesis. Structural knowledge at molecular and atomic-levels can be used to monitor aggregates *in vivo* for both diagnostics and drug-efficacy assessment. For instance, positron emission tomography (PET) imaging of protein aggregates is established in AD [33], and solid-state NMR spectroscopy (ssNMR) and cryogenic electron microscopy (cryo-EM) can be used to study amyloid-binding modes of drug candidates [34]. To understand how misfolding proteins contribute to disease, it is essential to understand the nature of the misfolded and aggregated state. In recent years there have been breakthrough discoveries that revealed atomic-level insights into various protein aggregates, enabled by cutting-edge techniques such as cryo-EM, ssNMR, and vibrational-spectroscopy techniques like two-dimensional infrared spectroscopy [35-38]. Excitingly, cryo-EM has even allowed the structure of protein fibrils from patients to be determined [37, 39]. In parallel, advances in microscopy combined with large-scale imaging dataset microscopy [40], optical photothermal infrared (OPTIR) microscopy [41], AFM-IR [42], and cryogenic electron tomography (cryo-ET) [35] are starting to reveal the subcellular localization, molecular composition, dynamics (oligomeric *vs*. fibrillar aggregation species), and morphology of protein aggregates in cellular contexts. These techniques offer a complementary view of the protein inclusions: techniques like cryo-EM and ssNMR excel at providing atomic-resolution structures, but they do so for isolated or purified fibrils, while their counterparts lack atomic resolution but offer a real-time view of their cellular context.

Although these methods have already provided important structural insights, many PAD-associated proteins remain largely unstudied and consequentially poorly understood in their aggregated or pre-aggregated state. For example, the structure of nuclear aggregates is unknown, as well as the structures of protein aggregates in many rare and ultra-rare diseases.

It is now well established that aggregating proteins do not just form one type of aggregates but are actually capable of forming many different types of aggregated structures (typically referred to as *polymorphs* or *strains* [43]). Among fibril-like aggregates, the concept of amyloid polymorphism describes the ability of a single polypeptide chain to form a diversity of fibril types, depending on environmental context or conditions [44]. This implies that structures solved with purified proteins, may or may not resemble those found in cells or in patients. This idea has been long held in the amyloid literature but has found further support from cryo-EM analysis of patient-derived amyloids, as can for example be seen for the structures of alpha-synuclein aggregates, which are different for those extracted from multiple-system atrophy patient's brains [39], as compared to test-tube generated ones [45]. In examining the structure of protein aggregates, researchers frequently focus on a specific protein domain rather than the entire molecule. This targeted approach aids in identifying the roles of distinct domains in the aggregation process. However, focusing solely on a specific domain rather than the full protein can also impact the outcome of the study, potentially affecting the interpretation of how these aggregates form and function in a biological context. Studying proteins in a test tube is highly useful, allowing for controlled experiments that can dissect the intricacies of protein behavior and interactions. However, this approach may not fully replicate the complex environment found within living organisms, potentially missing key elements of the protein's natural context. Therefore, while *in vitro* studies provide valuable insights, they must be complemented with in *vivo* experiments to fully understand protein functions and aggregation mechanisms in a biological setting. Thus, one of the key goals and challenges for the field must be to combine structural analysis with cellular or organismal expertise to test and evaluate the best ways to reproduce

disease-relevant misfolding and aggregation under laboratory conditions. Such validated models will be critical to develop a better understanding of molecular mechanisms but also test treatments.

**Therapeutical approaches to protein aggregation**

Developing effective treatments for PADs requires a deep understanding of the underlying genetics and molecular pathways. A multidisciplinary approach facilitates the identification of potential genetics and modifier targets, the design of therapeutic strategies, and the evaluation of treatment outcomes. Potential treatment for these disorders can be divided into: (1) gene therapies that either target a specific mutation (DNA- and RNA-targeting therapies) or replace the mutated gene with a normal gene; (2) pharmacological therapies that aim to dissolve the aggregating proteins or target molecules that are proteostasis-associated; and (3) stem cell replacement therapies.

Gene therapies that specifically target a disease-causing gene have the advantage of correcting the disease at its root but will require a unique approach for every disease or genetic variant. The successful first clinical trial of CRISPR-Cas9 for treating sickle cell disease marked a groundbreaking advance in gene editing, demonstrating its potential to correct genetic defects directly within the human body [46]. However, challenges such as off-target effects, where CRISPR-Cas9 inadvertently edits regions of the genome other than the intended target, raise concerns about safety and specificity. Additionally, efficient delivery of the CRISPR-Cas9 system to the desired cells and tissues remains a significant hurdle, limiting its therapeutic application across PADs. The gene silencing and replacement approach using viral system for delivery is in clinical trials for OPMD treatment [47]. RNA-targeting therapies offer the advantage of intervening in disease processes by modulating gene expression without altering the DNA, providing a potentially reversible and highly specific treatment option. However, their delivery to specific cells or tissues without degradation and eliciting unintended immune responses or off-target effects poses significant challenges [48, 49].

There are many different strategies that can target toxic downstream effects. Monoclonal antibodies can be designed to target and clear aggregated proteins, harnessing the immune system to recognize and remove pathological aggregates, as seen in experimental treatments for AD [50]. Two anti-amyloid human monoclonal antibodies were controversially granted FDA approval after demonstrating a reduction in Aβ; however, the ability to halt cognitive decline in all AD patients is unclear, and adverse events are common [51]. Protein disaggregation using small-molecule intrabodies refers to the Lama-expressed VHH, which are small in size, stable and easy to express intracellularly and therefore are promising molecules for therapeutics [52]. Vectorizing the VHH to achieve tissue, cell, or subcellular localization is specifically attractive to ensure expression specificity and minimize side effects. VHH as disaggregated molecules have been reported in preclinical models [53]. The ability to vectorize VHH for cell-specific expression and subcellular localization places VHH as prospect therapeutics for protein aggregation disorders [54]. Small-molecule inhibitors can target specific steps in the aggregation process, such as preventing protein misfolding or interfering with the formation of toxic aggregates, offering potential therapeutic avenues for diseases like AD and HD [55, 56]. Chaperone-based therapies aim to enhance the activity of molecular chaperones, specialized proteins that facilitate proper folding and prevent aggregation of misfolded proteins. These approaches hold promise for treating various PADs by addressing the fundamental pathology of protein misfolding and aggregation [57]. Heat-shock proteins are a class of chaperone proteins that help cells cope with stress and promote protein folding. Inducing the expression or activity of HSPs through pharmacological agents can enhance cellular proteostasis, reducing the burden of misfolded proteins and mitigating the progression of PADs [58]. Protein-degradation enhancers promote the clearance of misfolded proteins by enhancing cellular degradation pathways, such as the ubiquitin-proteasome system or autophagy. By increasing the removal of toxic protein aggregates, these therapies hold potential for treating a range of PADs.

Cell replacement studies for PADs involve transplanting healthy, functional cells into affected tissues to replace damaged or dysfunctional cells. Stem cells, such as neural stem cells or iPSCs, are promising candidates for cell replacement therapies, as they can differentiate into various cell types, such as neurons. While still in early stages, these studies hold potential for restoring proper cellular function and alleviating symptoms associated with PADs [59]. However, challenges such as immune rejection, cell survival, and integration into existing neural networks need to be addressed for the success of these therapies.

Despite several human clinical trials being conducted, only limited therapies for PADs have attained market authorization yet. Overcoming challenges in translating preclinical findings into clinical trials, designing effective clinical trials, and addressing issues such as off-target effects and immunogenicity are crucial for future therapeutics targeting PADs to successfully reach the clinic.

Additionally, future research into biomarkers and natural history that can accurately detect changes in patients well before the onset of clinical symptoms will be crucial for initiating clinical trials at earlier disease stages, potentially increasing the likelihood of beneficial treatment effects when tissue degeneration is less advanced [60, 61]. Therapeutic developments are closely associated with biomarkers for diagnostics, prognosis, or prediction. Biomarkers can directly report the presence of the aggregates using

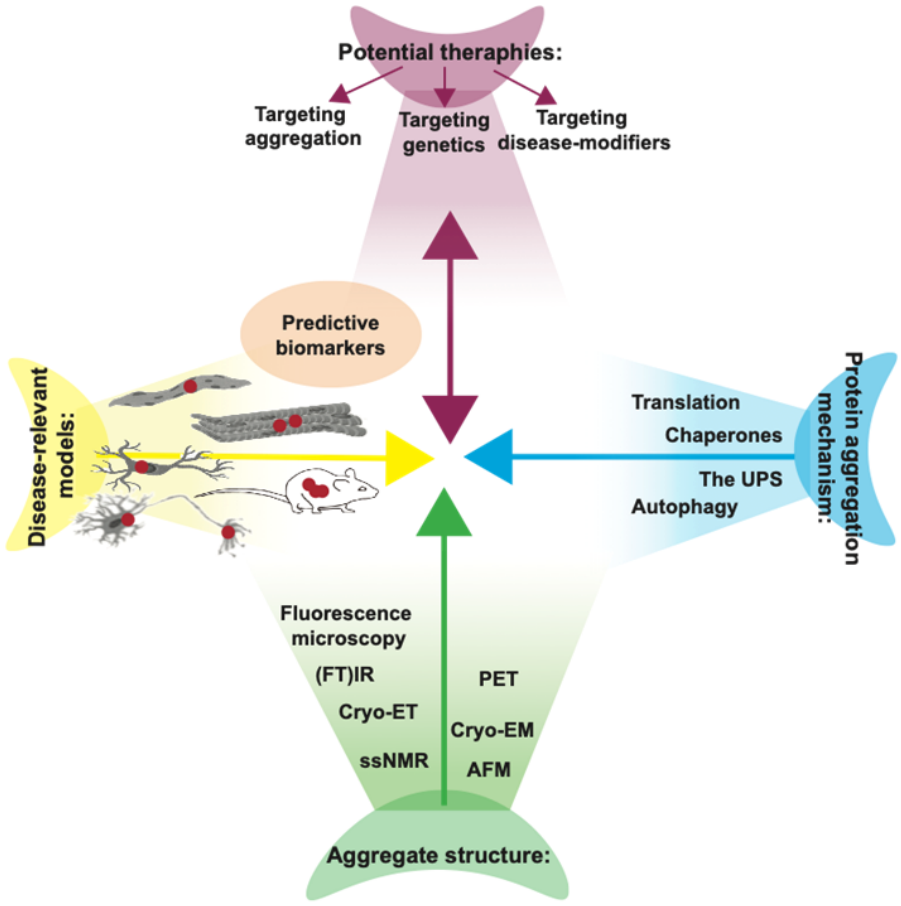


**Figure 1. A graphical summary of the multidisciplinary protein-aggregation research network approach**. Abbreviations: UPS = ubiquitin–proteasome system. (FT)IR = (Fourier-transform) infrared spectro-/microscopy, PET = positron emission tomography, cryo-ET = cryogenic electron tomography, cryo-EM = cryogenic electron microscopy, ssNMR= solid-state nuclear magnetic resonance, AFM = atomic force microscopy.

imaging or indirectly from biofluids, such as blood, plasma, cerebrospinal fluid, urine, or saliva [62, 63]. Circulating biomarkers are indirect reporters of proteins, metabolites, or small RNA molecules, and compared to imaging they are more sensitive and accurate. Considering the similarity between cellular mechanisms that drives protein aggregation across PADs, finding common biomarkers might provide a single diagnostic and/or prognostic platform and beneficial for the rare PADs. Combining common biomarkers with disease-specific biomarkers could be beneficial for disease variants.

To overcome the complex challenges listed here towards successful therapies, multidisciplinary research is essential.

## CONCLUSIONS

We advocate for a multidisciplinary protein aggregation research network encompassing complementary research fields that investigate cellular and animal models, protein-aggregate structure, molecular mechanisms, and therapy and biomarker development. The implication of protein aggregation fundamental research to the clinic and the patients can be gapped by an involvement of clinicians and the patient organization in the research network (Figure 1).

Key concepts behind such a protein aggregation research network are outlined here:

1. Protein-aggregation disorders are complex and multifaceted. Various proteins and cellular pathways may be implicated. They involve intricate interactions at molecular, cellular, and systemic levels. A multidisciplinary approach allows researchers to investigate these disorders from multiple angles.
2. Developing effective treatments for protein aggregation disorders requires a deep understanding of the underlying (structural) biology. A multidisciplinary approach facilitates the identification of potential drug targets, the design of therapeutic strategies, and the evaluation of treatment outcomes.
3. Most of the protein-aggregation disorders share similar underlying mechanisms. Such a forum can help share experiences between common and rare neurological disorders.

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## CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

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