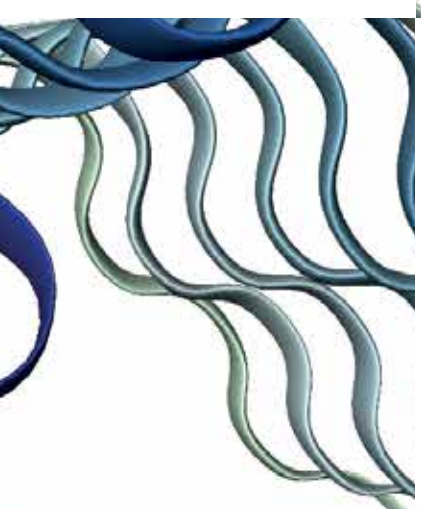
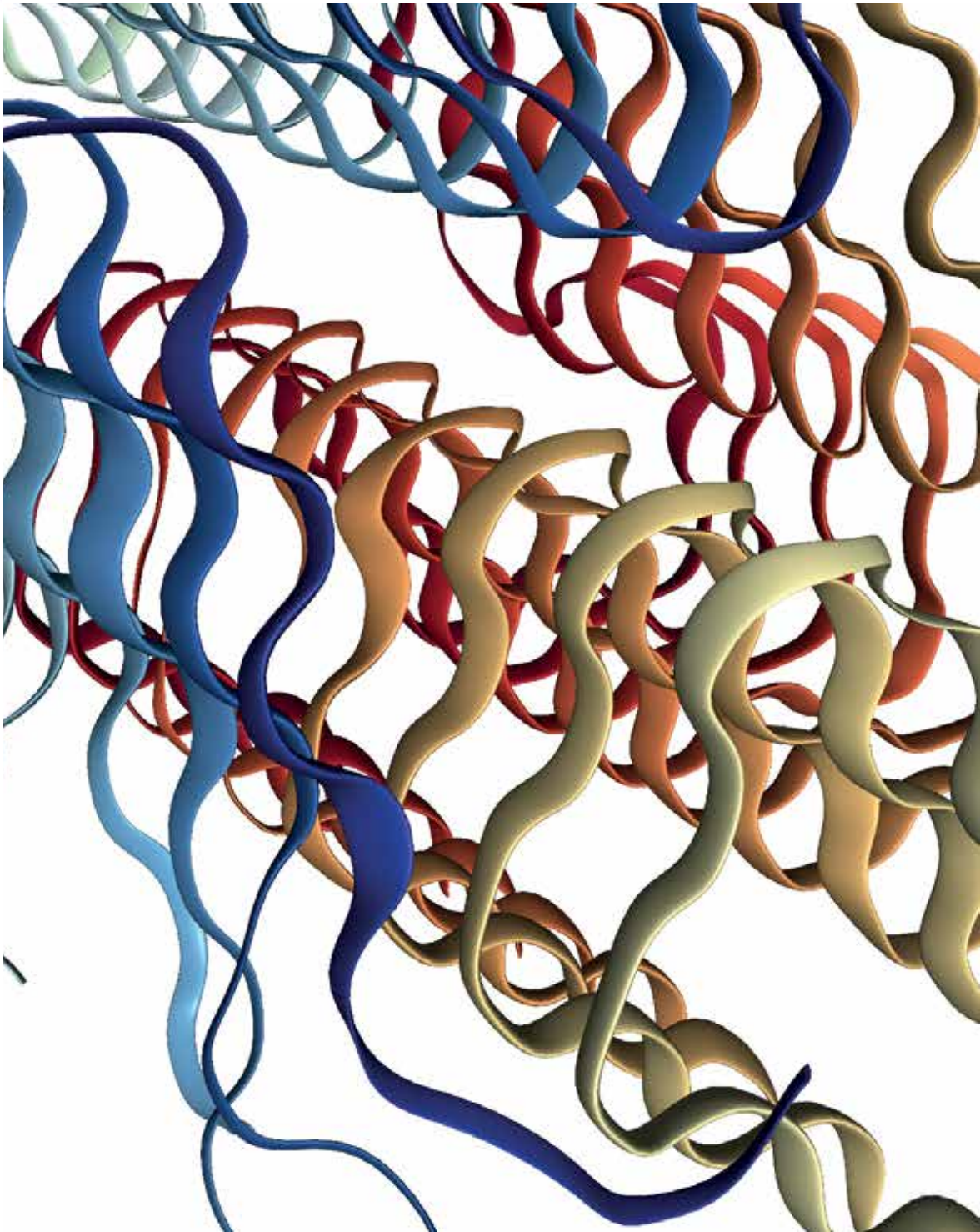


Aggregated proteins: are they infectious



CGM 2017-01

ONDERZOEKSRAPPORT

Aggregated proteins; Are they infectious?

COGEM onderzoeksrapport: CGM 2017-01

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Colophon:

Title	Aggregated proteins; Are they infectious?
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Date:	December 2016

Preface

In 2015 COGEM was asked for advice on the potential risks of exposure to so-called 'self-aggregating proteins', especially α-synuclein. Active researchers alerted the Dutch Ministry of Infrastructure and the Environment of a potential link between aggregation of certain proteins and the development of neurodegenerative diseases and that exposure and transmission thus may pose health hazards.

The best studied example of pathologic aggregating proteins are prions. Misfolding of these proteins causes transmissible fatal neural diseases (transmissible spongiform encephalopathies, TSEs), like Creutzfeldt-Jakob disease and kuru in humans, scrapie in sheep and goats, and BSE in cattle. Strict safety measures and rules are adopted in laboratories working with these proteins or infected materials.

In its advice COGEM concluded that a number of the proteins associated with degenerative diseases such as Parkinson or Alzheimer have properties that are partially similar to prions. They are native proteins that are not correctly folded, aggregate in the cell, and form 'seeds' which are capable of binding molecules of the same protein and inducing misfolding and aggregation. The aggregated proteins can spread to other cells but in contrast to prions had not been demonstrated to cause disease by natural transmission. Nevertheless, COGEM advised to take precautionary safety measures when working with α-synuclein, until more knowledge of properties of this protein was available.

In the aftermath of this advice, COGEM received three more requests for advice on permit applications dealing with α-synuclein and a request for advice on another aggregating protein, tau. In light of the scientific findings in recent years on the role of aggregating proteins in neurodegenerative diseases, the potential risks and possible need for safety measures when experimenting with these proteins and the increase in permit applications, COGEM commissioned a review of the scientific literature with focus on the protein aspects important for risk assessment and the need for safety measures.

The present report addresses the issues and discusses the potential prion-like properties such as self-propagation, infectivity, transmissibility and inactivation of a series of self-aggregating proteins potentially associated with neurodegenerative diseases. The authors selected eight proteins associated with human disease which are believed to 'self-propagate' and to spread to other cells. An extensive literature study and analysis of the obtained information was performed and the properties of these proteins are described in great detail.

The report offers a valuable overview and insight in the current knowledge of protein aggregation and pathology, possible infectivity and transmission of aggregating proteins, and the available inactivation procedures. The authors identify potential risks but also a serious lack of fundamental knowledge about this type of proteins that asks for more in-depth scientific research. The collected information is of interest to all who work with these types of proteins and is of great value to the COGEM as a knowledge base in the assessment of the potential risks of working with aggregating proteins and the need for precautionary safety measures.

Jos van Putten
Chair of the supervising committee

Summary

Background protein aggregation and prions

Abnormal protein aggregation is the process by which a protein misfolds due to sporadic, genetic or environmental factors. The protein adopts an aberrant conformation and causes its accumulation into oligomers, protofibrils and aggregates. These aggregates are called amyloid fibrils. During this process ''seeds'' or ''nuclei'' (=misfolded proteins) are formed. These seeds can accelerate conformational changes of normal proteins and the accumulation of other misfolded proteins resulting in the formation of aggregates. There are many proteins associated with abnormal protein aggregation that give rise to pathological conditions that are known as amyloidosis. There is one protein that is considered unique: prion protein. Prions are capable to transmit disease. The disease can be acquired through iatrogenic transmission (medical procedures) or by consuming bovine spongiform encephalopathy (BSE)-contaminated meat. Prion-related diseases, which are known as transmissible spongiform encephalopathies (TSEs), are fatal and treatments are currently not available.

Rationale for literature study on "aggregating proteins & infectivity"

Evidence from recent studies suggests that other neurodegenerative diseases (e.g. Parkinson's disease or Alzheimer's disease) can be developed in a similar prion-like mechanism by misfolded proteins that accumulate and induce misfolding of other proteins. The question rises if these suspicious prion-like proteins can cause transmission of disease. There are strict safety regulations when working with prions. If other aggregated proteins behave in a similar way, precaution measures are needed and inactivation and decontamination methods should be implemented.

Protein selection

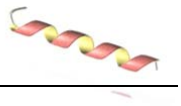
We performed a systematic literature study on the question: "Are aggregated proteins infectious?" The most prominent 'suspicious' proteins were included for review: α-synuclein, amyloid-β, tau, superoxide dismutase 1, huntingtin, TAR DNA-binding protein 43 (all involved in fatal neurodegenerative diseases), serum amyloid A and apolipoprotein A-II (involved in systemic amyloidosis).

Which protein characteristics were reviewed?

All selected proteins were reviewed in depth, resulting in a comprehensive overview on (i) protein function/biochemistry (incl. protein structure (native vs aggregated), disease association, and genetic mutations associated with disease), (ii) current knowledge on potential prion-like properties (i.e. seeding, cell-to-cell spreading, transmission routes), and (iii) current knowledge on inactivation/decontamination procedures.

Results from literature study

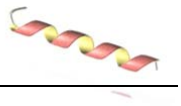
All eight proteins possess prion-like properties. They can self-propagate to form aggregates and are able to spread in experimental settings. Our analyses did not reveal shared biochemical properties (e.g. three-dimensional structure) that could be responsible for the protein's prion-like characteristics. We have classified each protein as low evidence, moderate evidence or high evidence on prion-like properties: α-synuclein: low/moderate; amyloid-β: moderate; tau: low/moderate;



superoxide dismutase 1: low/moderate; huntingtin: low; TAR DNA-binding protein 43: low, serum amyloid A: moderate/high, apolipoprotein A-II: low.
Scientific knowledge on inactivation/decontamination methods appeared limited; only eight manuscripts were identified in total, with results on α-synuclein, amyloid β, tau, serum amyloid A and apolipoprotein A-II. The publications were insufficient to propose appropriate measures, and more studies are definitely required.

Conclusion

We have identified several proteins that have been associated with aggregation and infectious properties. Although there is no solid evidence that these proteins are identical to prion proteins, being able to transmit disease between / to humans, the current knowledge raises concerns, at least for some of our identified proteins. Potential hazards may particularly occur in medical health care (e.g. disease transmission via blood transfusion or transplantation and exposure of medical personal to infectious material) and in the laboratory setting (e.g. when performing functional studies on a type of (mutated) protein). Further studies on suspicious prion-like proteins is recommended, to obtain better insights in the seeded aggregation/transmission properties and to validate methods for inactivation and decontamination.



Samenvatting

Achtergrond eiwitaggregatie en prionen

Abnormale aggregatie van eiwitten is een proces waarbij eiwitten verkeerd gevouwen worden door sporadische-, genetische- of omgevingsfactoren. Het eiwit neemt een afwijkende conformatie aan en accumuleert vervolgens als oligomeren, protofibrillen en aggregaten, genaamd amyloïde fibrillen. Tijdens dit proces worden ''zaadjes'' of ''kernen'' van verkeerd gevouwen eiwit gevormd. Deze zaadjes kunnen versneld voor misvouwing en ophoping zorgen van meerdere eiwitten, wat leidt tot een groeiend aggregaat. Er zijn veel eiwitten geassocieerd met abnormale eiwitaggregatie die leiden tot verschillende ziektes, ook wel amyloïdose genoemd. Er is een eiwit dat uniek wordt geacht voor eiwit-infectiviteit; het prion eiwit. Prionen kunnen ziekte overdragen door de overdracht van aggregerende eiwitten. Prion ziektes kunnen verkregen worden door iatrogene transmissie (tijdens medische handelingen) of door het eten van prion-besmet vlees. Prion-gerelateerde ziektes zijn fataal en er is geen behandeling beschikbaar.

Rationale voor literatuurstudie naar "Aggregerende eiwitten en infectiviteit"

Recentelijk wetenschappelijk onderzoek suggereert dat andere neurodegeneratieve ziektes (zoals de ziekte van Parkinson en de ziekte van Alzheimer) kunnen ontstaan op een prion-achtige wijze, ten gevolge van eiwitten die verkeerd vouwen en andere eiwitten aanzetten tot misvouwing en ophoping. Het is nu de vraag of deze eiwitten ook in staat zijn om niet alleen aggregatie, maar ook daadwerkelijk ziekte over te dragen. Er zijn strikte regels voor werkzaamheden met prionen. Als andere aggregerende eiwitten ook in staat zijn om ziekte over te dragen, dan zullen voor deze eiwitten ook voorzorgsmaatregelen en inactivatie/decontaminatie protocollen moeten worden opgesteld.

Eiwitselectie

Een systematische literatuurstudie is uitgevoerd omtrent de vraag: "Zijn aggregerende eiwitten infectieus?". De meest genoemde "verdachte eiwitten" zijn geselecteerd voor uitvoerig onderzoek: α-synucleïne, amyloid-β, tau, superoxide dismutase 1, huntingtin, TAR DNA-bindend eiwit 43 (allemaal geassocieerd met neurodegeneratieve ziektes), serum amyloid A and apolipoprotein A-II (geassocieerd met systemische amyloïdose).

Welke aspecten zijn uiteengezet in de literatuurstudie?

Voor alle geselecteerde eiwitten is een gedetailleerd overzicht verkregen omtrent (i) eiwitfunctie/biochemie (incl. eiwitvouwing (natuurlijk versus amyloïde), associaties met ziektes en ziekte-geassocieerde genmutaties), (ii) huidige kennis m.b.t. potentieel prion-achtige eigenschappen (eigenschap van "seeding", cel naar cel spreiding, transmissie), en (iii) huidige kennis m.b.t. inactivatie en decontaminatie procedures.

Resultaten van literatuurstudie

Alle acht eiwitten bezitten gedeeltelijk prion-achtige eigenschappen. Ze kunnen zelf-vermenigvuldigen en aggregaten vormen die in een experimentele setting verspreid kunnen worden. Onze analyse liet geen duidelijke correlaties zien tussen de verschillende eiwitten m.b.t. biochemische eigenschappen, zoals 3-dimensionale structuur en vouwingsmechanismen. N.a.v. onze studie hebben wij ieder eiwit geclassificeerd als weinig bewijs, aanzienlijk bewijs of sterk bewijs op

zijnde een eiwit met prion-achtige eigenschappen: α-synuclein: weinig/aanzienlijk; amyloid-β: aanzienlijk; tau: weinig/aanzienlijk; superoxide dismutase 1: weinig/aanzienlijk; huntington: weinig; TAR DNA-binding protein 43: weinig, serum amyloid A: aanzienlijk/sterk, apolipoprotein A-II: weinig.
De wetenschappelijke kennis omtrent inactivatie en decontaminatiemethodes bleek marginaal; de systematische literatuurstudie leverde slechts acht manuscripten op (met uitsluitend data omtrent α-synuclein, amyloid β, tau, serum amyloid A en apolipoprotein A-II). De publicaties waren niet afdoende om met zekerheid te kunnen zeggen welke inactivatie/decontaminatie methodes gebruikt kunnen worden, hetgeen verder onderzoek noodzakelijk maakt.

Conclusie

Wij hebben verschillende eiwitten geïdentificeerd die geassocieerd zijn met aggregatie en infectieuze eigenschappen. Hoewel er geen hard bewijs is dat deze eiwitten zich gedragen als prionen, d.w.z. de capaciteit hebben om ziekte over te brengen tussen/naar mensen, is enige vorm van ongerustheid op zijn plaats, met name voor sommige eiwitten. Speciale aandacht is vereist in het kader van mogelijke ziekte-overdracht in de medische zorg (bijv. via bloedtransfusie of handelingen met gecontamineerde instrumenten) en op laboratoria (bijv. bij functioneel onderzoek naar (gemuteerd) eiwit). Verder onderzoek naar eiwitten met mogelijk prion-achtige eigenschappen is aanbevolen, om meer inzicht te krijgen in de infectieuze eigenschappen van de eiwitten en om gevalideerde methodes ter inactivatie en decontaminatie vast te stellen.

Content

1 General introduction

1.1 Definitions

The report contains several key words related to protein aggregation (highlighted in bold throughout the entire document), which we define as follows:

- **Protein aggregation:** The process by which a protein misfolds due to sporadic, genetic or environmental factors, thereby adopting an aberrant conformation that causes its accumulation into **oligomers, protofibrils** and **aggregates** called **amyloid fibrils** (*Figure 1*).
  - **Oligomer:** Repetition of a few proteins of peptide molecules (<6) without formation of an α-helical/β-sheet ultrastructure.
  - **Protofibril:** Repetition of proteins of peptide molecules up to ~20 molecules, obtaining a β-sheet structure[1].
  - **Amyloid fibril:** Insoluble aggregates of proteins of peptide molecules, containing a common cross- β architecture. Amyloid fibrils are found either intracellularly (called inclusions) or extracellularly. They can be identified through a specific dye-binding method such as thioflavin T and Congo red[1].

- **Prion-like:** Sharing molecular mechanisms with prion proteins, i.e. ability to self-propagate aggregation and spreading[2].

- **Self-propagating:** Imposing the protein's aberrant conformation onto proteins with the normal configuration[3].

- **Seeding / Seeded aggregation:** By adding a preformed protein seed (a "nucleus"), the nucleation phase is shortened and protein aggregation is initiated and accelerated[4].

- **Nucleation phase:** The stage of aggregation in which nuclei are formed and the rate of aggregation increases. When a preformed seed/nucleus is added, the nucleation phase becomes shorter and aggregation occurs faster[4].

- **Cross-seeding:** The process in which presence of a misfolded protein leads to misfolding and aggregation of another type of protein[5]. The two types of protein can be completely unrelated (e.g. tau and amyloid β), can be two isoforms of the same protein (e.g. SAA1.1 and SAA1.2), or can be proteins from two different species (e.g. SAA from duck and SAA from mouse).

- **Transmission / Protein transmission:** Transfer of misfolded proteins from host to host, leading to aggregates.

- **Protein pathogenicity / Disease transmission:** Transmission of disease from one infected host to another host, individual or group.

- **Iatrogenic:** Induced inadvertently by a physician or surgeon, by a medical treatment or by a diagnostic procedure

- ***In vitro*:** Tube-only experiments.

- **Cell-to-cell spreading:** Spreading of self-propagating protein aggregates from cell to cell through several different mechanisms (e.g. exosomes, endocytosis/exocytosis, tunneling nanotubes).

- **Protein strains:** Different three-dimensional foldings of a single type of protein, e.g. observed for prion proteins. Each strain can yield distinct and reproducible clinical, pathological and molecular features[6].

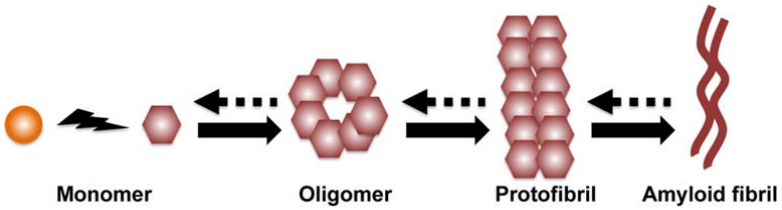
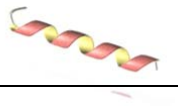


Fig.1 Schematic representation of different stages in the aggregation of proteins towards amyloid fibrils. (Obtained from Huang et al., 2013[7])



1.2 Introduction - Aggregating proteins can be infectious?

Protein aggregation is a common pathological feature in many diseases. The deposition of these aggregated proteins arises through misfolding of proteins that adopt an aberrant conformation. They form interactions with adjacent proteins that cause its accumulation into aggregates and insoluble fibrils, called **amyloid fibrils**[8, 9].

Protein aggregation is observed in many neurodegenerative diseases (e.g. Prion disease, Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS)), but it is not exclusive to the central nervous system. Type II Diabetes, cataract and hemodialysis-related amyloidosis are examples of diseases that have aggregated protein deposits in peripheral tissues[1].

Accumulation of the prion protein is well-known for causing prion-diseases, also termed transmissible spongiform encephalopathies (TSE). This includes Creutzfeldt-Jakob disease, Kuru disease (in humans), Scrapie (sheep), and Bovine Spongiform Encephalopathy (cattle).
Prion diseases are considered unique as they can be the result of genetic factors, may arise spontaneously (idiopathic) and can be the result of a transmitted infection. Prion proteins adopt an aberrant conformation that becomes **self-propagating**. These conformation-changed prion proteins can be transmitted in various ways, including transmission via medical devices, blood transfusion and zoonotic contacts[10-12].

Recent studies have suggested that other proteins than prion proteins may behave in a prion-like manner. In experimental settings, these proteins (e.g. alpha-synuclein, involved in Parkinson's disease) appear to have prion-like properties, i.e. ability to self-propagate and to spread from cell-to-cell[13].
These findings have raised serious concerns with regards to potential hazards for human health.

Detailed investigation is warranted, to obtain a complete and up-to-date overview on all 'suspect' proteins with prion-like properties. Insights into the potential hazard of these proteins is required, and it needs to be assessed whether additional procedures are required to inactivate the proteins.
If needed, biosafety considerations or public health control measures (e.g. surveillance, incineration of dead animals, banning certain risk materials and the use of inactivation methods) need to be taken, e.g. when working with these proteins in an experimental setting, during medical handlings or with regards to disposing potentially contaminated material.

In this report, we present the findings of our comprehensive literature study on the questions:

1. **Are aggregated proteins infectious?**
2. **Can aggregated proteins be inactivated?**

We have identified eight 'suspect proteins' with prion-like properties. For each protein, detailed information is provided on the natural biochemistry and known associations with disease. We summarize the types of evidence that are provided on shared mechanisms with prion proteins and provide insight on potential risk with regards to **protein pathogenicity**.
We present an overview and provide recommendations on the measures that can be taken to inactivate these proteins or to decontaminate instruments.

1.3 Prions

In 1982 Stanley Prusiner proposed the term prion, which is defined as ''a small proteinaceous infectious particle''. He found that this agent was solely a pure protein, which is resistant to inactivation by most procedures that modify nucleic acids, distinguishing them from viruses, plasmids and viroids[14]. The prion protein exists in two forms; the physiological form that is bound to the cell membrane (PrP^{C}) and the pathogenic form (PrP^{Sc}). PrP^{C} consists mainly of an alpha-helical structure (42%) and a relatively low content of beta sheets (3%), whereas PrP^{Sc} contains more beta sheets (>40%) and less alpha helices (30%)[15].

PrP^{Sc} causes prion diseases, also termed transmissible spongiform encephalopathies (TSE). In the human situation, this includes Creutzfeldt-Jakob disease, Gerstmann- Sträussler-Scheinker syndrome, fatal familial insomnia and Kuru disease. In animals, this includes scrapie (sheep), bovine spongiform encephalopathy (cattle), chronic wasting disease (deer and elk), feline spongiform encephalopathy (domestic cats) and transmissible mink encephalopathy (mink)[4].

The central event in prion diseases is the conformational change of the cellular prion protein ($PrP^{C)}$, which is localized in the outer layer of the plasma membrane, into the insoluble PrP^{Sc} that can self-propagate by imposing its aberrant conformation onto normal PrP^{C} proteins[3]. The "protein-only hypothesis" proposes two models that explain the conversion of PrP^{C} into PrP^{Sc} [16-18], as depicted in *Figure 2*.

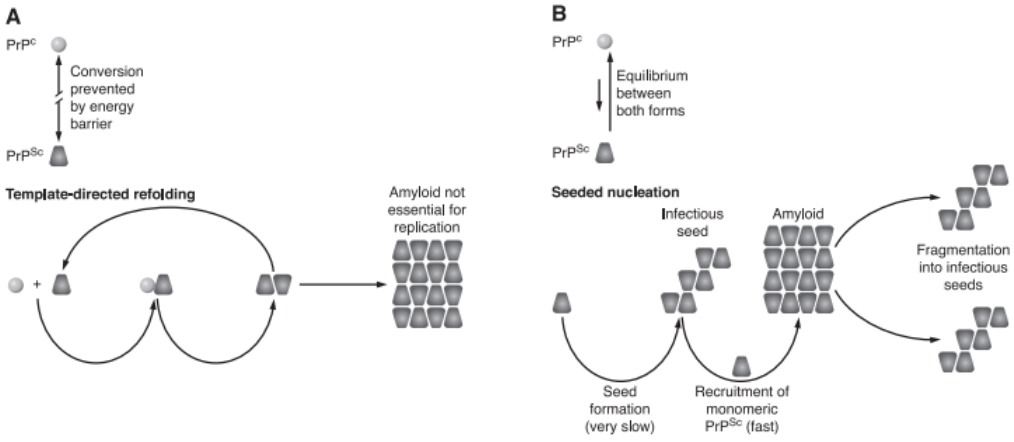
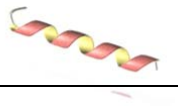


Fig.2 Two models for accumulation of the insoluble prion protein (PrP^{Sc}). (Obtained from Aguzzi et al., 2009[4]). (A) The "Template-directed refolding" model. Exogenously induced PrP^{Sc} interacts with endogenous PrP^{C}, which results in the crossing of an energy barrier and conversion of PrP^{C} into PrP^{Sc}. In this model, the aggregates of PrP^{Sc} (amyloids) are not essential for further replication. (B) The "Seeded nucleation" model, concerns slow formation of PrP^{Sc} seeds (nucleation phase), which is followed by rapid recruitment of monomers of PrP^{Sc} (exponential phase), resulting in amyloids. The amyloids can fragment into ***infectious seeds****, which continue to recruit monomers of PrP^{Sc}.*



Evidence for the accumulation of prion proteins according to the nucleation-polymerization model has been obtained in different experimental settings, including *in vitro* experiments (here defined as "protein-in-a-tube experiments"[19, 20], cell cultures[21, 22] and *in vivo* studies[23]. There are also different phenotypic TSE variants of prions, called prion **strains** that, after inoculation into distinct hosts, cause disease with different clinical, pathological and molecular characteristics[6]. It is unknown how a single type of prion protein can cause different types of diseases in humans and animals.

1.4 Set-up of our literature study

1.4.1 Search strategy ''Infectivity''

There are many proteins that are associated with **protein aggregation**[24]. This not necessarily means that these aggregated behave in a **prion-like** manner, i.e. with the ability to self-propagate and to cause spreading. We focused our search strategy on the question ''Are aggregated proteins infectious?"

Our search strategy (consulting MedLine Ovid, EMBASE, PubMed (publisher), Cochrane Library, Web of Science and Google Scholar, performed March 2, 2016) is depicted in **Table 1**. The search was based on two elements ("aggregated proteins" and "infectious"). After removal of duplicates, all publications were screened on title and abstract. Experimental studies (*in vitro*/cell culture/animal studies) that matched with the two elements were included. The inclusion strategy is shown in **Flowchart 1**. Subsequently, full text articles were screened, resulting in 18 proteins. The prevalence of each protein was counted, and scores were performed for prion-associated mechanisms (seeded aggregation, cell-to-cell spreading, different routes of transmission) (**Table 2**).

We selected eight proteins from this screening. These included the top-six scored proteins, α-synuclein, amyloid-β, tau, superoxide dismutase 1 (SOD1), huntingtin, TAR DNA-binding protein-43 (TDP-43), and two proteins that were linked with potential prion-like spreading or systemic amyloidosis in animals, serum amyloid A and apolipoprotein A-II. All identified proteins appear associated with human diseases, primarily neurodegenerative, but also including systemic diseases (*Figure 3*).

	Protein/peptide	Disease
Neurodegenerative diseases	α-synuclein	Parkinson's disease, Lewy body dementia, multiple system atrophy
	amyloid-β	Alzheimer's disease
	tau	Tauopathies (e.g. progressive supranuclear palsy, Pick's disease, frontotemporal lobar dementia with parkinsonism-17, Alzheimer's disease)
	superoxide dismutase	amyotrophic lateral sclerosis
	huntingtin	Huntington's disease
	TAR DNA-binding protein 43	amyotrophic lateral sclerosis, frontotemporal lobar degeneration
Systemic disease	apolipoprotein A-II	familial renal amyloidosis mouse senile amyloidosis
	serum amyloid A	Systemic AA amyloidosis

Fig.3 The selected proteins with prion-like aggregation properties are associated with different diseases.

1.4.2 Search strategy ''Inactivation''

To gain insight in decontamination/inactivation methods for the selected set of eight proteins, we performed a systematic literature search, focusing on the question: "Can aggregated proteins be inactivated?"

Our search strategy (consulting MedLine Ovid, EMBASE, and Cochrane, and Google Scholar, performed March 31, 2016) is depicted in **Table 3**. After removal of duplicates, all publications were screened on title and abstract. Experimental studies that matched with these two elements were included. The inclusion strategy is shown in **Flowchart 2**. Full text articles were screened, none of the articles that were found after screening of title and abstract were excluded. Three additional articles were identified after cross-referencing. This resulted in a total of eight articles on inactivation procedures for potentially infectious proteins. All identified articles will be discussed in *Chapter 3.2 Prion-like proteins: ''How to inactivate?''*.

1.5 Abbreviations

AA:	amyloid A
AApo A-II:	amyloid apolipoprotein A-II
Aβ:	amyloid-β
AD:	Alzheimer's disease
AEF:	amyloid enhancing factor
ALP:	autophagy lysosomal pathway
Apo A-II:	apolipoprotein A-II
Apo E:	apolipoprotein E
ALS:	amyotrophic lateral sclerosis
APP:	amyloid precursor protein
BSE:	bovine spongiform encephalopathy
CAA:	cerebral amyloid angiopathy
CJD:	Creutzfeldt-Jakob disease
CSF:	cerebrospinal fluid
DLB:	dementia with Lewy bodies
ER:	endoplasmatic reticulum
fALS:	familial ALS
FTD:	frontotemporal dementia
FTDP-17:	frontotemporal dementia with Parkinsonism linked to chromosome 17
FTLD:	frontotemporal lobar degeneration
HD:	Huntington's disease
LB:	Lewy bodies
LN:	Lewy neuritis
MAPT:	microtubule-associated protein tau
mHTT:	mutant huntington
MSA:	multiple system atrophy
NAC:	non-amyloid component
NaOH:	sodium hydroxide
NFT:	neurofibrillary tangles
PD:	Parkinson's disease
PHF:	paired helical filaments
PSP:	progressive supranuclear palsy
PTM:	post translational modifications
SAA:	serum amyloid A
sALS:	sporadic ALS
SOD1:	superoxide dismutase
TARDBP:	TAR DNA-binding protein
TDP-43:	TAR DNA-binding protein 43
TSE:	transmissible spongiform encephalopathies
UPR:	unfolded protein response
UPS:	ubiquitin proteasomal system
WT:	wild type
COGEM:	Commissie Genetische Modificatie
WUR:	Wageningen University Research [Bioveterinary Dept]

2 Prion-like proteins – Biochemistry, disease and infectivity

2.1 alpha-synuclein

2.1.1 Biochemistry and disease

Associated diseases

α-synuclein plays an important role in several neurodegenerative diseases, called α-synucleinopathies, which includes Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). They differ in clinical phenotypes, but are characterized by the formation of insoluble α-synuclein **aggregates** (inclusions), that can be found both intracellularly and extracellularly[25]. In PD and DLB, inclusions are observed in neurons (Lewy bodies (LB) or Lewy neuritis (LN))[25]. In MSA, inclusions occur in oligodendroglia and Schwann cells[25-27].

Genetics

About 20 years ago it was discovered that mutations in the α-synuclein gene (SNCA, synuclein a) are associated with rare forms of autosomal dominant inheritance of PD. In 1997 the first causal mutation, A53T, was identified [28], followed by the discovery of five other missense mutations (E46K, H50Q, G51D, A30P, A53E)[29-34]. In addition, also multiplications in the SNCA gene are associated with rare dominant inheritance of PD [35-38]. While duplication yields a similar form of late-onset PD, triplication causes a more severe phenotype, with earlier onset of disease[39]. SNCA gene multiplication is also associated with an increased risk for developing idiopathic (spontaneous) PD[40, 41]. MSA and DLB has not been linked with mutations in the SNCA gene but gene multiplications have been observed[42-44].

Structure

α-synuclein is a 14 kDa protein that exists in three isoforms, that arise through alternative splicing. The predominant isoform consists of 140 amino acids. The two other isoforms lack exon 3 (126 amino acid protein) and exon 5 (112 amino acid protein)[45].
The protein has three distinct structural domains (*Figure 4*). In its native form, α-synuclein is merely unstructured, i.e. without the presence of alpha helical or beta-sheet structures[46]. The amino-terminus (N-terminus) of the protein acquires an α-helical structure upon binding to phospholipid membranes[47], whilst the C-terminal region remains unstructured[48]. The N-terminal region contains seven 11-residue repeats[49] and has sequence similarity with apolipoproteins and other lipid carrying proteins[48, 50]. The central region of α-synuclein is a highly hydrophobic region and is believed to be the structural domain that triggers aggregation[51]. The C-terminal region is highly acidic, proline rich and is not known to form alpha helices or beta sheets[49]. The C-terminal region of the protein plays an important role in stabilization of the protein structure. Truncation of the C-terminus results in increased **aggregation** *in vitro* and pathological changes in dopaminergic nerve cells *in vivo*[52, 53]. Transgenic mice expressing truncated human WT α-synuclein developed **aggregates** in neurons, which had similar morphology as the aggregates found in human disease[54].

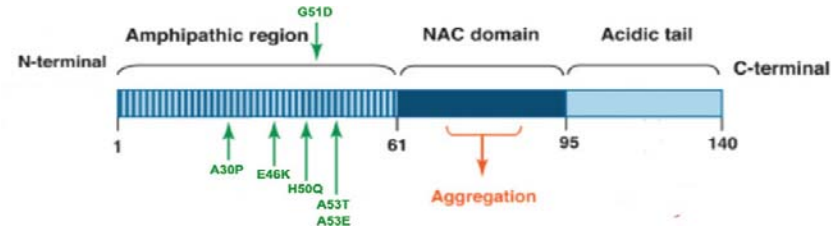


Fig.4 Schematic representation of the three domains of the α-synuclein protein. (Obtained from Xu et al., 2015[55].)

Function

α-synuclein is expressed throughout the brain[56], in particular in the neocortex, hippocampus, substantia nigra, thalamus and cerebellum[49], and is highly enriched in presynaptic terminals[57]. It's localization in the presynaptic nerve terminal suggest a regulatory role in the synapse. Different roles have been described: (I) interaction with tyrosine hydroxylase, a rate-limiting enzyme in the dopamine synthesis process[58], (II) molecular chaperone in the formation of SNARE complexes, which play an important role in neurotransmitter release[59], (III) preventing neurodegeneration by cooperating with cysteine-string protein-α[60], (IV) interaction with synaptic vesicles suggesting a role in neurotransmitter release and synaptic plasticity[61-63]. Expression of α-synuclein is not limited to the nervous system and is also detected in red blood cells and other tissues[64], suggesting additional physiological roles outside the brain.

Neurotoxicity

There are two observations that point towards a causal role for α-synuclein in neurodegeneration: (i) the association of SNCA gene mutations/multiplications with autosomal dominant PD inheritance, and (ii) the presence of large amounts of insoluble **fibrils** of α-synuclein (LB, LN and glial cytoplasmic inclusions). Although Lewy bodies are a hallmark of PD, accumulating evidence suggests that toxicity and pathology is not the result of these large insoluble **fibrils**, but caused by smaller, soluble protein **aggregates**, called **oligomers**.
This would explain why LBs are found in normal elderly without showing any PD pathology[65]. α-synuclein oligomers were initially found in *in vitro* experiments where recombinant α-synuclein accelerated **fibril** formation[66]. Similar results were obtained in cell culture and animal assays[67-70]. These studies also pinpointed the oligomers as causative factors for cellular toxicity. Further evidence on the putative role of α-synuclein oligomers in neurodegeneration is provided from studies on PD or DLB patients, which have increased α-synuclein **oligomer** levels in comparison to healthy individuals[71]. This is in line with studies on mice: A53T transgenic mice display increasing levels of α-synuclein **oligomers** followed by **fibril** formation, while this was absent in transgenic mice expressing wild type α-synuclein[72]. All together, these studies indicate an important role for α-synuclein **oligomers** in neurodegeneration, but their exact role in protein accumulation and protein infectivity needs to be elucidated.

Oligomeric or fibrillary α-synuclein conformations may induce neurotoxicity via several mechanisms. Firstly, the **aggregates** may bind lipid membranes, thereby interfering with natural binding, penetrate the membrane bilayers and form pore-like structures[69]. Secondly, the **aggregates** may disrupt the normal function of α-synuclein in neurotransmission release. In addition, intracellular structures may be affected, such as the mitochondria or the ER-Golgi transport machinery, which can lead to endoplasmatic reticulum (ER) stress[73]. Also, the aggregates may cause failure of cellular machineries involved in protein homeostasis, such as the unfolded protein response (UPR), the ubiquitin proteasomal system (UPS) and the autophagy lysosomal pathway (ALP), which can lead to neurodegeneration[69].

Still, the majority of patients with α-synuclein-related pathology are sporadic and come without mutations in the SNCA gene. These patients display large insoluble α-synuclein **fibrils**. The question remains what alterations of α-synuclein occur during the disease process and at which aggregation state WT α-synuclein becomes toxic.

Cellular and environmental factors

Posttranslational modification (PTM) may promote pathological structural changes in α-synuclein and influence the **aggregation** process to form **oligomers** and **fibrils**. Phosphorylation of α-synuclein at Serine 129 promotes aggregation *in vitro* and in animal studies[74, 75]. Ubiquitination of α-synuclein has an opposite effect and is associated with a decrease in **oligomer** levels[69]. Nitration of α-synuclein appears to be prominent in LB pathology[51]. *In vitro* studies have shown that nitration can promote **fibril** formation and induce cell death[76, 77]. Yamin et al. showed that **fibril** formation was blocked by nitration, while the propensity to form stable soluble **oligomers** was increased[78].

Environmental factors, such as pesticides or heavy metals, may also accelerate deposition of α-synuclein[79]. Epidemiological and clinical observations indicate exposure to pesticides and herbicides (especially paraquat and rotenone) or insecticide (dieldrin) as an environmental risk factor for PD[80-82]. Subcutaneous exposure and oral administration of paraquat and rotenone leads to overexpression of α-synuclein, fibril formation and dopaminergic degeneration in mice and rats, presumably caused by oxidative stress[83, 84].
Exposure to heavy metals may also play a role in the etiology of PD, as suggested by epidemiological studies. Several studies performed in the North America in the early 90's show an increased risk for PD in industries with heavy metal use (e.g. manganese, aluminum, iron or copper-related) or in the paper industry[85]. The effect of various metals was studied *in vitro*. Aluminum, copper, iron, cobalt and manganese induced conformational changes of α-synuclein and significant accelerations in the rate of fibril formation[85]. Epidemiological studies in the UK and Japan revealed a correlation between magnesium intake and higher risk of PD. Still, adding magnesium *in vitro* did not accelerate fibril formation[85].

2.1.2 Infectivity α-synuclein

2.1.2.1 Seeded aggregation

In vitro

It was first discovered in 1998, that α-synuclein can form **fibrils** *in vitro* (that is, in a tube)[66, 86) (87]. Conway *et al.* showed that WT and mutations protein (A53T and A30P) were able to form LB-like fibrils with **amyloid**-like characteristics[87]. The **seeding** was initiated via the **nucleation dependent aggregation** process, by adding **seeds** to purified WT or mutant α-synuclein[88]. **Seeding** was also demonstrated by using the "Protein-misfolding cyclic amplification *in vitro* model", which is also used for amplification of prion **aggregates**[89, 90].

Cell culture

The ability to form fibrils has been shown in several cell culture models (e.g. HEK293, SH-SY5Y and primary neurons)[91-94]. Fibril formation was shown for endogenous α-synuclein or exogenous α-synuclein (WT or mutated), which was applied to the cells or expressed via transfection. A study by Sacino *et al.* was particularly interesting, by showing that the morphology of the formed **fibrils** was similar to the morphology of the original seed-donating **fibrils**, even when different mutant forms of α-synuclein were expressed (A53T or E46K)[95]. This resembles the **self-propagating** properties of **prion strains**. Peelaerts and collaborators showed that different well-defined α-synuclein assemblies (e.g. **oligomers** and two distinct **strains**) cause different synucleinopathies after injection in rat brain. Apparently, these **strains** have different functional and biochemical properties and result in specific pathological phenotypes[96].

In vivo

In vivo evidence for **seeded aggregation** was obtained after injection of brain homogenates from older transgenic mice overexpressing A53T-mutated α-synuclein into younger asymptomatic α-synuclein transgenic mice. Formation of LB/LN-like inclusions was accelerated and early onset symptoms were developed[97]. Similar results were obtained in the study by Luk *et al.*[98, 99].

2.1.2.2 Cell-to-cell spreading

Cell culture

Intercellular transfer of α-synuclein has been explored in many studies to determine if an altered form of α-synuclein can be released from a donor cell to be subsequently taken up by a recipient cell[92, 94, 100-102]. α-synuclein, both **monomeric** and **oligomeric**, has been detected in cerebrospinal fluid and plasma samples of PD patients and healthy people[103, 104], which implicates that α-synuclein can be found in the extracellular space. Hansen *et al.* showed through co-culture experiments that α-synuclein, coupled with a fluorescent label, transferred between cells[94]. They showed that the **aggregates** can already be formed before transfer into the cell, but also after entering the recipient cell.

In vivo

Cell-to cell transfer of α-synuclein has also been demonstrated in animal models. Please refer to Dehay *et al.* for a comprehensive review[105]. Desplats *et al.* investigated on the possibility of α-synuclein to propagate to transplanted stem cells[106]. They implanted GFP-labeled mouse cortical neuronal stem cells (MCNSCs) into the hippocampus of transgenic mice expressing human WT α-synuclein. After four weeks, 15% of the MCNSCs were positive for human α-synuclein and inclusions were found in the cytoplasm of cells. Hansen *et al.* performed a similar study, by implanting mouse fetal mesencephalic neurons into the striatum of transgenic mice expressing human WT α-synuclein[94]. Approximately 5% of the stem cells contained human α-synuclein after 6 months. Using a rat model, in which recombinant α-synuclein was expressed via an Adeno Associated Virus (AAV) vector, Angot *et al.* showed transfer of α-synuclein to 23% of grafted embryonic ventral mesencephalic neurons[107]. Reyes *et al.* studied oligodendrocytes in cell- and animal models to investigate whether these cell types take up α-synuclein species (e.g. **monomeric, oligomers and fibrils**) from the extracellular space[108]. In a cell culture model, it was found that oligodendrocytes are able to take up **monomers** and **oligomers**, and to a lesser extent **fibrils**. *In vivo* results showed the presence of α-synuclein species within oligodendroglial cells after they were grafted into the striatum of rats that had received rAAV- α-synuclein injection. Sastry *et al.* investigated α-synuclein-expressing transgenic mice, that specifically express human WT α-synuclein in neuron populations[109]. Interestingly, these studies showed absence of α-synuclein propagation between neurons.

Recasens *et al.* injected nigral LB fractions containing α-synuclein (purified from postmortem brain of PD patients) into WT mice and macaque monkeys[110]. Both WT mice and macaques showed progressive nigrostriatal neurodegeneration. Control animals that received non-LB fractions from the same PD brains did not show signs of neurodegeneration.

2.1.2.3 Risk of transmission

In 2008, two independently published studies suggested a possible **prion-like transmission** of α-synuclein. Postmortem brain of PD patients who received grafts of fetal mesencephalic dopaminergic nerve cells, more than a decade prior to death, were examined[111, 112]. Both studies found LB-like inclusions in grafted cells, in three individuals in total. It is not known how these grafts were able to develop these **aggregates**. A too short period of time had passed for the grafts to develop Lewy-like pathology spontaneously, because it normally takes several decades for these bodies to become apparent[113]. This led to the hypothesis that these **aggregates** were transferred by host-to-graft propagation, suggesting a **''prion-like'' mechanism**. It is important to mention that in contrast to these two studies, Mendez *et al.* did not observe pathology in the grafted cells of five subjects[114].
Pathogenic transmission of prion proteins has occurred after blood transfusions with cadaveric human growth hormone (c-hGH), with development of CJD in 200 out of 30,000 recipients. Irwin *et al.* have investigated whether similar transfer via c-hGH can occur for α-synuclein, exposing a risk to develop PD. The result appeared negative: of the 796 recipients of c-hGH, none developed PD[115].

The potential **prion-like propagation** of α-synuclein has attracted a lot of attention within the scientific community and the general public. Still, scientific knowledge is insufficient; many reviews and expert opinions have been published but actual investigational studies are limited. More studies are required to gain full insights.

2.1.2.4 Summary

α-synuclein

Associated diseases: Parkinson's disease, Lewy body dementia, Multiple system atrophy

Changes protein structure: α-synuclein is largely unstructured. The central region of the protein is highly hydrophobic and can trigger aggregation. This aggregation is inhibited by the C-terminal region, but still prion-like aggregation can occur. Oligomers (and not fibrils) seem to be the toxic and pathogenic component. Exact mechanisms of aggregation are unknown.

- **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: YES NHP: x
Cell-to-cell transfer:		Cells: YES	R: YES NHP: YES
Induced pathology:			R: YES NHP: x
Transmission:	R: YES (injection)		NHP: YES (injection)
	H: SUGGESTIVE (YES; Host-to-graft, NO; cadaveric hormone)		

- **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: Mutations A53T, E46K, H50Q, G51D, A30P, A53E / Gene multiplications / Truncation C-terminal region

Cellular/environmental factors: Phosphorylation at Serine 129, Nitration / pesticides, heavy metals

- **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: Treatment with 1N NaOH or 0.2% SDS/0.3% NaOH for 1h at RT may be sufficient.
Ureum + heating is currently advised by COGEM but is questionable.

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW / MODERATE**

- **Monitor literature**
- **Suggestive evidence on transmission in NHP, also (but less pronounced) in humans**
- **Gene mutations and/or exogenous factors may be supportive (→ screening in clinically faithful model systems)**

R = rodent models, NHP = Non-Human Primate, H = Human

x = unknown

2.2 amyloid-β

2.2.1 Biochemistry and disease

Associated diseases

Alzheimer's disease (AD) is the most common form of dementia and is characterized by neuronal degeneration and disruption of synaptic function throughout the brain, particularly in the hippocampus, that plays an important role in memory formation[116]. Histopathologically, AD is characterized by extracellular senile plaques comprising amyloid-β (Aβ) peptides and intraneuronal neurofibrillary tangles (NFT) containing hyperphosphorylated forms of the microtubule-associated tau protein[117]. Tau will be discussed in chapter 2.3.
Aβ plays a central role in the onset and progression of AD. Aβ is naturally found in the human brain, cerebrospinal fluid (CSF) and plasma of healthy individuals[118], but in certain circumstances it can aggregate, form **dimers**, **oligomers** and long insoluble **amyloid fibrils** and lead to disease progression of AD[119, 120]. Aβ can also form similar plaques in the walls of cerebral blood vessels, known as cerebral amyloid angiopathy (CAA)[121]. Accumulation of Aβ is also found in inclusion body myositis, the most common cause of muscle degeneration among the elderly, where it accumulates exclusively intracellularly[122].

Structure

Aβ is a product of the transmembrane amyloid precursor protein (APP), which is present in neurons. The APP protein can vary in length (695-770 amino acids) depending on alternative splicing. APP can undergo cleavage by one of two pathways; the non-amyloidogenic pathway and the amyloidogenic pathway, with the formation of different fragments after secretase cleavage (*Figure 5*).

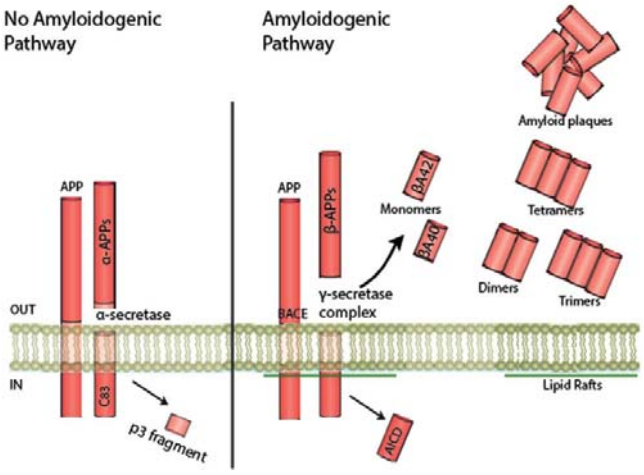


Fig.5 Schematic representation of the formation of amyloid-β aggregates. (Obtained from Hernández-Zimbrón et al., 2015.[123])

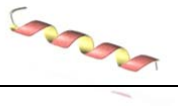
In the predominant non-amyloidogenic pathway, APP is cleaved by α- and γ- secretase. In the amyloidogenic pathway, APP is cleaved by β- secretase and subsequently cleaved by γ- secretase to result in the Aβ peptide (38-43 amino acids, ~4kDa)[122]. The most common isoform of Aβ is the $A\beta_{40}$ peptide (40 amino acids) (~90%), followed by the $A\beta_{42}$ peptide (42 amino acids) (~10%). $A\beta_{42}$ is hydrophobic and is more prone to form **fibrils**. It is the major component of senile plaques[122, 124]. Aβ can aggregate and deposit in senile plaques or is degraded by enzymes such as neprilysin, insulin degrading enzyme or endothelin converting enzyme[125]. The N-terminus of Aβ is hydrophilic and exposed to the aqueous environment, while the C-terminus is hydrophobic and found in the membrane. Due to the lack of solubility of Aβ, many studies use hydrophobic solvents to increase solubility. It is believed that $A\beta_{42}$ has two α-helical segments in the central part of the peptide. The length of the α-helices varies, depending on the solvents used[126, 127].

Genetics

Different genetic mutations are linked with autosomal dominant early-onset AD: mutations in the APP gene and mutations in the genes encoding Presenilin 1 (PSEN 1) and Presenilin 2 (PSEN 2)[120] . Both presenilin proteins are member of the γ-secretase complex. The PSEN 1 and PSEN 2 mutations (occurring in 5% of AD cases) play a critical role in APP cleavage and enhance formation of amyloidogenic Aβ[120]. There are 52 pathogenic mutations reported in APP, 238 mutations in PSEN1 and 45 mutations in PSEN 2[128]. Mutations in these three genes lead to increased levels of the total population of Aβ peptides or $A\beta_{42}$ specifically. Mutations in the APP gene near the α, β, γ secretase cleavage sites lead to favorable cleavage by β- secretase and γ-secretase, thereby producing more Aβ[129]. Natural variants of the gene encoding for apolipoprotein E (APOE), which plays a role in lipid metabolism, appear to be associated with late-onset AD[130]. APOE has three allelic variants (E2, E3 and E4). The presence of an APOE4 allele is the major genetic risk factor for late-onset AD. In contrast, the presence of APOE2 has a protective effect[131]. The APOE isoforms can exert different effects on Aβ **aggregation** or Aβ clearance, but the exact pathological mechanisms are not fully understood[132]. APP is located on chromosome 21. As a consequence, patients with Down syndrome (with an extra copy of chromosome 21) have increased levels of APP, resulting in increased Aβ levels[120].

Function

Low levels of Aβ are found in CSF and plasma of individuals without signs of dementia which indicates that Aβ also has a role in normal physiology[133]. Three functions have been ascribed to Aβ (but the exact roles need to be elucidated):
(I) Aβ levels may regulate synaptic activity in an endocytosis-dependent manner, depressing synaptic function[134].
(II) Aβ is a metallo-protein that can capture redox metal ions such as Cu, Fe and Zn. This prevents these ions from participating in redox cycling and subsequently protects cells against oxidative stress. Aβ may exhibit its antioxidant properties in different cells (e.g. neurons, astrocytes, neuroblastoma cells, hepatoma cells, fibroblasts and platelets)[135].
(III) Aβ may play a role in neuronal survival. Inhibition of endogenous Aβ production in primary neurons resulted in neuronal cell death. The underlying mechanism is not clear, but may be due to changes in the expression of K+ channels[136].



Neurotoxicity

Hardy and Higgins proposed in 1992 the "**amyloid** cascade hypothesis", where the insoluble Aβ **fibril** was pointed as the main toxic species in the pathogenesis of AD[137]. Several cell culture studies supported this hypothesis[138, 139].
Still, the density and distribution of senile plaques did not correlate with the cognitive impairment in AD patients and Aβ plaques were found to be present in cognitively normal people[140]. Also, in APP transgenic mouse models neuronal loss was seen before plaque formation and Aβ **fibrils** purified from AD brain did not cause toxicity in different cell culture studies. These findings showed that Aβ **fibrils** can't be the sole toxic species[141]. It is currently believed that the Aβ **oligomers** are the key player in neurotoxicity, especially in the pre-symptomatic stage of AD. The small size of Aβ **oligomers** allows diffusion through the brain parenchyma[142]. Aβ **oligomers** can disrupt cognitive function[143] and can also induce (in absence of **fibrils**) synaptic dysfunction, enhance long-term depression and inhibit long term potentiation, which subsequently leads to disruption of learning and memory[144, 145]. Recent studies suggest that neuronal toxicity in AD relies on interactions between soluble Aβ and soluble hyperphosphorylated tau[146].

Although soluble Aβ **oligomers** appear the main cause for toxicity, similar effects of the **fibrils** cannot be excluded. It has been proposed that Aβ **oligomers** cause an inflammatory state in the early stages of pathology, and that **fibrils** cause chronic inflammation in later stages[141].

Cellular and environmental factors

Only a small proportion of AD cases is caused by genetic mutations. The underlying causes for sporadic accumulation/misfolding of Aβ are not known. There are many factors that could play a role in AD development[147]. Environmental factors could potentially play a role in developing AD, as suggested by epidemiological and experimental studies[148]. Increased concentrations of certain metals (aluminum, iron, copper and cobalt) have been found in post-mortem brains of individuals with AD. These metals can induce the formation of amyloid fibrils *in vitro* and in rodents[149]. Other environmental factors, such as air pollutants, antimicrobials, insecticides and pesticides have also been lined with AD[149]. However, experimental studies have not yet been performed, to test whether these factors induce Aβ **aggregation**[149].

2.2.2 Infectivity amyloid-β

2.2.2.1 Seeded aggregation

In vitro

Two decades ago, it was first described that Aβ **aggregates** are able to **self-propagate** through the **nucleation-dependent aggregation** process[19]. The **seeding** ability of Aβ can be initiated by preformed Aβ **aggregates**, also known as **seeds**[19, 150]. It was shown that Aβ can very rapidly form **oligomers** *in vitro* and subsequently convert slowly to **fibrils** by **nucleation-dependent aggregation**[151].

Cell culture

Several different cell culture models have been used to show the concept of **seeded aggregation**[152-155]. Of particular interest are the hippocampal slice culture (HSCs) models, which closely mimic the *in vivo* brain environment. The plaques that are formed in HSCs show similarities with plaques that are formed in the brains of transgenic mice[156]. HSCs and similar types of organoid models offer novel opportunities to study **aggregation** of Aβ and other 'suspect' **prion-like** proteins.

In vivo

Most evidence for **seeded aggregation** comes from studies where **seeds** are inoculated in transgenic mice. It is important to know that AD is a human-specific disorder and other mammals show only marginal signs of Aβ accumulation in the brain. This may be caused by the shorter life expectancy of other mammals[157]. Animal studies on the **aggregation** of Aβ are in general performed by inoculating APP transgenic mice with brain extracts from AD patients, brain extracts from aged APP transgenic mice, or with purified Aβ protein. Please refer the review by Walker and Jucker[158]. These studies have shown that the formation of Aβ plaques, cerebral amyloid angiopathy and other related accumulations can be induced through **seeding** in young APP transgenic rodents[158]. Control injections from healthy patients or wild-type (WT) mice did not result in plaque formation. It seems likely that seeds were the causative entity, since immunodepletion, denaturation by formic acid and immunization abolished plaque formation[159]. The **nucleation phase** can be prolonged, and thereby accumulation slowed down, by reducing the availability of soluble Aβ peptides[157].

Aβ **seeds** also possess **strain**-like properties which has been shown both *in vitro* and *in vivo*[158]. Different types of assemblies can lead to distinct phenotypes of AD. As an example, in two people with AD, strains of Aβ **fibrils** with different 3D structures were shown to cause different disease progression[160].

Peripheral routes of inoculation, like intravenous, oral, intraocular or intranasal, did not result in seeding Aβ **aggregation** in APP transgenic mice[161]. However, Aβ rich extracts delivered through intraperitoneal inoculation did induce Aβ plaque formation, but they were more prominent in cerebral blood vessels[162]. Implantation of stainless steel wires, contaminated with Aβ-rich extracts, also lead to plaque formation in APP transgenic mice[161].

2.2.2.2 Cell-to-cell spreading

Cell culture

Cell-to-cell spreading of Aβ has been shown in neuronal cell culture models[163, 164]. The exact mechanism of transfer is unknown. Possibly, Aβ **aggregates** were transferred via extracellular vesicles (exosomes)[165].

In vivo

Spreading of Aβ from cell-to-cell has been shown in APP transgenic mice, which received neuronal grafts from transgenic and WT mice. Transfer of diffuse plaques into the recipients was observed[166]. In animals, protein deposits are initially seen locally at the inoculation site. After prolonged incubation, plaques also form along anatomically connected distant brain areas[157]. However clinical signs and symptoms have not been reported. Spreading of Aβ from peripheral tissue (Aβ rich extracts delivered through intraperitoneal inoculation) towards the brain, with formation of plaques in blood vessels, has also been observed[162].

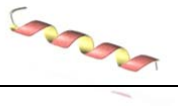
2.2.2.3 Risk of transmission

It has been demonstrated that misfolded Aβ proteins, known as **seeds**, can induce Aβ **aggregation** of endogenous Aβ when injected into brains of rodents, suggesting a prion-like template-assisted accumulation mechanism.

Several epidemiological studies have been conducted to investigate on transmission between humans. Blood transfusion appears to be no risk factor for developing AD[167]. O'Meara *et al.* performed a similar study, comparing 326 newly diagnosed AD patients with 330 control subjects. This also showed that blood transfusion is not associated with an increased risk for AD[168].

Transplantations with cadaveric human growth hormone (c-hGH) have caused transmission of **iatrogenic** Creutzfeldt-Jakob disease (iCJD), which was reported in 200 out of 30,000 recipients of c-hGH[115]. Remarkably, autopsies on eight of the iCJD patients, revealed that four had moderate to severe formation of Aβ plaques as well as deposits in the wall of cerebral blood vessels. Other signs of AD were not observed in these patients, i.e. no clinical deficits prior to death and no hyper-phosphorylated tau NFT in the post-mortem brain[169]. Transplantation of dural grafts is also a major route of transmission of iCJD. Autopsy study on 7 patients with iCJD after receiving dural grafts, showed that 5 patients had combined vessel and parenchymal Aβ depositions[170]. No further clinical deficits and symptoms of AD were observed.

These studies suggest that **iatrogenic** transmission of Aβ may occur. This warrants further investigation, e.g. increasing the number of brains analyzed. Symptoms of AD and tau pathology were not observed, and it is well possible that Aβ accumulation was the result of other factors, unrelated to AD. Still, these findings warrant special care in medical practice. Even though c-hGH is nowadays replaced with synthetic growth hormone and dural grafts are generally performed with synthetic/nonhuman material. Screening of donor patients and dural grafts may be advisable in the future.



2.2.2.4 Summary

amyloid-β (Aβ)

Associated diseases: Alzheimer's disease, inclusion body myositis (muscle degeneration)

Changes protein structure: Aβ (cleavage product of the amyloid precursor protein, APP) has different isoforms: $A\beta_{40}$ (~90%) and $A\beta_{42}$ (~10%). $A\beta_{42}$ is hydrophobic and is prone to form fibrils. Both oligomers and fibrils may be the toxic and pathogenic component. Exact mechanisms of aggregation are unknown.

- **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: YES	NHP: x
Cell-to-cell transfer:		Cells: YES	R: YES	NHP: x
Induced pathology:			R: NO	NHP: x

Transmission: R: YES/NO (dep. on route) NHP: x
H: SUGGESTIVE (YES; dura graft/cadaveric hormone, NO; epidemiological)

- **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: 52 pathogenic mutations in APP, 238 mutations in PSEN1 and 45 mutations in PSEN 2 (http://www.alzforum.org/mutations). The E4 allelic variant of APOE (APOE4) is a major genetic risk factor for late-onset AD.

Cellular/environmental factors: Phosphorylated tau, metals

- **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: Treatment with 1N NaOH or 0.2% SDS/0.3% NaOH for 1h at RT may be sufficient. Plasma sterilization can block Aβ deposition *in vivo* (heating cannot). Aβ seeds resist inactivation by formaldehyde.

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **MODERATE**

- **Monitor literature**
- **Suggestive evidence for human transmission after dura graft or blood transfusion → Pre-screening tissue and blood / inactivation medical devices may be advisable in the future**
- **Rodent models are poor models for AD. Studies in NHP or human brain slices / organoids recommended**

R = rodent models, NHP = Non-Human Primate, H = Human

x = unknown

2.3 tau

2.3.1 Biochemistry and disease

Associated diseases

More than 20 different neurodegenerative diseases are characterized by **aggregation** of tau known as tauopathies[171]. The most common tauopathies are Alzheimer's disease (AD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), corticobasal degeneration, Pick's disease, corticobasal degeneration, and argyrophilic grain disease[171]. Tau is associated with microtubules in neurons and (at lower levels) in glial cells. Extracellular presence is also observed[172]. **Aggregation** of tau can lead to the formation insoluble filaments, known as neurofibrillary tangles (NFT) that are primarily composed of structures called paired helical filaments (PHF)[173]. NFT are found throughout the brain, in general in the cytosol of neurons but sometimes in glial cells[172]. The exact mechanisms behind tau **aggregates** and neuronal loss are largely unknown.

Structure

Tau is a natively unstructured protein, even though there are some secondary structures present. The C-terminal region of tau is the microtubule-binding domain consisting of two hexapeptide motifs that are able to form β-sheets and are important for **aggregation**. The N-terminal region is the projection domain and does not bind to microtubules. Even though tau is known as a natively unstructured protein, binding to other proteins can trigger the formation of a β-hairpin structure, in which both termini approach each other. This may protect tau from **aggregation**[172].

Genetics

Tau has six different isoforms, which are a result of alternative splicing in exons 2, 3, and 10 of the *MAPT* gene (*Figure 6*). The six isoforms are: 2N4R (longest isoform), 1N4R, 0N4R, 2N3R, 1N3R, 0N3R (shortest isoform)[172]. "N" indicates N-terminus, and "R" indicates the number of repeats at the C-terminus. The adult brain expresses all six isoforms. The 4R isoforms are found in **aggregates** present in PSP, CBD and AGD, whereas 3R isoforms are present in tau inclusions in Pick's disease. In AD, all six isoforms are present[172].

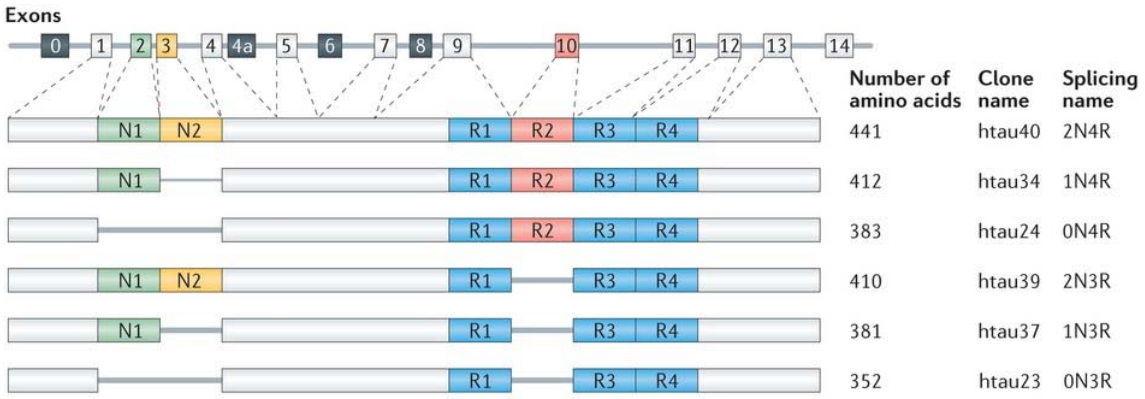


Fig.6 Tau consists of six isoforms, as a result of splicing in exons 2, 3 (N-terminal region) and 10 (C-terminal region). The N-terminus is the projection domain and does not bind to microtubules. The C-terminus is the microtubule-binding domain, which can form β-sheets and plays a major role in aggregation. (Obtained from Wang and Mandelow, 2016.[172])

To date, more than 100 mutations are known to be associated with tauopathies, including FTDP-17, Pick's disease, progressive supranuclear palsy, argyrophilic grain disease and corticobasal degeneration (*http://www.alzforum.org/mutations*). Most of these mutations are missense mutations and are located in or nearby the microtubule-binding domain of the protein that contains the repeat domains. These mutations reduce the protein's ability to bind to microtubules and increases its propensity to form **aggregates**[172]. Also, various mutations are known near exon 10, leading to changes in splicing ratios[174]. These changes in ratio can induce hyperphosphorylation, which is abundantly observed in AD[175].

Function

Tau mainly functions as an intracellular protein in neurons. The protein is also released into the extracellular space and is found in cerebrospinal fluid and interstitial fluid[176]. Intracellularly, tau binds to microtubules and plays a role in a variety of processes. These include regulation of microtubule dynamics, influencing axonal transport, (by interacting with cargo-carrying proteins dynein and kinesin), axonal elongation and maturation, and regulation of neuronal activity, neurogenesis, export of iron, and synaptic plasticity (reviewed by Wang *et al.*, 2016[172]).

Neurotoxicity

It was initially believed that NFTs were the toxic species causing neurodegeneration. However, evidence suggests otherwise (reviewed by Wang *et al.*, 2016[172]). Neuronal loss occurs without development of NFT. This is observed in individuals with AD, as well as in several transgenic mouse models. Possibly, NFTs could even have a protective role by scavenging toxic species. This may particularly occur in the early stages of disease, while the NFTs may compromise cellular functions in later stages.

Aggregated tau causes neurotoxicity in different ways: (i) loss of microtubule binding and cellular functions, (ii) formation of prefibrillar aggregate species (soluble **oligomers**) that form **aggregates** with other proteins, such as amyloid β, (iii) induction of neuroinflammation[172, 177].

Cellular and environmental factors

Tau is highly phosphorylated with approximately 30 (Serine and Threonine) phosphorylation sites on the longest tau isoform. In its unbound form, tau is prone to hyperphosphorylation. Abnormal hyperphosphorylation also suppresses assembly of tau on microtubules[175]. In human brains, tau contains on average two phosphates per molecule, which is increased to eight phosphates per molecule in AD brains[172]. In AD brains, both PHF and oligomerized tau are hyperphosphorylated. Similarly, tau aggregates in FTDP-17 are hyperphosphorylated[175]. Other posttranslational modifications may influence **aggregation** of tau as well, such as ubiquitination, glycosylation, nitration, lipoperoxidation, sumoylation, acetylation and truncation[175]. For instance, truncation of tau, which occurs in several tauopathies, results in fragments that are more prone to **aggregation**[172]. As mentioned in the previous chapter on amyloid-β, several environmental factors may play a role in AD. Not much is known about environmental factors that could play a role in other tauopathies.

2.3.2 Infectivity tau

2.3.2.1 Seeded aggregation

In vitro

Tau **aggregation** follows **a nucleation-dependent aggregation** process. By adding **seeds** from preformed PHF[178, 179] or isolated from AD brain[179] the **nucleation phase** is circumvented to accelerate fibril formation. During *in vitro* preparation, different types of fibrils are observed[178]. Frost *et al.* showed that WT tau can **self-propagate**; fibrils are formed, which can act as seeds to re-initiate accumulation[180]. Tau is involved in more than 20 phenotypically different neurodegenerative diseases. Similar to prion proteins, different **strains** occur, which give rise to distinct pathologies.

Cell culture

Seeded aggregation of tau has been shown in multiple cell culture studies[93, 181, 182]. **Aggregates** of tau can enter cells through micropinocytosis, subsequently inducing the accumulation of endogenous tau[183]. Distinct tau **strains**, retrieved from patients with five different tauopathies, can give rise to distinct tauopathies in cell culture experiments[184].

In vivo

Multiple *in vivo* studies have revealed accumulation of tau according to the **seeded aggregation** mechanism. For instance, injection of brain extract from mutant P301S tau-expressing mice into the brain of transgenic wild-type tau-expressing animals induces assembly of wild-type human tau into filaments and spreading of **aggregates** from the site of injection to neighboring brain regions[185]. This also occurs after injection of human brain extracts (consisting of oligomers or fibrils) from tauopathy patients[186, 187]. Tau **oligomers** induced memory deficits, which were not observed in control mice and tau knockout mice[186]. Also, *in vivo* studies show that brain extracts from patients with different tauopathies induce distinct pathology in mutant tau expressing mice[184, 188].

Various animal studies point towards a cross-seeding mechanism of tau and amyloid-β. For instance, inoculation of mutant-tau mice with amyloid β fibrils can increase formation of NFT[189]. However, these findings are not confirmed by other studies[187].

2.3.2.2 Cell-to-cell spreading

Cell culture

Several cell culture models have shown that tau can be secreted into the extracellular medium and can be taken up as **aggregates** in recipient cells[190]. Tau **aggregates** may be released by exocytosis and internalization may occur by endocytosis or membrane penetration. Alternatively, transfer via exosomes or tunneling nanotubes has been reported[191, 192].

In vivo

Spreading of tau **aggregates** has been shown in many *in vivo* models[191]. Tau pathology is not restricted to the injection sites. It can progress and spread over time to neighboring brain regions and to more distant located regions. This process can be blocked when transgenic mice are

immunodepleted from tau[191]. Intraperitoneal injection of tau-rich homogenate can also induce intracerebral tauopathy, however less effective than intracerebral injection. The underlying mechanism in which tau **aggregates** can reach the central nervous system from the periphery remains to be determined but it is possible that it can be carried by blood cells[193].

2.3.2.3 Risk of transmission

At the moment there is no proof that tau can be infectious like prions. As mentioned in the previous section about amyloid-β, blood transfusion does not increase the risk for AD. Together with amyloid-β, tau plays a significant role in AD. The autopsy results[169] that were discussed in the amyloid-β section (2.2) showed that tau inclusions were not found in patients with iCJD that had received c-hGH. It is worth mentioning that in these patients amyloid-β plaques were identified. There is no way to tell if NFT would have developed if these individuals had not succumbed from prion disease[169].

2.3.2.4 Summary

tau

Associated diseases: Tauopathies (e.g. progressive supranuclear palsy, Pick's disease, frontotemporal lobar dementia with parkinsonism-17, Alzheimer's disease)

Changes protein structure: Tau is a natively unstructured protein. Mutations in the C-terminal region induce the formation of β-sheets and increase the propensity to form aggregates. Both oligomers and fibrils may be the toxic and pathogenic component.

❖ **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: YES	NHP: x
Cell-to-cell transfer:		Cells: YES	R: YES	NHP: x
Induced pathology:			R: YES	NHP: x

Transmission: R: YES (injection) NHP: x
H: NO (cadaveric hormone)

❖ **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: >100 mutations in the tau gene (*MAPT*) associated with tauopathy (located in the microtubule-binding domain) (http://www.alzforum.org/mutations).

Cellular/environmental factors: hyperphosphorylation (hallmark of all taupathies), amyloid-β (cross-seeding mechanism), metal exposure (only linked with AD, unknown for other tauopathies)

❖ **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: Treatment with 1N NaOH or 0.2% SDS/0.3% NaOH for 1h at RT may be sufficient.

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW/MODERATE**

- **Monitor literature**
- **No evidence for human transmission**
- **Rodent models are poor models for AD. Studies in NHP or human brain slices / organoids recommended**

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown

2.4 superoxide dismutase 1

2.4.1 Biochemistry and disease

Associated diseases

Superoxide dismutase 1 (SOD1) is associated with amyotrophic lateral sclerosis (ALS). ALS is a fatal neurodegenerative disorder that targets upper and lower motor neurons. Its clinical course is characterized by progressive paresis, leading to respiratory failure within 3 to 5 years[194]. Pathologically, neurons and glial cells in the brain of ALS patients show ubiquitinated proteinaceous inclusions. Since these inclusions are not stainable with classical amyloid stainings, they are classified as amyloid-like **aggregates**[195, 196].

Familial ALS (fALS) comprises 5-10% of ALS[197, 198]. In these familial cases, SOD1 mutations are found in approximately 20-25% of the cases[199]. Within the group of sporadic ALS (sALS) (~90% of ALS) SOD1 mutations are found in up to 7% of the patients[200]. In patients with SOD1 mutations, SOD1 is the primary component of the amyloid-like aggregates[201]. The primary component of cytosolic inclusions in sALS is the TAR DNA-binding protein 43 (TDP-43) (*see also section 2.6*). Though, recent evidence shows that misfolded SOD1 inclusions can also be present in sALS cases[202].

Genetics

In 1993, 11 mutations were found in the SOD1 gene in 11 families with fALS[199]. Since then, 183 mutations have been identified within the SOD1 gene (*http://alsod.iop.kcl.ac.uk/*). fALS-linked mutations are not localized at specific regions, mutations cover all coding regions of the SOD1 gene. Different types of mutations are present (e.g. missense, deletions, frameshift) but the majority of the SOD1 mutations result from a single amino acid substitution[200]. The most severe mutation is the A4V mutation, which is associated with dramatic prognosis of only 1 year after diagnosis. In contrast, patients with the H46R mutation have an average survival of 18 years after diagnosis[203]. Not all SOD1 mutations cause disease. Patients can be carriers and remain asymptomatic throughout their entire life[200].

Structure

SOD1 is a typical metalloenzyme, and plays a role in the removal of superoxide radicals. SOD1 is highly abundant in the cellular cytoplasm, but also penetrates the nucleus, lysosomes and the intermembrane space of mitochondria[204]. SOD1 acts as a homodimer (*Figure 7*). Each monomer (153 amino acids, 16kDa) contains one copper and one zinc molecule and is stabilized by an intramolecular disulfide bond. SOD1 forms a so-called beta barrel core. SOD1 converts superoxide radicals to oxygen, water and hydrogen peroxide[202, 205].

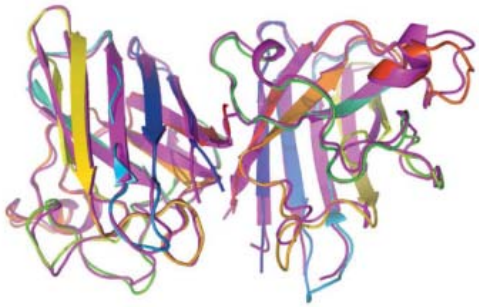


Fig.7 Crystal structure of a SOD1 dimer. (Obtained from Auclair et al., 2013[206])

Function

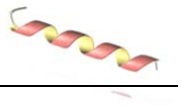
SOD1 is best known for its role as an anti-oxidant. Additional functions are emerging: repressing respiration through casein-kinase 1 signaling, nitration of proteins, functioning as a buffer agent for copper, activation of calcineurin (a phosphatase protein), role in zinc metabolism and activation of the immune system, in particular macrophages[204].

Cellular toxicity

Mutations in SOD1 can lead to de-metalation and reduction of the disulfide bond and destabilization of the protein[205]. This induces misfolding and **aggregation**. Presence of misfolded SOD1 is believed to be a cause for toxicity, which may occur via disruption of the cytoskeleton, disruption of mitochondria, activation of caspase-1 and apoptosis, activation of microglia and inducing abnormalities in axonal transport[200, 202, 207]. It has not yet been determined whether toxicity of SOD1 is caused by **amyloid**-like **fibrils** or a pre-fibrillary state[200]. Deficiencies in the ubiquitin-proteasome system (which has a role in refolded or degradation of misfolded proteins) are also common in ALS, which may induce or support **aggregation** of misfolded SOD 1[202].

Environmental factors

Many environmental factors are believed to be risk factors for the development of ALS, such as lifestyle factors and risk factors related to occupation, such as exposure to electromagnetic fields, metals and pesticides. However, studies on the effects of these factors on **aggregation** of SOD1 have not been performed[208-210].



2.4.2 Infectivity superoxide dismutase 1

2.4.2.1 Seeded aggregation

In vitro and cell culture

SOD1 is a highly stable dimer and resistant to proteolytic degradation. SOD 1 (WT and mutated forms) can form oligomers under physiological conditions, which is strongly increased in denaturing conditions[211-216]. Mutated forms of SOD1 have increased tendency to form **fibrils**, as compared to WT SOD1[212, 215]. Small amounts of SOD1 **aggregates** can act as seeds, inducing the accumulation of WT SOD1 into **fibrils**, in accordance with a **template-dependent mechanism**[212]. This has also been shown in cell culture studies, where mutated SOD1 was able to trigger **aggregation** of endogenously expressed WT protein[214][217, 218].

In vivo

It has been demonstrated that ALS disease progression, in transgenic mice expressing mutant hSOD1, can be accelerated by overexpressing WT hSOD1, resulting in a shorter lifespan[219-221]. More recently, evidence was obtained on SOD1 propagation in a **prion-like** manner. Ayers *et al.* showed that extracts from the spinal cord of paralyzed mutant SOD1 mice can accelerate ALS pathology in mutant SOD1 mice[222]. Bergh *et al.* showed that **aggregates** in mutated hSOD1 mice possess two different **strains[223]**. When both strains were injected into the lumbar spinal cords of transgenic hSOD1 mice distinct disease progression was found[224]. This confirmed results of *in vitro* studies[212].

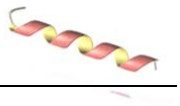
2.4.2.2 Cell-to-cell spreading

Cell culture

Cell-to-cell propagation of SOD1 has been shown in a variety of cell culture studies[214, 217, 225]. SOD1 **aggregates** can be released to the extracellular space and transferred to other cells. SOD1 protein aggregates can be taken up by cells through micropinocytosis, or SOD1 can be transferred via packaging in extracellular vesicles/exosomes[214, 218, 226]. Interestingly, Munch *et al.* demonstrated that endogenous **aggregates** of SOD1 are still present in neurons after 30 days, whilst the original **seeds** already dissipated after a few passages[214]

In vivo

Two reports describe spreading of SOD1 in a mouse model in a prion-like manner[222, 224]. Spreading was observed throughout the spinal cord via particular neuroanatomical pathways. Another study in rats suggests that SOD1 spreading could be essential for disease propagation. SOD1 mutated glial-precursor cells (that are capable to differentiate into astrocytes) were injected into the cervical spinal cord of WT rats. These grafts were able to induce motor neuron death and other symptoms that are seen in ALS (e.g. motor and respiratory dysfunction). The mechanism behind these malfunctions is not clear[227]. More studies are definitely needed to gain full insight in the seeding and spreading mechanistic of SOD1.



2.4.2.3 Risk of transmission

Current evidence on SOD1 as a prion-like protein in ALS disease is exclusively based on rodent studies. Still, SOD1-rich **amyloid**-like **aggregates** are abundant in ALS patients, particularly in cases of familial ALS. Further research is definitely warranted to obtain full insights. It also needs to be mentioned that several studies report on SOD1 inclusions in the liver and kidney, suggesting that SOD1 pathology is not restricted to the CNS[228, 229]. These findings have already led to concerns and debate with regards to harvesting organs from ALS patients, which may require special caution[230].
Taken together, it is highly recommended to carefully monitor novel research findings on these matters and to take appropriate actions if required.

2.4.2.4 Summary

superoxide dismutase 1 (SOD1)

Associated diseases: Amyotrophic lateral sclerosis (ALS)

Changes protein structure: SOD1 is a dimer protein (two monomers of 16kDa) and forms a beta barrel core. Mutations destabilize the protein and trigger aggregation to oligomers and amyloid-like fibrils.

- **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: YES NHP: x
Cell-to-cell transfer:		Cells: YES	R: YES NHP: x
Induced pathology:			R: YES NHP: x

Transmission: R: YES (injection) NHP: x
H: x

- **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: 183 mutations have been identified within the SOD1 gene, randomly distributed (http://alsod.iop.kcl.ac.uk/). Some cause ALS, but not all. Most severe is the A4V mutation, least severe is the H46R mutation.

Cellular/environmental factors: Unknown (ALS has been linked with certain factors, e.g. lifestyle, occupation, metal or pesticide exposure. But effect of these factors on SOD1 aggregation is unknown.)

- **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: x

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW / MODERATE**

- **Monitor literature.**
- **Few experimental data available, only *in vitro*, cell culture and rodent data. Still, these findings raise considerable concern → more exp. highly recommended.**
- **ALS patients have SOD1 fibrils also outside CNS (liver and kidney) → special caution in organ transplant.**

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown

2.5 huntingtin

2.5.1 Biochemistry and disease

Associated diseases

The huntingtin protein is associated with Huntington's disease (HD). This disease was described by George Huntington in 1872 and is a devastating neurodegenerative disease, caused by mutated huntingtin protein. Mutated huntingtin leads to dysfunction of neurons in the basal ganglia. The striatum is mainly affected, but it can cause neuronal cell death throughout the entire brain[231, 232]. One of the mechanisms responsible for neuronal damage in HD may be the formation of protein aggregates, known as neuronal inclusions[232].

Genetics

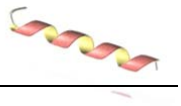
HD is an autosomal dominant disorder, although sporadic cases do exist in 5-8% of the cases. A mutation in the huntingtin gene results in an expansion of the CAG repeats (>35 repeats) (polyQ). Development of HD is determined by the length of the CAG repeat. Expansion of CAG to 35-39 repeats results in incomplete penetrance (only some individuals develop disease), while expansion to more than 40 repeats results in complete penetrance. The time of onset is determined by the allele with the longest CAG repeat[232].

Structure

Huntingtin (348 kDa, 3,144 amino acids) is a highly soluble, almost exclusively unfolded protein (except for the HEAT domains, which may obtain an alpha-helical structure). It is expressed throughout the body, but most pronounced in neurons of the central nervous system and in the testes[231]. The N-terminus contains its most distinct region, characterized by a CAG repeat, also called polyQ. In healthy people, polyQ ranges from 6 to 35 repeats. The huntingtin protein contains various HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A and TOR1) domains of 40 amino acids, which are binding sites for other molecules. In addition, multiple post translational modification (PTM) sites are present[231, 232]. PTM includes proteolytic cleavage, which leads to a variety of N-terminal fragments. In addition, aberrant splicing can occur, leading to the smallest huntingtin fragment (HTT exon1), only containing the first 100 amino acids of the N-terminus. This fragment is highly prone to **aggregation**. Many different forms of **aggregates**, different in size and morphology, have been visualized, both *in vitro* and *in vivo*[232].

Function

Huntingtin is produced throughout the body and is associated with many cellular organelles. Fragments of huntingtin are found in the nucleus but also in the cytoplasm. It is also found in neurites and synapses and plays a role in important processes such as embryonic development, inhibition of apoptosis, intracellular transport of vesicles by interacting with microtubules, and regulation of gene transcription in neurons via binding to transcription factors[231, 233].



Neurotoxicity

Different studies, in cells, animals, and post mortem human brain tissue, suggest a pathological role of mutated huntingtin (mHTT) **aggregates** in HD[233]. **Aggregates** are found both in the nucleus and the cytoplasm. Nuclear **aggregates** are mostly composed by the smallest fragment of huntingtin (HTT exon 1), whereas cytoplasmic inclusions contain both full-length mutant huntingtin and smaller huntingtin fragments[233]. **Seeded aggregation** of mHTT has been shown, which appears to differ between mHTT genes with different lengths of polyQ[232]. Longer polyQ segments have different PTM and delayed formation of **amyloid fibrils**.
Regardless these solid findings on mHTT's ability to form **amyloid fibrils**, it remains uncertain how mutated huntingtin protein causes cellular toxicity. mHTT may cause toxicity in different ways, e.g. by interfering with transcription, intracellular signaling, secretory pathways, endocytic recycling, axonal transport, mitochondrial function, or by activating innate and adaptive immune responses[232]. Toxicity of **aggregates** was shown in cell culture studies, where inclusions led to cell death[234, 235]. However, other studies point towards a protective role of mHTT **aggregates**, instead of a toxic role[236]. For instance, formation of inclusion bodies was found to reduce the levels of mutant huntingtin and to lower the risk of neuronal death[237]. In animals, abundance of **aggregates** did not lead to behavioral changes and neurodegeneration[238]. Possibly, **amyloid fibrils** of HTT are formed by cells to protect them against soluble **pre-fibrillary forms** that are toxic[239-242]. It remains enigmatic whether HTT **aggregates** are inducers of pathology, are protective or are just incidental phenomena without pathological role.

Cellular and environmental factors

Malfunctioning of the ubiquitin-proteasome system (UPS) (which refolds or degrades ubiquitin-tagged proteins) has been linked with increased aggregation of mHTT[232]. Studies show that these **aggregates** are over-abundant in ubiquitin and are linked with chaperones and proteasome subunits, suggesting UPS dysfunction[233].

2.5.2 Infectivity huntingtin

2.5.2.1 Seeded aggregation

In vitro

Mutated huntingtin (mHTT) is able to form **oligomers**[243] and insoluble **aggregates** *in vitro*, with aggregation depending on the length of the polyQ sequence[244]. The mHTT's can form distinct **aggregates** under different circumstances. This suggests **strain**-like properties for mHTT, which may be the reason for HD's heterogeneity[245]. Busch *et al.* demonstrated that synthetic mHTT proteins can act as **seeds** and can initiate **aggregation** when added to (WT) huntingtin proteins[246]. Nenooki-Machidi *et al.* showed similar results, using protein from brains of HD mice[245].

Cell culture

Similar results were obtained in cell culture models. Synthetic fibril-like **aggregates** of huntingtin protein can be taken up by cells, followed by **aggregation** of endogenous, soluble polyQ proteins in a **template-dependent manner**[234, 247]. Intracellular transfer of the **aggregates** into the nucleus (resulting in toxicity) was shown to depend on the presence of an nuclear localization signal (NLS) in the protein[234]. The degree of **aggregation** appears to be stronger when adding synthetic **oligomers** of mHTT or cerebrospinal fluid from HD mice and HD patients[248].

In vivo

Transgenic mice expressing mHTT form **oligomers** and **fibrils**, in a polyQ length-dependent manner[243, 244]. Besides the evidence on mHTT's property to induce **seeded aggregation**, it has also been reported that the protein has the ability to propagate throughout the brain. When a lentiviral vector expressing mHTT was injected into WT mice, transneuronal propagation was observed of mHTT **aggregates**, that were found in the cytoplasm and nucleus of neurons[249].

2.5.2.2 Cell-to-cell spreading

Intercellular spreading of HTT **aggregates** has been demonstrated in neurons[249-251]. This occurs (at least in part) via tunneling nanotubes[251]. *In vivo*, uptake of mHTT **aggregates** has been shown in transplanted neurons, after implantation in mutated HD mice[249]. Moreover, interneuronal propagation of mHTT **aggregates** has been described in WT mice, after local expression of mHTT by lentiviral vector transduction[249].

2.5.2.3 Risk of transmission

Post-mortem analysis on intracerebral fetal neural allografts in 3 HD patients, who received their transplant 1 decade earlier, showed the presence of mHTT **aggregates** in the extracellular matrix of the grafted tissue. This differed from non-grafted parts of the brain, where mHTT **aggregates** were detected in neurons, extracellular matrix and blood vessels[252]. This suggests that mHTT **aggregates** in humans can be released from cells, can be transferred to surrounding brain parenchyma, and can possibly enhance or induce disease. Still, the amount of experimental data is poor and further investigations are needed to obtain insight in the potential **prion-like** properties of mHTT proteins.

2.5.2.4 Summary

huntington

Associated diseases: Huntington's disease (HD)

Changes protein structure: Huntingtin (348 kDa, 3,144 amino acids) is a highly soluble protein, almost exclusively unfolded. Proteolytic cleavage and splicing result in smaller variants of Htt (e.g. HTT exon1), which are prone to aggregation. Both oligomers and fibrils are formed. Exact mechanisms of aggregation are unknown.

❖ **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: YES NHP: x
Cell-to-cell transfer:		Cells: YES	R: YES NHP: x
Induced pathology:			R: NO NHP: x

Transmission: R: YES (infection) NHP: x
H: MINOR (YES; host-to-graft)

❖ **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: Length of the polyQ region of Htt (<35 repeats in normal situation) determines disease penetrance: 35-39 is incomplete penetrance, >40 repeats is complete penetrance.

Cellular/environmental factors: Malfunctioning of the ubiquitin-proteasome system (UPS)

❖ **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: x

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW**

- **Monitor literature**
- **Seeded aggregation and cell-to-cell transfer in experimental models, no signs of induced pathology in rodents. NHP data lacking.**
- **Minor evidence on spread in human brain (host-to-graft).**

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown

2.6 TAR DNA-binding protein 43

2.6.1 Biochemistry and disease

Associated diseases

TAR DNA-binding protein 43 (TDP-43) was identified as major component of protein inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD)[253]. The inclusions are ubiquitin-positive, hyperphosphorylated and consist of full-length TDP-43 or cleaved TDP-43 (C-terminal fragments)[254]. TDP-43 is normally localized to the nucleus, but is abnormally redistributed to the cytoplasm in both ALS and FTLD, where it **aggregates** to form inclusions[254]. These inclusions are present in 50% of the FTLD cases and in almost all ALS cases, except for the familial forms of ALS (fALS) with SOD1 mutations[255]. Similar to SOD1, the cytoplasmic inclusions are not classified as **amyloids**, because negative staining results with standard **amyloid** staining methods, and are therefore referred to as **amyloid**-like **aggregates**[256].

Genetics

In 2008, mutations were found in the gene encoding TDP-43 (*TARDBP*) in patients with sporadic and familial ALS[257, 258]. Since then, 53 mutations in *TARDBP* have been found in sporadic and familial ALS patients (*http://alsod.iop.kcl.ac.uk/*). *TARDBP* mutations are present in 4% of fALS cases and 1-5% of sporadic ALS (sALS) cases[254]. Mutations are also observed in (a low percentage) of FTLD patients[254, 259]. Most of the mutations in *TARDBB* are located in exon 6, resulting in TDP-43 proteins with increased propensity to **aggregate** and obtain **prion-like** properties[202, 260, 261].

Structure

TDP-43 (414 amino acids) belongs to the ribonucleoprotein family[202]. (See *Figure 8* for a schematic representation.) The N-terminal domain of TDP-43 contains a nuclear localization signal and a nuclear export signal, allowing protein transfer between the cytoplasm and the nucleus. The protein also contains two RNA recognition motifs, RRM 1 and RRM 2, that are involved in nucleic acid binding. The C-terminal domain is structurally disordered[262] and is important for interactions with other proteins[220]. This domain is also called the **prion-like** domain as it can cause misfolding and aggregation, which is enhanced by ALS-linked mutations. Proteolytic cleavage results in C-terminal fragments that are redistributed into the cytoplasm where they **aggregate**[202]. The two RNA recognition motifs contain β-sheets, β-hairpins and α-helices, while the other parts of the protein lack these secondary structures[263].

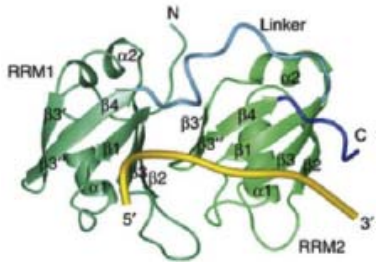


Fig.8 Ribbon representation of TDP-43 protein bound to an RNA molecule. (Obtained from Luvavsky et al., 2013[263])

Function

TDP-43 is a DNA and RNA-binding protein, which primarily targets intron sequences, but also non-coding RNAs and 3'UTR sequences. TDP-43 is well known for its role in RNA metabolism, such as pre-mRNA splicing, transcription, miRNA synthesis, mRNA translation, mRNA transport and stability[264]. Knock-down of TDP-43 leads to cell death[253] and TDP-43 knockout mice display embryonic lethality, demonstrating the essential role of the protein[260].

Cellular toxicity

The exact role of TDP-43 in the pathogenesis of ALS is not clear[254]. It is possible that the large cytoplasmic inclusions (**aggregates**) are toxic to neurons, for instance via depletion of normal-functioning proteins. However, the TDP-43 inclusions may also be merely epiphenomenal or could even be protective, by sequestering toxic **pre-fibrillary** forms of TDP-43. Contradicting results have been obtained in cell- and animal studies. Overexpression of WT TDP-43, without the presence of mutations, has been found to increase the formation of **aggregates** and to induce cell death. The presence of **aggregates**, however, appeared to be not essential for inducing toxicity[254]. Some studies suggest that **amyloid**-like **fibrils** of TDP-43 are the main toxic agent[261], while others suggest that **pre-fibrillary** forms are the causative form[256]. Protective properties of aggregates have also been reported[265]. TDP-43 is also able to sequester other proteins, but it is unknown what the influence is on pathogenesis[264].

Cellular and environmental factors

Similar to other **aggregation** processes, the **aggregation** process of TDP-43 is very complex and many cellular or environmental factors could play a role[266]. Extensive ubiquitination and inhibition of the proteasome system has been associated with the formation of TDP-43 inclusions[254, 266].
As already mentioned in the chapter on SOD1, many environmental factors seem to be a risk factor for the development of ALS, such as certain lifestyles, types of occupation or exposure to metals. However, it is unknown whether these factors affect accumulation of TDP-43[208-210].

2.6.2 Infectivity TAR DNA-binding protein 43

2.6.2.1 Seeded aggregation

In vitro

Experiments with purified proteins have revealed that TDP-43 has the ability to form **aggregates** through **template-dependent propagation**, driven by the C-terminal domain of TDP-43 and enhanced by particular mutations[261, 267, 268]. Truncated TDP-43 shows increased **aggregation** *in vitro*, which also occurs in cell cultures[269]. The C-terminal fragments of TDP-43 are the main product in the cytoplasmic inclusions in ALS patients, and deletion of the C-terminus prevents the formation of **fibrils**[270]. Recently, Shimonaka *et al.* demonstrated (*in vitro* and in cell culture) that TDP-43 can aggregate as different **strains**, similar to the **strain**-property of prions[271].

Cell culture

The **seeding** ability of TDP-43 has been studied primarily in cell culture models. Furukawa *et al.* introduced *in vitro*-created **fibrils** into cells that overexpressed TDP-43, resulting in the aggregation of endogenous TDP-43 into **fibrils**[267]. Similar results were obtained when TDP-43 **fibrils** from ALS and FTLD-TDP patients were introduced to cells, also demonstrating **aggregation** in a **template-dependent manner**[272].

In vivo

There are currently no reports on *in vivo* **seeding** ability of TDP-43. Such studies, which can make use of suitable mouse models[189], are highly recommended to obtain better insight in the **aggregation** / **prion-like** properties of TDP-43.

2.6.2.2 Cell-to-cell spreading

TDP-43 can be transmitted between cultured cells[273]. The presence of TDP-43 **oligomers** has been shown in exosomes, which can mediate cell-to-cell transfer[272].

2.6.2.3 Risk of transmission

There is currently no evidence that TDP-43 aggregation causes **transmission** of disease. More studies are needed, particularly in animals, to investigate on the potential **prion-like** mechanisms of TDP-43.

2.6.2.4 Summary

TAR DNA-binding protein 43 (TDP-43)

Associated diseases: Amyotrophic lateral sclerosis, frontotemporal lobar degeneration

Changes protein structure: The C-terminal domain of TDP-43 is structurally disordered and is important for interactions with other proteins. This is the "prion-like domain" as it can cause misfolding and aggregation (enhanced by ALS-linked mutations). Both oligomers and amyloid-like fibrils are formed. It is unclear which of these forms causes toxicity to neurons.

❖ **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: YES	R: x	NHP: x
Cell-to-cell transfer:		Cells: YES	R: x	NHP: x
Induced pathology:			R: x	NHP: x

Transmission: R: YES (injection) NHP: x
H: x

❖ **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: 53 mutations identified in the *TARDBP* gene in patients with sporadic and familial ALS (http://alsod.iop.kcl.ac.uk/). Most of the mutations are located in exon 6, resulting in TDP-43 proteins with increased propensity to aggregate and obtain prion-like properties.

Cellular/environmental factors: Unknown (ALS has been linked with certain factors, e.g. lifestyle, occupation, metal or pesticide exposure. But effect of these factors on TDP-43 aggregation is unknown.)

❖ **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: x

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW**

- **Monitor literature**
- **Seeded-aggregation and cell-to-cell spreading of TDP-43 has been observed in cell cultures, but is unknown in animals and humans. → Studies in mice recommended.**

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown

2.7 serum amyloid A

2.7.1 Biochemistry and disease

Associated diseases

Serum amyloid A (SAA) proteins are lipid-binding proteins (apolipoproteins) that are predominantly produced by the liver during states of inflammation[274]. Four SAA isoforms, expressed by four genes, have been identified (SAA1 to SAA4). Accumulation of SAA1 proteins can cause amyloid A (AA) amyloidosis[275]. The symptoms of AA amyloidosis are highly variable and primarily depend on the major organ of AA accumulation. The AA **aggregates** disrupt the normal tissue architecture and damage the function of tissues and organs.

The plasma concentration of SAA1 (under control of interleukin-1, interleukin-6 and tumor necrosis factor-α) is normally very low, but can increase up to 1,000-fold during acute inflammation[274]. This can lead to amyloid A (AA) amyloidosis, where the N-terminal fragment of SAA1 (usually residues 1 to 76) forms extracellular **amyloid** depositions in different organs. **Amyloid** deposits are primarily seen in the kidney, but also in the liver and spleen[276]. AA amyloidosis can be caused by chronic inflammatory diseases, chronic infections, auto-inflammatory syndromes and certain malignant tumors[277]. AA amyloidosis is less common in the Western world, as a result of successful treatments of chronic diseases, e.g. using blockers of interleukin-6 or tumor necrosis factor-α receptor[275]. AA amyloidosis can also occur secondary to chronic inflammation in domestic, laboratory and wild animals[278]. AA amyloidosis can be induced by injection of stimulants, such as silver nitrate, casein or lipopolysaccharide, that increase the concentration of SAA[279].

Genetics

There are currently no genetic mutations linked with AA amyloidosis. Throughout the population, the SAA1 gene comes as five different alleles that do not show major differences in amino acid sequence and tendency to aggregate[275]. Still, the minor differences could possibly effect the lipid binding ability of SAA and contribute to the formation of **amyloid fibrils**[280]. Japanese individuals that are homozygous for SAA 1.3 have a higher risk to develop AA amyloidosis[281], whereas homozygosity for SAA 1.1 is a risk factor for Caucasian individuals[275].

Structure

In the natural situation, inflammation increases the levels of SAA1 in the plasma, where it binds to high density lipoprotein (HDL)[280]. (Please refer to *Figure 9* for a schematic representation of SAA1.) The N-terminus of the protein contains two amyloidogenic segments (residues 2-9 and 53-55), which are prone to aggregation[280]. If bound to HDL, the amyloidogenic segments are docked with HDL and the N-terminus obtains an amphipathic α-helical structure[280]. In the unbound format, however, the N-terminus is solvent exposed and prone to misfolding[282]. The fibrillary aggregates of AA amyloidosis merely consist of shortened variants of SAA1, which lack the C-terminus[275].

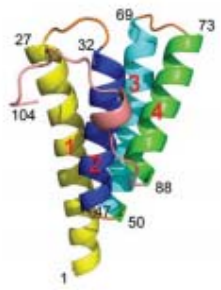


Fig.9 Ribbon representation of human SAA1. (Obtained from Lu et al., 2014[282])

Function

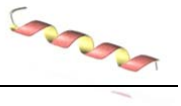
The SAA protein is evolutionary highly conserved, suggesting a critical role in different species. However, the function of SAA remains poorly understood. The protein is evidently involved in inflammatory reactions, but also plays important roles in cholesterol metabolism by displacing the apo A-II protein from HDL[280]. Such effects on apo A-II may lead to cardiovascular disease. SAA may also stimulate the oxidation of low-density lipoprotein (LDL), which may lead to atherosclerosis[283]. SAA can bind to many different ligands, such as lipoproteins (e.g. HDL), cell membranes, liposomes, cholesterol, retinol, charged ligands, extracellular matrix proteins and different cell receptors[284]. It is unknown whether and how these functions relate to SAA's involvement in AA amyloidosis.

Cellular toxicity

Systemic AA amyloidosis is characterized by **amyloid** depositions in different organs. It is believed that this results in cellular toxicity, leading to organ failure. While SAA1 oligomers can act as a **seed** to form **amyloid** deposits[285]. There is currently no evidence that **pre-fibrillary** states lead to cell toxicity. Still, cell culture studies need to be done, to obtain insight on SAA1 **aggregation**, spreading and toxicity.

Cellular and environmental factors

It is currently unknown which endogenous and exogenous factors influence the aggregation of SAA1. Binding to certain ligands, e.g. heparin sulfate, can displace SAA from HDL. Heparin sulfate is abundantly present in the extracellular matrix, which may cause result in high concentrations of free SAA and formation of amyloids[280]. Aggregation of SAA1 may also be linked with deficiencies in the protein degradation system[280].



2.7.2 Infectivity serum amyloid A

2.7.2.1 Seeded aggregation

In vitro

In contrast to the manifold *in vivo* studies, only a few *in vitro* studies have been done on SAA aggregation. These studies have demonstrated the ability of SAA1 to form **amyloid fibrils**, according to a **nucleation-dependent mechanism**. Self-**seeding** was shown, as well as cross-seeding with different SAA isomers[286].

Cell culture

SAA aggregation has not been studied in cell culture systems.

In vivo

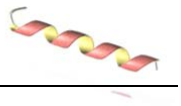
Already in the sixties, it was discovered that a particular type of protein aggregates (obtained from spleens of casein-treated mice) were capable to induce amyloidosis in inflamed recipient mice[287]. The unidentified **seeding** agent, which later on appeared to be **fibrillary** SAA, was initially termed Amyloid Enhancing Factor (AEF), a term that still appears in literature.

Later on, the **seeding** ability of SAA, in the form of **protofibrils**, **oligomers** or **fibrils**, has been demonstrated in multiple mouse studies. Of importance, this also included studies in which SAA-containing **fibrils** were administered intravenously, intraperitoneally or orally[288, 289]. Induction of SAA overexpression appeared necessary in these mouse experiments. This was established via classical methods to induce inflammation or via the use of genetically engineered mice, which overexpress IL-6 (and consequently SAA)[189].

SAA-containing **fibrils** also have strong cross-seeding capability, as has been observed in the context of SAA isoforms or SAAs from different species[189, 290]. Mice receiving SAA-containing **fibrils** from foie gras of duck or goose display accelerated systemic disease[291]. Similar results are obtained (observed in rabbits and mice) with bovine SAA-containing **fibrils**[292, 293]. It is worth mentioning that when mice receive mouse-derived SAA **fibrils**, amyloid formation is enhanced as compared to the mice that receive bovine-derived SAA **fibrils**. This points towards a cross-species barrier of AA amyloidosis transmission, which is also seen in prion diseases[278, 293]. Cross-**seeding** is also seen between avian species and from swans to mice[288].

Not only SAA-containing **fibrils**, but also other amyloid structures that are found in nature (e.g. silk fibrils, Sup35 (a yeast protein) and curli (important protein component in the extracellular matrix of Enterobacteria))[294] can cross-**seed** in mice[295]. Still, it needs to be stressed that these (cross) **seeding** mechanisms only occurred when pro-inflammatory reactions were induced in the recipient animals.

In humans, there are at least two distinct phenotypes of SAA-containing deposits in AA amyloidosis patients, especially seen in the kidney. This may indicate **strain**-like properties of SAA, similar to what is observed in prion diseases[296].



2.7.2.2 Cell-to-cell spreading

Cell-to-cell propagation has been observed in the majority of animal experiments, where systemic amyloidosis was accelerated in many organs (predominantly in the spleen, kidney and liver)[278]. Accumulation usually starts within the spleen and presumably spreads to other organs via the blood. Blood monocytes contain AA **seeds** and could play a role in transporting these to other organs[297].

2.7.2.3 Risk of transmission

AA amyloidosis is a problematic disease in several mammals and birds. Epidemic outbreaks of AA amyloidosis occur in avian species, such as fowls[278]. AA amyloidosis also causes fatal disease in hunting falcons[298], in geese farms[299] and in cheetah species that are in danger of extinction[300].

It is hypothesized that **horizontal transmission** may occur via ingestion of food or feces[301]. In an experimental setup, intravenous injection of AA fibril-containing cheetah feces resulted in rapid appearance of **amyloid** deposits in mice organs. Such preliminary findings increase the possibility that cheetahs transmit AA amyloidosis, e.g. upon licking during mutual grooming[302].

There is currently no epidemiological data that points towards transmission of AA amyloidosis to or between humans. Still, preliminary experimental findings raise some concern. First, recent findings draw the attention towards blood transfusion as a potential routing for transmission of AA amyloidosis. Blood cells from humans with AA amyloidosis appear to accelerate pathology in mice, which does not occur with blood cells from healthy individuals[296] Sponarova *et al.* found that monocytes contain AA-amyloid **seeds** and can induce AA-amyloidosis in susceptible mice after intravenous injection[303]. Second, consumption of meet was raised as a potential concern. AA amyloidosis is found in slaughtered cattle[304], Pekin ducks and foie gras of duck or goose liver[291]. This also warrants special attention, since these substances are known to be capable to transfer AA amyloidosis in an experimental setting in mice. However, it will be challenging to obtain clear answers on the horizontal transmissibility of AA to (healthy) human beings. Transfer of AA fibrils to mice only results in AA amyloidosis in combination with pro-inflammatory stimuli[296]. Amyloidosis in healthy settings may occur at a longer-term, which makes mouse experiments, as well as epidemiological analyses on humans, difficult.

2.7.2.4 Summary

serum amyloid A (SAA)

Associated diseases: Systemic AA amyloidosis

Changes protein structure: SAA1 proteins can bind to HDL in plasma, which shields the N-terminal amyloidogenic segments (residues 2-9 and 53-55) from aggregation. If unbound, SAA1 has a tendency to aggregate. SAA1 fibrils merely consist of shortened variants of SAA1, which lack the C-terminus. Both oligomers and fibrils are formed. Exact mechanisms of aggregation are unknown.

- **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: x	R: YES	NHP: x
Cell-to-cell transfer:		Cells: x	R: YES**	NHP: x
Induced pathology:			R: NO	NHP: x

Transmission:	R: YES (injection, oral)**	NHP: x
	H: x	

- **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: Disease-causing mutations in the SAA1 gene have not been described. Five allelic variants are known. Some alleles are associated with increased AA amyloidosis (i.e. homozygosity SAA 1.1 (Caucasian population) and homozygosity SAA 1.3 (Japanese population)).

Cellular/environmental factors: Unknown

- **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: Treatment of AA fibrils with NaOH (0.1N or 1N) plus autoclaving (1h, 132°C) resulted in complete inactivation, while mono-treatment with 0.01N NaOH; 0,001 NaOH; pepsin; trypsin; pronase or proteinase K was insufficient.

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **MODERATE / HIGH**

- Seeded aggregation and spreading of SAA fibrils in mouse models, incl. oral transfer
  Still, inflammation is required.
- Cross-seeding in animal models (e.g. duck to mouse)
- Outbreaks of AA amyloidosis in animals (mouse, goose, cheetah, fowl, falcon)
- Speculations about horizontal transfer to humans (blood transfusion or food intake)

➔ **Studies recommended to clarify molecular/biological mechanisms of SAA aggregation and infectivity**

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown
** = Includes cross-seeding of SAA from one species to another species, including non-rodents

2.8 apolipoprotein A-II

2.8.1 Biochemistry and disease

Associated diseases

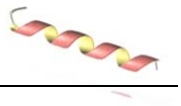
Misfolded apolipoprotein A-II (apo A-II) causes a rare hereditary form of amyloidosis, called Familial Amyloidosis. Mutated apo A-II proteins form **amyloid** deposits (AApo A-II), primarily in the kidney, which ultimately leads to kidney failure in humans. **Amyloid** depositions are also seen in other organs such as heart, liver, spleen and adrenals[305].
In mice, spontaneous formed AApo A-II leads to senile mouse amyloidosis. Senile mouse amyloidosis is characterized by extracellular deposition of AApo A-II in several organs, such as the liver, tongue, heart, stomach, small intestine, spleen, kidney and walls of blood vessels[306].

Structure

Apo A-II is a highly hydrophobic member of the apolipoprotein family. It is secreted by the liver and is present in human plasma as a disulfide-linked homodimer, where it is bound to the surface of mature high density lipoprotein (HDL). HDL removes excess cholesterol from peripheral tissues and has a protective role against cardiovascular disease[307]. Each monomer contains 77 amino acids and has a molecular weight of approximately 9 kDa. It is believed that Apo A-II is only partially folded in solution, but forms amphipathic α-helices when bound to HDL. These helices have a highly apolar surface on one side, which makes it suitable for binding to the phospholipid membrane of HDL. The apo A-II protein has two amyloidogenic regions, located at both ends of the protein (residues 10-18 and 60-70). These regions overlap with the α-helical regions. Therefore, and this is similar to the SAA protein (see previous section), the apo A-II protein is protected from aggregation when bound to HDL, but becomes prone to aggregation in its free, unbound format[307].

Genetics

The rare autosomal dominant form of apo A-II-associated familial amyloidosis is caused by mutations in the stop codon of the protein. This leads to an extension of the C-terminus by 21 residues (residues 78-98)[305]. It is not clear why extension of the C-terminus leads to the aggregation of apo A-II into **amyloid fibrils**. Possibly, extension may hamper the formation of amphipathic α-helices, thereby losing the ability to bind to HDL[305-307]. Also, it could be that extension leads to the addition of an additional amyloidogenic segment, which may act synergistically with the other amyloidogenic regions[307].
In mice, three different apo A-II alleles (apo A-II types A, B and C) can be present, which encode different **strains** of apo A-II. These three variants differ in amino acid substitutions at four positions. apo A-II type C is known to be the most amyloidogenic strain, as it contains a glutamine at position 5, which stabilizes β-sheet formation. apo A-II types A and B contain a proline at position 5, which disrupts β-sheet formation[308]. Murine apo A-II, however, exists as a monomer and its sequence differs approximately 40% from human apo A-II[309]. Murine aggregation of apo A-II can be accelerated by introducing mutations, but can also happen spontaneously[307].



Function

Apo A-II plays a role in several different processes[310]. Apo A-II plays a major role in lipid metabolism, for instance inhibiting or activating proteins, such as cholesteryl ester transfer protein, phospholipid transfer protein, lecithin:cholesterol acyltransferase, and lipases[310]. Apo A-II also regulates the metabolism of free fatty acids and cholesterol. As a result the protein has been associated with insulin resistance as changes in free fatty acid metabolism and accumulation of cholesterol result in reduced insulin secretion and insulin resistance[310].

Cellular toxicity and environmental factors

AApo A-II amyloidosis is characterized by **amyloid** depositions that are found in the extracellular spaces of many different organs. It is believed that the deposition of **amyloid fibrils** impairs the physiological function of the organs and leads to cell toxicity. Pre-fibrillary forms of AApo A-II may be major cause for cellular toxicity, similar to other amyloidogenic proteins[311]. However, research performed on pre-fibrillary forms of AApo A-II (e.g. **oligomers**) is scarce.

2.8.2 AApo A-II infectivity

2.8.2.1 Seeded aggregation

In vitro

In 1991, first evidence was obtained on the **seeding** ability of AApo A-II, by adding AApo A-II **fibrils** to apo A-II in a test tube. The **nucleation phase** was aborted, which support **nucleation-dependent aggregation**[312].

Cell culture

Apo A-II **aggregation** has not been studied in cell culture systems.

In vivo

When AApo A-II **fibrils** were injected intravenously or intraperitoneally into young mice carrying the apo A-II gene type C, disease progression was accelerated dramatically[313-315]. When AApo A-II fibrils were denatured or when native apo A-II was used, **aggregation** did not occur[315]. Intravenous injections of AApo A-II from mice carrying the apo A-II genes type B and C both resulted in **fibril** deposits. However, differences were observed in the rate and degree of amyloidosis, which suggests **strain**-like phenomena[316]. Cross-**seeding** can occur with AApo A-II and SAA and vice versa[279]. Cross-seeding with many other types of fibrils has also been observed after intravenous injection in mice[317].

2.8.2.2 Cell-to-cell spreading

Cell-to-cell transfer of AApo A-II has been observed in mouse experiments, including intravenous, intraperitoneal, and intragastric delivery[318]. Special focus has also been on feces, since ingestion of faces is common behavior of mice. Injection of feces with AApo A-II **fibrils** intraperitoneally into mice resulted in AApo A-II depositions. These findings suggest that exogenous amyloid **fibrils** could act as a **seed** for **fibril** formation and disease propagation[318]. AApo A-II deposits also occurred in many organs after implantation of AApo A-II containing grafts into the liver or under the skin of mice. This was not observed after implanting healthy tissue grafts[319].

2.8.2.3 Risk of transmission

Oral transmission of AApo A-II can occur in mice, after drinking distilled water containing AApo A-II[318]. Transmission between mice has also been observed, when young mice expressing the apo A-II type C were sharing a cage with older mice expressing apo A-II with severe **amyloid** deposits. Transmission may have occurred via the ingestion of feces, but this has not been experimentally validated[318]. Intraperitoneal injection of feces containing AApo A-II fibrils has been shown to induce the formation of AApo A-II deposits[318].

Vertical transmission through the placenta seems not to occur in mice. However, AApo A-II deposits are detected in the mice after being nursed by apo A-II **fibril** injected mothers[320]. It was suggested that deposit forming factors are transmitted during the nursing period to accelerate **fibril** formation[321].

2.8.2.4 Summary

apolipoprotein A-II (apo A-II)

Associated diseases: Human: Familial amyloidosis, Mouse: Mouse senile amyloidosis

Changes protein structure: Apo A-II is partially folded in solution, but contains amphipathic α-helices when bound to HDL. These helices (located at both ends of the protein) have an apolar surface, which allows binding to HDL. The α-helices contain amyloidogenic regions, which are protected from aggregation upon binding to HDL. In its unbound format, apo A-II is prone to aggregation. This mechanism is similar to the SAA protein (as described in the previous section). Apo A-II fibrils merely consist of extended apo A-II proteins, caused by a mutation in the gene's stopcodon. Exact mechanisms of aggregation are unknown.

❖ **Evidence on prion-like properties (see also Table 4 & Table 5):**

Seeded aggregation:	In vitro: YES	Cells: x	R: YES	NHP: x
Cell-to-cell transfer:		Cells: x	R: YES	NHP: x
Induced pathology:			R: YES	NHP: x

Transmission: R: YES (injection, graft-to-host, oral, nursing) NHP: x
H: x

❖ **Endogenous / Exogenous factors as supporters of aggregation?**

Gene: Mutations in the stop codon of the apo A-II gene cause Familial amyloidosis (autosomal dominant inheritance) The aberrant protein has a C-terminal extension of 21 residues (residues 78-98). In mice, three apo A-II alleles occur; type C is amyloidogenic, while this is not the case for type A and B. (This allelic variation is unknown in humans.)

Cellular/environmental factors: Unknown

❖ **Inactivation method (see also Chapter 3):**

No validated method.
Recommended to test inactivation methods (incl. prion-standards; NaOH & heating).
Literature search: Treatment of apo A-II fibrils with 88% formic acid, 6M guanidine hydrochloride and autoclaving in 1N NaOH resulted in complete inactivation, while treatment with proteinase K, antibiotics, freeze/thaw, 6M urea was insufficient.

OVERALL EVIDENCE ON PRION LIKE PROPERTIES: **LOW**

- Aggregated apo A-II causes disease in humans (but extremely rare), no evidence on infectious properties
- Aggregated apo A-II causes disease in mice, and may be infectious (no evidence in other animals though)

Recommended: Monitor literature

R = rodent models, NHP = Non-Human Primate, H = Human
x = unknown

3 Inactivation methods

3.1 Current protocols to inactivate prions

Prion proteins, characterized by their ability to transmit transmissible spongiform encephalopathies (TSEs), are a major threat for human (and other species) health. The dangerous properties of prion proteins became particularly evident upon the occurrence of patients with Variant Creutzfeldt–Jakob disease, which had consumed BSE-contaminated food. Based on guidelines provided by the European parliament[322], Dutch government stressed the necessity of implementing protection measures to reduce the risk of exposure of employees to prion proteins[323]. Accordingly, the Workgroup Infection Prevention of the RIVM (WIP, Werkgroep Infectie Preventie) has presented guidelines[324] (2008, revised in 2013), based on guidelines of the United Kingdom[325].

The WHO[326] recommends the following measures when working with TSE-containing (or potentially containing) patients or tissues:
- Discard medical instruments that have been used on patients that are diagnosed with CJD (or other form of TSE) or at risk (dura mater or human derived hormones recipients, or people with a family history or relatives (parents, brothers, sisters, children, grandparents and grandchildren). In case of suspected patients, instruments can be held in quarantine until diagnosis has been done[325].
- Discard medical instruments in case of exposure to tissues with infectious risk (both high and low risk) (See table below, obtained from Rutala *et al*., 2010[327]).

TABLE 1. Comparative Frequency of Infectivity in Organs, Tissue, and Body Fluids of Humans with Transmissible Spongiform Encephalopathies (Creutzfeldt-Jakob Disease)

Infectious risk[a]	Tissue
High	Brain (including dura mater), spinal cord, posterior eye, pituitary tissue
Low	Cerebrospinal fluid, liver, lymph node, kidney, lung, spleen, placenta, olfactory epithelium
No risk	Peripheral nerve, intestine, bone marrow, whole blood, leukocytes, serum, thyroid gland, adrenal gland, heart, skeletal muscle, adipose tissue, gingiva, prostate, testis, tears, saliva, sputum, urine, feces, semen, vaginal secretions, milk, sweat

NOTE. Modified from Brown[14] and Brown et al,[15] with information from other studies.[16-20]
[a] High risk indicates a rate of transmission to inoculated animals of >50%; low risk indicates a rate of transmission to inoculated animals of ⩾10%–20% (except for lung tissue, for which transmission is 50%); no risk indicates a rate of transmission to inoculated animals of 0% (several tissues in this category had few tested specimens).

When working with high and low risk tissues, transcutaneous exposure (non-intact skin, mucous membranes, splashes to the eye, inoculations via needle, scalpel or other surgical instruments) imposes a higher risk as compared to cutaneous exposure. Still, all types of exposures should be avoided[325].

The safest way to ensure that there is no risk of infection by prion contaminated instruments (or other materials) is through incineration (extensive heating). However, in some situations, another method may be preferred or more practical. In that case, it is important to follow general measures[326]:

- Instruments should be kept moist until decontaminated, to prevent drying and attachment of tissue (use of disinfection methods that can lead to fixation, such as dry heat, aldehydes or alcohols should be avoided).

- Avoid the use of potentially contaminated instruments on non-infected tissues
- Work surfaces should be covered with disposable material which can be removed and incinerated
- Recycle materials according to specialized prion-inactivation methods

The WHO suggests the following prion inactivation/decontamination measures (provided in 1999)[328]:

- Disposable materials, instruments and waste: incineration (extensive heating).

- Re-usable instruments (heat-resistant):

1. Immerse in 1 N NaOH and autoclave at 121°C for 30 min (gravity displacement autoclave), rinse in water. Clean and subject to routine sterilization.
2. Immerse in 1 N NaOH or sodium hypochlorite (20,000 ppm) for 1 hr, transfer to water and autoclave at 121°C for 1 hr (gravity displacement autoclave). Clean and subject to routine sterilization.
3. Immerse in 1 N NaOH or sodium hypochlorite (20,000 ppm) for 1 hr, rinse in water, heat at 121°C or 134°C (in case of porous load) for 1 hr. Clean and subject to routine sterilization.
4. Immerse in 1 N NaOH and boil for 10 min (atmospheric pressure). Clean and subject to routine sterilization.
5. Immerse in 1 N NaOH or sodium hypochlorite for 1 hr. Rinse in water and subject to routine sterilization.
6. Autoclave at 134°C (pre-vacuum autoclave) for 18 minutes.

(All six are considered sufficient)
It is recommended to consult the manufacturer's recommendations, as some handlings may be deleterious.

More recently, comprehensive testing has been done on different inactivation methods for prion-contaminated materials. Rutala *et al.* demonstrate that methods 1,3,6 are most stringent. However, in some cases incomplete inactivation was observed[327]. The USA Center of Disease Control and Prevention suggests that methods 1,2,3 are most appropriate and should be used[329]. Wageningen Bioveterinary Research (WUR) in The Netherlands recommends the use of 2 M NaOH or sodium hypochlorite (20,000 ppm) to disinfect instruments, before autoclaving for 1 hr at 136°C (*personal communication*).

- Re-usable instruments (heat sensitive) and contaminated surfaces:

Flood with 2 N NaOH or undiluted sodium hypochlorite for 1 hour. Soak up the chemicals and rinse with water afterwards.

Prion-related measures have been implemented in the Netherlands (*personal communication*). The WUR decontaminates surfaces either with 2 M NaOH or with sodium hypochlorite (20,000 ppm), depending on the type of material of the surface. The Dutch Prionlab (Nederlands Surveillance

Centrum voor Prionziekten, Dept. Pathology, University Medical Center Utrecht) decontaminates its instruments by using 2 M NaOH (*personal communications*).
The advisory committee on dangerous pathogens spongiform encephalopathy (United Kingdom) advises to not use sodium hypochlorite on open surfaces (e.g. benches) due to possible release of chlorine gas[330].

- Liquid waste:

All liquid waste should be treated with sodium hypochlorite (20,000 ppm) for 1 hr, and should be offered as infectious waste.

This is also recommended by the U.K.'s advisory committee on dangerous pathogens spongiform encephalopathy[325]. The CVI treats liquid waste with 20,000 ppm hypochlorite and subsequent autoclaving for 1 hr at 121°C. Afterwards, the waste is neutralized with hydrochloride.

- Histological examined tissues:

Immerse in 96% formic acid for 1 hour (unless exposed to phenol, as the interaction can be deleterious).

The UMCU Prionlab uses 100% formic acid for 1 hour for cassettes with infective tissue. When it involves tissues sections with only 6µm thickness, it is immersed in 100% formic acid for only 5 min (formic acid penetrates tissue at approximately 1 mm per hour. (*Personal communication*)

Additional considerations:

As indicated above, sodium hypochlorite can be effective for prion-decontamination. However, it should not be used on open surfaces (e.g. benches) as it can induce the release of chlorine gas, and it can corrode metal and steel (and autoclaves). When using sodium hypochlorite, it must be rinsed thoroughly from the instrument before autoclaving. Hypochlorite is incompatible with formaldehyde, alcohol and acids. NaOH is an irritant and should not be used on aluminum or zinc. It can be damaging to the human body and it can be harmful as dust[330].

There is no evidence that TSEs are transmitted by aerosols from contaminated material. However, literature has recently suggested on mice transmission via aerosols[331]. To prevent possible inhalation or splashing of infected material, the use of a microbiological safety cabinet or other primary enclosure is recommended.

There are currently no documented cases of laboratory-associated or occupational transmission of prion disease to a healthcare worker[332]. But maintenance of stringent measures (as described in this paragraph) will be essential.

3.2 Prion-like proteins: ''How to inactivate?''

We have identified several proteins that have been associated with **aggregation** and infectious properties. Although there is no solid evidence that these proteins are identical to prion proteins, being able to transmit disease between / to humans, the current knowledge raises concerns, at least for some of our identified proteins. It is advisable that scientific literature on these matters remains carefully monitored. There are currently no concerns with regards to spreading and disease transmission via oral routes, sexual routes, or consumption of "**amyloid**-contaminated" food. Potential hazards may be more prominent in medical health care (e.g. disease transmission via blood transfusion or transplantation and exposure of medical personal to infectious material) and in the laboratory setting (e.g. when performing function studies on a type of (mutated) protein *in vitro*, in cell culture or in animals). It will be important to obtain clear insights into methods to inactivate / decontaminate the candidate **prion-like** proteins, to provide advice to health care- and laboratory workers, and to implement as obligatory inactivation and containment measures in case this is necessitated by advanced insights on the proteins.

To gain insight in decontamination/inactivation methods for our selected set of proteins, we performed a systematic literature search on the question: "Can aggregated proteins be inactivated?" (searched per March 31, 2016). Please refer to **Table 3** for the search strategy and **Flowchart 2** for the exclusion/inclusion criteria. Our search resulted in eight articles, which we will separately (and grouped per protein) discuss in this chapter.

❖ α-synuclein / amyloid β / tau

- Bousset et al. An Efficient Procedure for Removal and Inactivation of Alpha-Synuclein Assemblies from Laboratory Materials. J Parkinsons Dis. 2016;6(1):143-51.

The authors compared different cleaning solutions to remove α-synuclein oligomers and fibrils from different surfaces. It was found that washing with 1% SDS removes **oligomers** and **fibrils** from the surfaces, but not entirely from plastic. The commercial detergents TDF4 and Hellmanex were able to remove aggregates more efficiently from all surfaces. Methods that are regularly used for prion removal (sodium hypochlorite and sodium hydroxide) did not remove α-synuclein assemblies from these surfaces.

Our remark: Although the authors show that α-synuclein can be displaced from surfaces with SDS or commercial detergents, this does not necessarily mean that the proteins are inactivated. Also, sodium hypochlorite and sodium hydroxide may not remove α-synuclein from surfaces, but is likely to inactivate the proteins.

- Paslawski et al. High stability and cooperative unfolding of alpha-synuclein oligomers. Biochemistry 2014 53: 6252-6263

The authors show that purified α-synuclein **oligomers** can resist heating to 120°C and remain stable at a broad pH range (2.5-11). The oligomers can be dissociated to monomers using high concentrations of urea (≥6 M), but the primary structure remains intact by this procedure.

- Eisele et al. Induction of cerebral beta-amyloidosis: intracerebral versus systemic Abeta inoculation. Proc Natl Acad Sci U S A. 2009 Aug 4;106(31):12926-31.

The authors demonstrate that plasma sterilization can completely block amyloid-β deposition *in vivo*, while heating (in PBS, 10 min, 95°C) cannot.

- Fritschi et al. Aβ seeds resist inactivation by formaldehyde. Acta Neuropathol. 2014 Oct;128(4):477-84.

The authors showed that extracts from formaldehyde-fixed tissues of AD brain or mouse brain can induce amyloid-β deposition in young transgenic APP (amyloid precursor protein) mice. Fixed extracts from different transgenic mice showed **seeding** activity *in vitro* and were also able to maintain their **strain**-like properties when injected into young transgenic APP mice. This implies that amyloid-β deposition is not inactivated by formaldehyde.

- Thomzig et al. Decontamination of medical devices from pathological amyloid-β-, tau- and α-synuclein aggregates. Acta Neruopathol commun 2014 Oct 25:2:151

The authors intended to mimic decontamination of medical devices by using stainless steel wire grids as alternates for medical instruments. Grids were contaminated with brain tissue from AD patients and depletion of **aggregated** α-synuclein, Aβ and tau was tested through an *in vitro* carrier assay. Depletion of all three **aggregates** was observed with 1 N NaOH, 1 hr, RT, or with a mixture of 0.2% SDS/0.3% NaOH (with or without subsequent pre-vacuum steam sterilization).

- serum amyloid A (SAA)

- Omoto et al. Inactivation of amyloid-enhancing factor (AEF): study on experimental murine AA amyloidosis. Med Mol Morphol. 2007 Jun;40(2):88-94.

The authors tested different inactivation methods for AA fibrils (also known as fibril-amyloid enhancing factor, F-AEF), including guanidine compounds and standard sterilization procedures for CJD materials (1 N NaOH and autoclaving for 1 hr at 132°C). Treatment of AA **fibrils** with NaOH (0.1 N or 1 N) plus autoclaving (1 hr, 132°C) resulted in complete inactivation, as evidenced by the absence of amyloid deposits after intraperitoneal injection into mice. Heat led to incomplete inactivation. Mono-treatment of the AA **fibrils** with heat, 0.01 N NaOH; 0.001 N NaOH; pepsin; trypsin; pronase or proteinase K had no effect.

- Ogawa et al. Effect of heating on the stability of amyloid A (AA) fibrils and the intra- and cross-species transmission of AA amyloidosis. Amyloid. 2015;22(4):236-43.

The authors tested the effect of heating (60°C or 100°C) and autoclaving (121°C or 135°C) and showed that AA fibrils are heat stable until 100°C and autoclaving is required to disrupt the structure and pathogenicity of the AA **fibrils**.

❖ apolipoprotein A-II (apo A-II)

- Zhang et al. Transmissibility of mouse AApoAII amyloid fibrils: inactivation by physical and chemical methods. FASEB J. 2006 May;20(7):1012-4.

The authors analyzed the effect of different inactivation methods (physical, chemical and organic) on the ability of AApo A-II fibrils to act as a seed in recipient mice. Treatment with 88% formic acid, 6 M guanidine hydrochloride and autoclaving in 1 N NaOH led to complete disruption of the **fibril** structure and prevented transmission *in vivo*. AApo A-II **fibrils** were found to be resistant to proteinase K, similar to prions. Based on suggestive evidence that antibiotics may disrupt **amyloid** depositions, these were tested as well. This showed *in vitro* disruption of **fibrils**, but marginal effects on **amyloid** deposition *in vivo*. Multiple freeze/thaw cycles showed marginal effects on AApo A-II fibrils as well. This was also the case for 6 M urea; after 144 hr of treatment the transmitted **fibrils** were still able to induce **amyloid** deposition in the recipient mice.

Key points & summary on inactivation of prion-like proteins:

Last years have witnessed a growing demand from clinic and laboratory workers to be advised on inactivation and decontamination, for instance with regards to α-synuclein (*personal communication with the Commissie Genetische Modificatie, COGEM*). The COGEM has previously released an advisory report on how to process materials containing α-synuclein[333, 334]. The COGEM currently recommends to inactivate α-synuclein-containing liquid waste with 8 M urea treatment, followed by autoclaving for 30 min at 121°C. After communication with several biological safety officials (BVF Erasmus MC, BVF Platform) in The Netherlands it is clear that the COGEM's recommendations on α-synuclein are implemented (*personal communication*). Since ammonia is released during autoclaving urea, the BVF of the University Utrecht has added minor changes to the protocol by adding a coagulant to the liquid waste (thereby making it solid), followed by incineration (*personal communication*). It also remains to be seen whether the COGEM's advised inactivation of α-synuclein, consisting of co-treatment with urea and heating, is sufficient. Paslawski *et al.* showed that α-synuclein is heat stable (up to 120°C) and that treatment with urea results in dissociation to monomers, but maintenance of the primary structure[335]. Combining urea and heating, as advised by the COGEM, may be sufficient. If not, the standard inactivation procedures for prion proteins (primarily consisting of NaOH treatment and heating) will probably be suitable. Investigation on this matter is urgently recommended.

As revealed by our in-depth systematic literature search, there is limited data available on methods to inactivate /decontaminate our identified set of proteins with potentially infectious properties. The published research is rather scattered, and contains marginal data with regards to some of the candidate infectious proteins (only for α-synuclein, amyloid β, tau, serum amyloid A and apolipoprotein A-II). Different experimental setups are used (e.g. testing of seeding capacity *in vitro* vs testing in mice) and different types of starting material are used (e.g. purified proteins vs tissue-derived fibrils). **More studies are definitely required**.

4. Conclusions and recommendations

❖ Conclusions:

- **Scientific literature points towards 18 proteins that could possibly be infectious**

In total 18 proteins were revealed by a systematic literature search on the question: "Are aggregated proteins infectious?" (See **Table 2**) The majority of the identified proteins is linked with neurodegenerative diseases.

We have selected the most prominent proteins for our in-depth study:

- **amyloid-β, tau, α-synuclein, huntingtin, superoxide dismutase 1 and TAR DNA-binding protein 43** (associated with neuro-degenerative diseases)
- **serum amyloid A, apolipoprotein A-II**
(associated with systemic amyloidosis in humans and animals)

- **The selected proteins have (at least partly) prion-like characteristics (i.e. seeding, cell-to-cell spreading, strain like properties, transmission)**

Only for TDP-43, seeded aggregation and cell-to-cell spreading has not yet been studied *in vivo*. For serum amyloid A and apolipoprotein A-II, seeded aggregation and cell-to-cell spreading has only been shown *in vitro* and *in vivo*, while cell culture studies have not been done yet. A detailed overview on prion-like properties of the proteins is provided in **Table 4** and **Table 5**.

- **Biochemical properties (e.g. structure, function) of the potentially infectious proteins are different**

All eight proteins selected in this report differ from each other in many ways. They differ in length, structure, function and number of mutations associated with disease. The end product of the aggregations are amyloid structures that primarily consist of beta sheets. The underlying mechanisms that cause misfolding and that provide seeding capabilities (that is, the capability of a misfolded protein to induce misfolding of native proteins) are largely unknown. Misfolding may occur when aggregation-prone domains (that may naturally be hidden, for instance by binding to lipid structures) become exposed. This can be triggered or enhanced by genetic mutations and/or by (largely unknown) external factors.

- **The early stage of aggregation (monomer to oligomer) is believed to be the main factor for cellular toxicity**

Many studies point towards prefibrillar aggregates, rather than mature fibrils, as the cause of cellular toxicity. However, this is still subject of intense research.

- **Both oligomers and fibrils can act as a seeding agent**

- **For the selected proteins, evidence has been obtained in animal or human context on transmission of amyloid proteins that can serve as a seed in the recipient**

Different lines of evidence for transmission have been reported: human-to-graft transmission (α-synuclein, huntingtin, apolipoprotein A-II, amyloid-β), oral transmission (by adding fibrils to water), via cadaveric-growth hormone supplements (amyloid-β), via dura transplantation (amyloid-β) or via vertical transmission through nursing (apolipoprotein A-II). A detailed overview on the different transmission routes is provided in **Table 5**.

- **For the selected proteins, there is currently no scientific evidence that transmission of disease occurs in humans**

For our selected proteins, epidemiological studies on humans (e.g. on patients that received blood transfusions or dura transplantations) do not point towards disease transmission.
At the moment, there is no reason for major concern that any of the eight prion-like proteins are able to cause disease transmission. However, it is important to realize that they potentially could. This may be particularly in laboratory settings, where high concentrations of (mutated) proteins are used.

- **There is no consensus on inactivation methods for prion-like proteins**

As revealed by our in-depth systematic literature search, there is limited data available on methods to inactivate and decontaminate our identified set of proteins. The published research is rather scattered, and contains marginal data with regards to some of the candidate infectious proteins (only for α-synuclein, amyloid β, tau, serum amyloid A and apolipoprotein A-II). More studies are definitely required. Taking together all current knowledge, is seems plausible that treatment based on NaOH (>1 N) (sodium hydroxide) or NaClO (>20,000 ppm) (sodium hypochlorite), followed by heating/autoclaving is sufficient to inactivate all the identified proteins. Such a procedure is in line with the standard procedures used for prion proteins. (Please refer to paragraph 3.1 for detailed descriptions on prion inactivation and decontamination.) Possibly, heating/autoclaving may be left out of the protocol and/or the concentration of NaOH may be lowered. For instance, 0.2% SDS with 0.3% NaOH has been reported as sufficient for α-synuclein, Aβ and tau. Single treatments with other compounds (commonly used in laboratory practice), in particular urea and SDS, seems not sufficient. Still, validation studies are required to obtain definite answers on the appropriate inactivation methods for all proteins identified in our study.

❖ **What's the current evidence for the selected proteins to be prion-like?**

For all eight 'suspicious' proteins, we have summarized the current evidence on prion-like characteristics, by providing a scoring as low, moderate or high evidence. It needs to be emphasized that these scorings are rather subjective. Since current knowledge is insufficient, exact side-by-side comparison between all proteins is currently impossible. Also, comparison is hampered by the proteins' differences in pathological manifestation, i.e. proteins associated with neuro-degenerative diseases versus proteins associated with systemic amyloidosis disease.

- Scorings for the neuro-degenerative disease-associated proteins:

α-synuclein: low/moderate; amyloid-β: moderate; tau: low/moderate; superoxide dismutase 1 (SOD1): low/moderate; huntingtin: low; TAR DNA-binding protein 43 (TDP-43): low

Major scoring criteria: Amyloid-β was scored highest, as it has been associated with iatrogenic transmission in humans (observed after autopsy on patients receiving human growth hormone or dural graft). α-synuclein and tau were scored slightly lower, primarily based on the current minimal evidence in human settings. Still, aggregates of α-synuclein and tau have shown clinical pathology in animal experiments, which warrants careful evaluation in the future. For all proteins, upscaling or downscaling of scoring may occur in the future. Scoring of TDP-43 and SOD1, both associated with ALS, was done side-by-side. Prion-like aggregation, including clinical pathology, has been observed for SOD1 in experimental settings in rodents. TDP-43 has only been tested *in vitro* and in cell cultures (demonstrating seeded aggregation and cell-to-cell spreading), but it remains to be investigated how this protein behaves *in vivo*. Also, SOD1 aggregates are observed in the liver and kidney of ALS patients, which has raised concern with regards to harvesting organs of patients. For these reasons, SOD1 has been scored slightly higher than TDP-43. The huntington protein was scored as 'low evidence', since marginal experimental data is available and there are no severe points of concern from NHP or human settings, despite minimal signs of mutant huntington spread in a host-to-graft direction (observed in a few HD patients).

- Scoring for the systemic amyloidosis-associated proteins:

serum amyloid A (SAA): moderate/high, apolipoprotein A-II (apo A-II): low

Major scoring criteria: SAA is scored relatively high ('moderate / high evidence'), which is primarily based on the knowledge that (i) SAA aggregation can cause disease (systemic amyloidosis) in both humans and animals, (ii) cross-seeding occurs between species and (iii) oral transmission has been demonstrated in animal models, which has raised speculations with regards to horizontal transfer to humans, for example via consumption of contaminated food. These aspects put aggregated SAA protein on the radar as highly-suspicious protein with potentially pathogenic infectious properties. It The apo A-II protein has been scored as 'low evidence', as current knowledge on this protein is very poor. Animal experiments have not been performed and it is unknown whether cross-seeding between species occurs. Also, aggregation of this protein is rarely observed (familial amyloidosis). Still, all scorings are based on current evidence and further research is required to obtain definite answers and to facilitate thorough risk assessment.

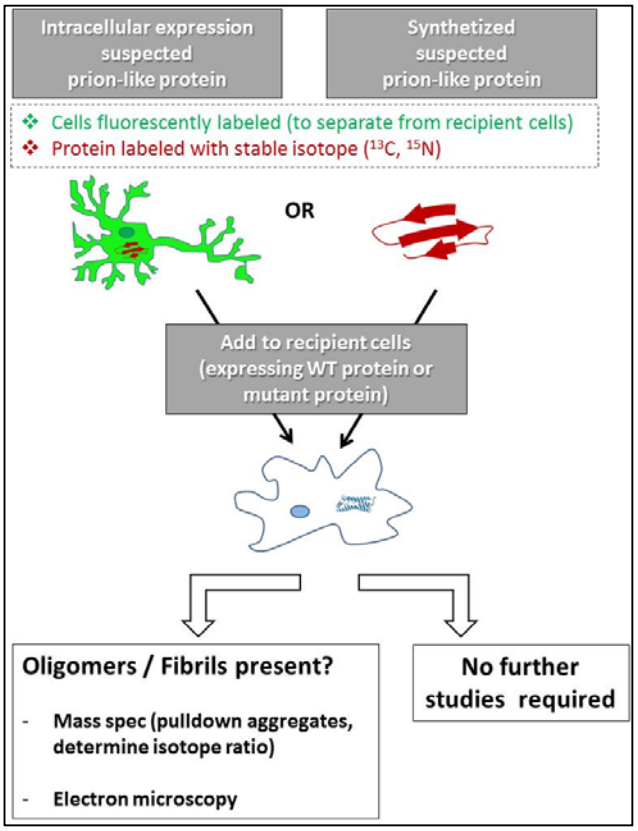
❖ **Recommendations:**

- **Routinely monitor literature**

Our literature study has identified a set of proteins that shows some degree of prion-like characteristics. Although there is no evidence that these proteins are identical to prion proteins, i.e. being able to transmit disease between / to humans, the current knowledge raises concerns, at least for some of our identified proteins. It is advisable to routinely monitor scientific literature. For this, our carefully defined search strategy can be used.

- **To develop a test to define the risk of protein infectivity**

Although major concern is not justified by current scientific knowledge, it is highly recommended to perform further studies on proteins with potential prion-like characteristics. Current research on prion-like proteins lacks standard testing models. Often, proteins are introduced into mice. Although this provides highly valuable information, it also comes with limitations, particularly mouse vs human differences, non-screening compatibility, and high costs. We recommend development of a robust, easy to use, screening-compatible system to define the risk of protein infectivity. Example of such a system, based on cell culture screening and mass-spectrometry-based analysis on protein aggregation is provided below:



Recommended test to define the risk of protein infectivity.

The protein of interest is expressed in cells or prepared as a purified protein and transfer to recipient cells is analyzed. The proteins are labeled with stable isotopes, to discriminate them from endogenous protein in the recipient cells. The capacity of the transferred proteins to act as a seed in the recipient cells, thereby inducing oligomer/fibril formation can be quantified. A protein is scored as highly-suspicious prion-like when both cell-to-cell transfer and oligomer/fibril accumulation occur.

Such a system provides the opportunity to test large numbers of suspect proteins or peptides, and to analyze the effect of particular mutations. Also, effects of cellular factors or external factors can be analyzed. Besides its primary value in “scaling” of suspicious prion-like proteins, the model system may also provide novel insights into the biochemical mechanisms of protein infectivity, which is currently largely unknown.

Cell culture screening may also incorporate the use of human organotypic cultures as recipient cells, to more realistically mimic the natural situation. This could be cerebral organoids (of relevance for proteins related to neurodegeneration) or organoids of systemic organs, such as liver, heart or kidney (of relevance for proteins associated with systemic amyloidosis, such as SAA and apo A-II).

In case a protein is characterized as highly-suspicious prion-like (i.e. both cell-to-cell transfer and oligomer/fibril accumulation occurs) the system and detection equipment can be used to screen the effect of inactivation methods (e.g. NaOH, heat).

This type of experiments will be carried out in specific laboratories that have implemented all necessary measures required for working with prion-like material.

Table 1: Search strategy ''Are aggregated proteins infectious''

Database	Search strategy	Hits
Medline Ovid	("Protein Aggregation, Pathological"/ OR "Protein Aggregates"/ OR "Protein Folding"/ OR ((protein* ADJ6 (aggregat* OR misfold* OR folding OR folded))).ab,ti.) AND ("infection"/ OR "Disease Transmission, Infectious"/ OR (infectious* OR "prion like" OR (transmis*)).ab,ti.) NOT ("Microscopy, Electron, Transmission"/ OR (Transmission electron*).ab,ti.) NOT (letter OR news OR comment OR editorial OR congresses OR abstracts).pt. AND english.la.	1735
Embase	('protein aggregation'/exp OR 'protein aggregate'/de OR 'protein folding'/de OR ((protein* NEAR/6 (aggregat* OR misfold* OR folding OR folded))):ab,ti) AND ('infection'/de OR 'disease transmission'/de OR (infectious* OR 'prion like' OR (transmis*)):ab,ti) NOT ('transmission electron microscopy'/exp OR ('Transmission electron*'):ab,ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim	2379
Cochrane Library	(((protein* NEAR/6 (aggregat* OR misfold* OR folding OR folded))):ab,ti) AND ((infectious* OR 'prion like' OR (transmis*)):ab,ti) NOT (('Transmission electron*'):ab,ti)	0
PubMed (Publisher)	("Protein Aggregation, Pathological"[mh] OR "Protein Aggregates"[mh] OR "Protein Folding"[mh] OR ((protein*[tiab] AND (aggregat*[tiab] OR misfold*[tiab] OR folding OR folded))) AND ("infection"[mh] OR "Disease Transmission, Infectious"[mh] OR (infectious*[tiab] OR "prion like" OR (transmis*[tiab]))) NOT ("Microscopy, Electron, Transmission"[mh] OR (Transmission electron*[tiab])) NOT (letter[pt] OR news[pt] OR comment[pt] OR editorial[pt] OR congresses[pt] OR abstracts[pt]) AND english[la] AND publisher[sb]	44
Web of Science	TS=((((protein* NEAR/5 (aggregat* OR misfold* OR folding OR folded)))) AND ((infectious* OR "prion like" OR (transmis*))) NOT (("Transmission electron*"))) AND DT=(article) AND LA=(english)	1615
Google Scholar	protein aggregation|aggregated|misfolding|misfolded infectious|"prion like"|transmission -"Transmission electron"	175

Flowchart 1: Inclusion strategy: ''Are aggregated proteins infectious?''

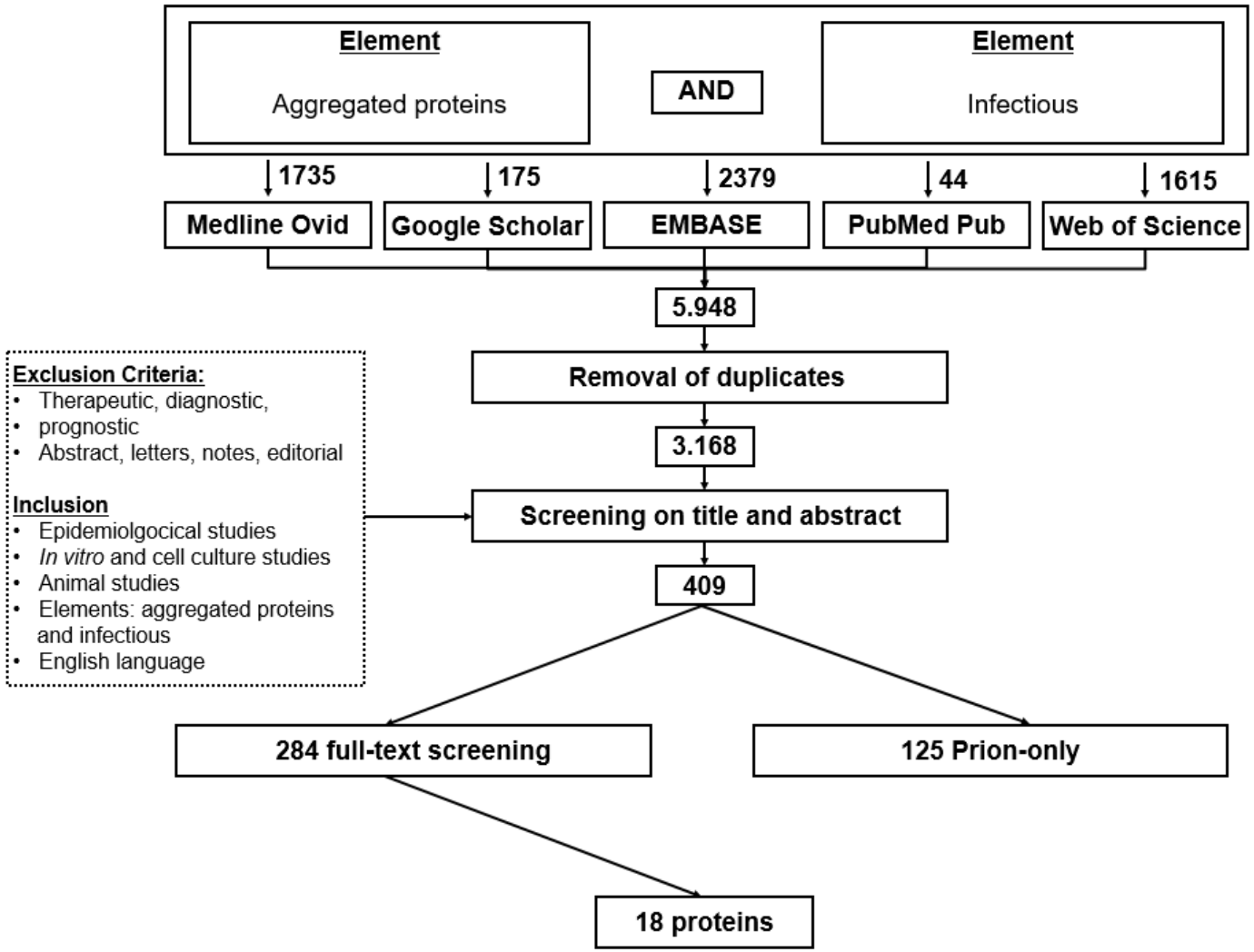


Table 2: Protein hits after systematic literature search on "Are aggregated proteins infectious?"

Proteins														Counted prevalence (after full-text screening)
	Seeded aggregation			Cell-to-cell spreading		Routes of transmission*								
	In vitro	Cell culture	In vivo	Cell culture	In vivo	Oral/fecal	Surgical tools	Organ transplant	GH	Dural graft	Blood transfusion	Vertical transmission	Sexual transmission	
prions	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NF	198
a-synuclein	Yes	Yes	Yes	Yes	Yes	NF	NF	NF	NF	NF	NF	NF	NF	112
amyloid-β	Yes	Yes	Yes	Yes	Yes	NF	NF	NF	Yes	Yes	NF	NF	NF	85
tau	Yes	Yes	Yes	Yes	Yes	NF	NF	NF	NF	NF	NF	NF	NF	69
SOD-1	Yes	Yes	NF	Yes	NF	NF	NF	NF	NF	NF	NF	NF	NF	32
mutated huntingtin	Yes	Yes	NF	Yes	NF	NF	NF	NF	NF	NF	NF	NF	NF	24
TDP-43	Yes	Yes	NF	Yes	NF	NF	NF	NF	NF	NF	NF	NF	NF	19
amyloid apolipoprotein A-II	NF	NF	Yes	NF	Yes	Yes	NF	NF	NF	NF	NF	Yes	NF	5
serum amyloid A	NF	NF	Yes	NF	Yes	Yes	NF	NF	NF	NF	NF	NF	NF	3
P53	NF	Yes	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	7
IAPP	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	7
FUS	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	6
transthyretin	NF	NF	NF	NF	NF	NF	NF	Yes	NF	NF	NF	NF	NF	3
C90RF72	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	2
B2-microglobulin	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	2
OPTN	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	1
UBQLN2	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	1
myelin basic protein	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	1

All proteins identified after a systematic literature search on: ''Are aggregated proteins infectious'' (see Table 1 and Flowchart 1 for details about the search strategy. Yes: prion-like mechanism mentioned in screened articles. NF: Route of transmission not found after full-text screening (NF).

Table 3: Search strategy ''Can aggregated proteins be inactivated''

Database	Search strategy	Hits
Medline Ovid	("Amyloid beta-Peptides"/ OR "alpha-Synuclein"/ OR "TDP-43 Proteinopathies"/ OR "tau Proteins"/ OR "Serum Amyloid A Protein"/ OR "Apolipoprotein A-II"/ OR ((amyloid ADJ1 (beta)) OR ((alpha) ADJ3 synuclein*) OR TDP-43 OR TDP43 OR ((htt OR huntington OR hd) ADJ3 protein*) OR tau OR superoxide-dismutase-1 OR sod1 OR sod-1 OR sods-1 OR superoxide-dismutase-i OR sodi OR sod-i OR sods-i OR CuZnSOD OR Cu-ZnSOD OR CuZn-SOD OR Cu-Zn-SOD OR (Superoxide-dismutase ADJ3 (Cu-Zn OR Zn-Cu OR copper-zinc OR zinc-copper)) OR huntingtin* OR serum-amyloid-A OR serum-amyloid-protein-A OR saa OR ((apolipoprotein OR apo) ADJ (A2 OR A-2 OR Aii OR A-ii))).ab,ti.) AND ("Decontamination"/ OR "Detergents"/ OR "Disinfection"/ OR (Decontaminat* OR reagent* OR detergent* OR Deactivat* OR inactivat* OR cleaning OR cleansing OR disinfect*).ab,ti.) NOT (letter OR news OR comment OR editorial OR congresses OR abstracts).pt. AND english.la.	2279
Embase	('amyloid beta protein'/de OR 'amyloid beta protein[1-42]'/de OR 'amyloid beta protein[1-40]'/de OR 'alpha synuclein'/de OR 'TDP 43 proteinopathy'/de OR 'tau protein'/de OR 'copper zinc superoxide dismutase'/de OR 'huntingtin'/de OR 'serum amyloid A'/de OR 'apolipoprotein A2'/de OR ((amyloid NEAR/1 (beta OR β)) OR Aβ OR ((alpha OR α) NEAR/3 synuclein*) OR TDP-43 OR TDP43 OR ((τ OR htt OR huntington OR hd) NEAR/3 protein*) OR tau OR ((τ) NEAR/3 (human OR isoform* OR inactiv*)) OR superoxide-dismutase-1 OR sod1 OR sod-1 OR sods-1 OR superoxide-dismutase-i OR sodi OR sod-i OR sods-i OR CuZnSOD OR Cu-ZnSOD OR CuZn-SOD OR Cu-Zn-SOD OR (Superoxide-dismutase NEAR/3 (Cu-Zn OR Zn-Cu OR copper-zinc OR zinc-copper)) OR huntingtin* OR serum-amyloid-A OR serum-amyloid-protein-A OR saa OR ((apolipoprotein OR apo) NEXT/1 (A2 OR A-2 OR Aii OR A-ii))):ab,ti) AND ('reagent'/de OR 'detergent'/de OR 'cleaning'/de OR 'disinfection'/de OR (Decontaminat* OR reagent* OR detergent* OR Deactivat* OR inactivat* OR cleaning OR cleansing OR disinfect*):ab,ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim	2762
Cochrane Library	(((amyloid NEAR/1 (beta OR β)) OR Aβ OR ((alpha OR α) NEAR/3 synuclein*) OR TDP-43 OR TDP43 OR ((τ OR htt OR huntington OR hd) NEAR/3 protein*) OR tau OR ((τ) NEAR/3 (human OR isoform* OR inactiv*)) OR superoxide-dismutase-1 OR sod1 OR sod-1 OR sods-1 OR superoxide-dismutase-i OR sodi OR sod-i OR sods-i OR CuZnSOD OR Cu-ZnSOD OR CuZn-SOD OR Cu-Zn-SOD OR (Superoxide-dismutase NEAR/3 (Cu-Zn OR Zn-Cu OR copper-zinc OR zinc-copper)) OR huntingtin* OR serum-amyloid-A OR serum-amyloid-protein-A OR saa OR ((apolipoprotein OR apo) NEXT/1 (A2 OR A-2 OR Aii OR A-ii))):ab,ti) AND ((Decontaminat* OR reagent* OR detergent* OR Deactivat* OR inactivat* OR cleaning OR cleansing OR disinfect*):ab,ti)	8

Flowchart 2: Inclusion strategy: ''Can aggregated proteins be inactivated?''

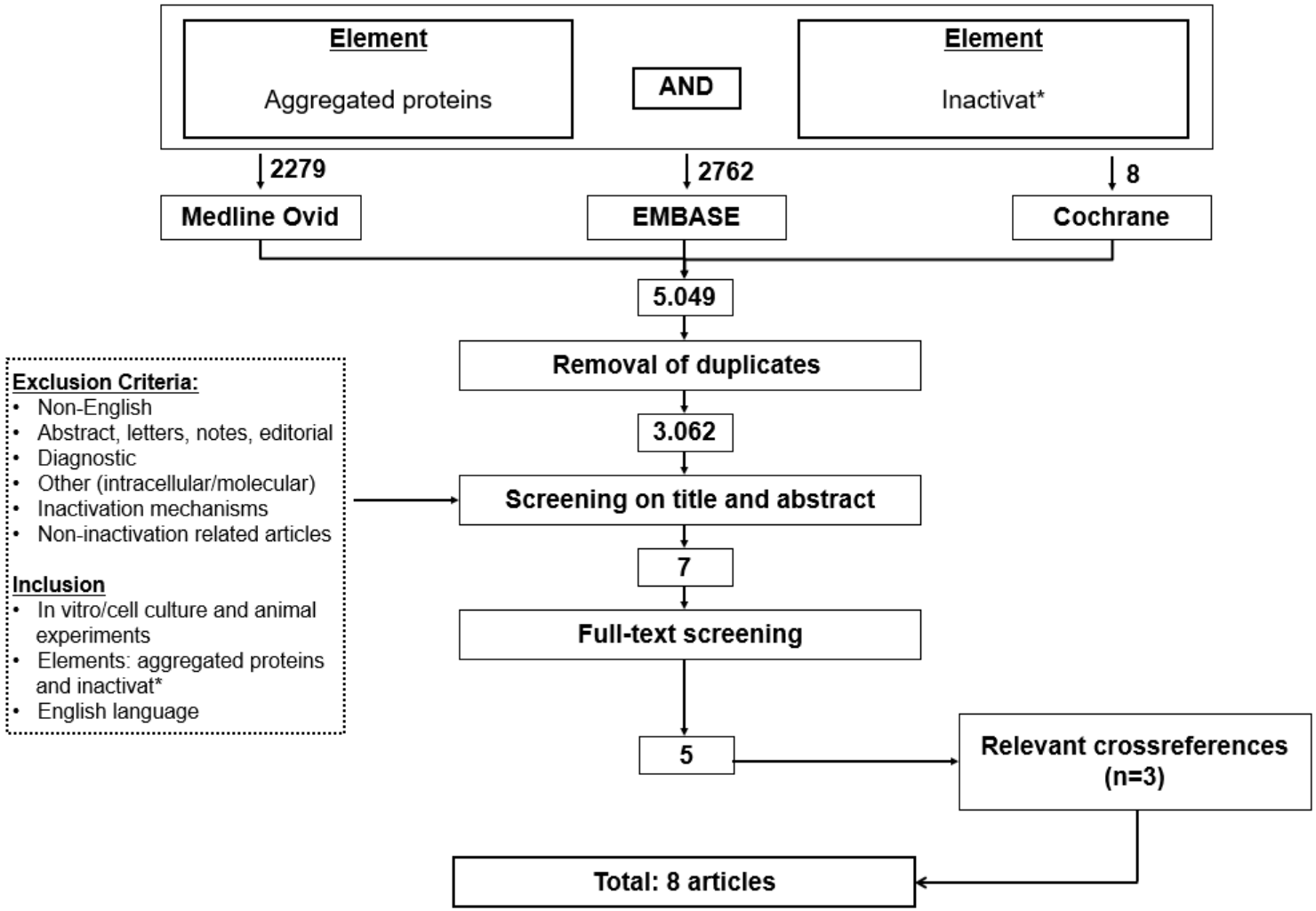


Table 4: Experimental evidence on prion-like mechanisms

			Main localization		**Seeded aggregation**			**Cell-to-cell spreading**		**Strains-like properties**	**Inducible clinical pathology**
Native protein	Aggregated form	Associated diseases in humans and animals	Native	Aggregates	In Vitro	Cell Culture	In vivo*	Cell culture	In vivo*	In vitro/ cell culture/ in vivo	In vivo*
PrP^{c}	PrP^{sc}	Creutzfeldt-Jakob (H) Kuru (H) Fatal familial insomnia (H) Gerstmann-Straussler Scheinker (H), Scrapie (A), Bovine spongiform encephalopathy (A), Chronic wasting disease (A) Feline spongiform encephalopathy (A), Transmissible mink encephalopathy (A)	Plasma membrane (anchored)	Mostly extracellular	Yes[19,20]	Yes[21,22]	Yes[23]	Yes[21,22]	Yes[4,23]	Yes[6]	Yes[4]
α-synuclein	α-synuclein	Parkinson (H), Lewy body dementia (H), Multiple system atrophy (H)	Presynaptic	Mostly cytoplasmic	Yes[88-90]	Yes[91-94]	Yes[97, 98]	Yes[92, 94, 100-102, 108]	Yes[110] (NHP) Yes[105]	Yes[95, 96]	Yes[97, 98]
APP	amyloid-β	Alzheimer (H)	Trans-membrane	Mostly extracellular	Yes[19,150, 151]	Yes[152-155]	Yes[158]	Yes[163,164]	Yes[158,159,161,162]	Yes[158]	No
tau	tau	Frontotemporal lobar dementia (H), Alzheimer (H)	Cytoplasmic	Cytoplasmic	Yes[178.179(	Yes([93,181-183]	Yes[185-187,191]	Yes[181]	Yes[185,187,191,193]	Yes[178,183, 184,188]	Yes[186] (memory deficit)
SOD1	SOD1	Amyotrophic lateral Sclerosis (H)	Cytoplasmic	Cytoplasmic	Yes[212]	Yes[213,214,218]	Yes[222.223.224,227]	Yes[214, 217,225]	Yes[222,224,227]	Yes[223,224]	Yes[227]
huntingtin	PolyQ	Huntington (H)	Nuclear	Nuclear	Yes[245,246]	Yes[245,247,248]	Yes[249]	Yes[249-251]	Yes[249]	Yes[245]	No
TDP-43	TDP-43	Amyotrophic Lateral Sclerosis (H), Frontotemporal lobar degeneration (H)	Nuclear	Mostly cytoplasmic	Yes[2,271,	Yes[267,271,272]	ND	Yes[272,273]	ND	Yes[271]	ND
SAA	AA	Systemic (AA) Amyloidosis (H and A)	Plasma	Extracellular	Yes([286]	ND	Yes[2,278,288,290]	ND	Yes[2,278,290]	Yes[296]	No
apo A-II	AApo A-II	Familial amyloidosis (H) Mouse senile amyloidosis (A)	Plasma	Extracellular	Yes[312,315]	ND	Yes[313-315,319,321]	ND	Yes[313-315,316,318,321]	Yes[316]	Yes[321]

Summary on the proteins with reported prion-like characteristics. Information is provided on type of disease and clinical/subcellular localization, and presence or absence of different prion-like characteristics. PrP^{c}: cellular prion protein**,** PrP^{sc}: pathological prion protein, APP: amyloid precursor protein, PolyQ: polyglutamine, SOD1: superoxide dismutase 1, TDP-43: TAR DNA binding protein 43, SAA: serum amyloid A, apo A-II: apolipoprotein A-II, AApo A-II: amyloid apolipoprotein AII. ND: not determined (there is no conclusive experimental or epidemiological data available/performed yet) H: humans, A: Animals, NHP: Non-human primates. *In vivo models are rodent models unless indicated otherwise.

Table 5: Evidence on different transmission routes of disease

(Routes of transmission observed in experimental settings in rodents, unless indicated otherwise (NHP=non-human primate, H=human)

Disease related protein	Exogenous injection	Oral/Saliva/ Aerosol	Surgical tools	Organ transplant	Host- to-graft (H-G)/ graft-to-host (G-H) transplant	c-hGH	Dural Graft	Blood transfusion	Vertical	Sexual
PrP^{sc}	Yes Intracerebral[4] Intravenous[4] Intraperitoneal[4] Intranasal[4] Intraocular[4]	Yes[4](oral) (Zoonotic) Yes[4] (saliva) Yes[331] (aerosol)	Yes[4]	Yes[4]	Yes[4]	Yes[4]	Yes[4]	Yes[4]	Yes[336, 337]	ND
α-synuclein	Yes Intracerebral[105,110,] (NHP) intramuscular[338]	ND	ND	ND	(H) Yes H-G[111,112] (H) No H-G[114]	(H) No[115] (epidemiological)	ND	ND	ND	ND
amyloid-β	Yes Intracerebral[158] Intraperitoneal[162] No Intravenous[161] Intraocular[161] Intranasal[161]	No[161] (oral)	Yes[161] (steel wires)	ND	H-G Yes[166]	(H) No[115] (epidemiological) Yes[169] (autopsy)	(H)Yes[170] autopsy	(H) No[167.168]	ND	ND
tau	Yes Intracerebral[183-187,191] Intraperitoneal[193]	ND	ND	ND	ND	(H) No[169] (autopsy)	ND	ND	ND	ND
SOD1	Yes Spinal cord([222, 224]	ND	ND	ND	Yes[227] G-H	ND	ND	ND	ND	ND
Huntingtin	Yes Intracerebral[249]	ND	ND	ND	(H Yes[252] H-G) Yes[249] H-G	ND	ND	ND	ND	ND
TDP-43	No	ND	ND	ND	ND	ND	ND	ND	ND	ND
AA	Yes Intravenous[278,291-293,295,296,302] Intraperitoneal[293]	Yes[278,289,391,293] (oral)	ND	ND	ND	ND	ND	ND	ND	ND
ApoAII	Yes Intravenous[313,314,316,317] Intraperitoneal[315,318] Intragastric[315]	Yes[318](oral)	ND	ND	Yes[319] G-H (1x)	ND	ND	ND	Yes[321]	ND

Summary of current evidence on different routes of transmission. ND: Not Determined (no scientific reporting available). PrP^{sc}: pathological prion protein, SOD1: superoxide dismutase 1, TDP-43: TAR DNA binding protein 43, AA: amyloid A, ApoAII; apolipoprotein-AII. Host-to-graft: "Clean" grafted material (cells or implant) obtains amyloid accumulation . Graft-to-host: "Clean" host tissue obtains amyloid accumulation from "contaminated" (amyloid-containing) grafts.

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