Targeting Cytotoxic Species in Amyloid Diseases

Malin Lindhagen Persson
Till min familj.
För att vi har sett nog av den där sjukdomen.
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AMERICAN UNIVERSITY IN CAIRO

ABSTRACT

Amyloid diseases, a group of more than 25 disorders including Alzheimer’s disease (AD) and Parkinson’s disease (PD), are a world-wide problem causing great human suffer and a large economical cost. Consequently, it is urgent to deepen the understanding of the etiology behind these diseases and to find new potential therapies. Amyloid deposits, fibrillar assemblies of β-sheet rich proteins or peptides, are a common denominator in all amyloid disorders. Although amyloid deposits are detrimental to the surrounding tissue, there is a poor correlation between total amyloid burden and clinical symptoms. Prefibrillar soluble assemblies, denoted soluble oligomers, are much more potent to exert a tissue damaging effect. The aim of this thesis was to study cytotoxic species in amyloid diseases: why and how are the cytotoxic species formed and how can their cytotoxic potential be omitted?

AD is strongly linked to self-assembly of the amyloid-β (Aβ) peptide. Studies have shown that the most cytotoxic species is a soluble oligomer with a composition varying from dimer/trimer to 12/24-mer. Antibodies selectively targeting these cytotoxic species are useful both for understanding oligomer formation and for their therapeutic abilities. AD mice-models can be fully attenuated upon passive immunization with anti-Aβ antibodies and a number of clinical trials are ongoing in humans based on this rational. However, the mechanism by which certain antibodies gain selectivity for oligomeric species is not completely understood, making it difficult both to produce and to improve these antibodies. We have noted that the binding of antibodies is significantly enhanced through the effect of avidity, where a synergistic effect of binding strength is acquired as a result of multiple binding sites. Oligomeric assemblies, per definition, always represent a multimeric antigen. We hypothesized that the effect of avidity would compensate for a low single site affinity and be enough to selectively target oligomers. To evaluate this hypothesis, we focused on the IgM isotype having ten antigen-binding sites. In accordance with the hypothesis, the IgM isotype effectively bound oligomeric Aβ also in presence of a vast excess of its monomeric counterpart, clearly illustrating the potentiating effect of avidity. The antibody could moreover rescue neuroblastoma cells from Aβ induced cytotoxicity at highly substoichiometric ratios. As a continuation of this work, we have shown that the avidity effect from a bivalent binding is enough to induce oligomer specificity. This finding facilitates a direct application on the clinically more useful IgG isotype, where the binding properties now can be controlled in detail. The method is general and we have, using this technique, also designed oligomer specific antibodies targeting α-synuclein associated with PD.
Transthyretin (TTR) is an amyloidogenic protein involved in familial amyloidotic polyneuropathy, familial amyloidotic cardiomyopathy, and senile systemic amyloidosis. The cytotoxicity of TTR is intriguing since studies have shown cytotoxic potential from oligomers, tetramers and even monomers. Elucidation of the molecular properties associated with TTR cytotoxicity is hence of interest.

By preventing tetramer dissociation, TTR aggregation and TTR-induced cytotoxicity is abolished. Based on this rationale, a current therapeutic strategy is to stabilize the TTR tetramer with small molecules. The kinetic stability within the spectra of known TTR mutations spans more than three orders of magnitude. However, although the most stable mutants are inert, a poor correlation within the group of cytotoxic variants exists where the cytotoxic effect is not potentiated in proportion to their kinetic stability. Through analysis of a large spectra of TTR variants, our results indicate that TTR induced cytotoxicity requires an intermediate stability of the TTR molecule. The kinetic stability should be low enough to permit tetramer dissociation and the thermodynamic stability high enough to prevent instant aggregation and to allow formation of the cytotoxic fold.
This thesis is based on the following papers, which will be referred to by their roman numerals (Paper I-IV).


III. \textbf{Lindhagen-Persson M}, Vestling M, Reixach N, Olofsson A (2008) Formation of cytotoxic transthyretin is not dependent on inter-molecular disulphide bridges commonly found within the amyloid form. \textit{Amyloid} 15, 240-5

Contributions have also been made to the following papers, not included in this thesis:


<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid Beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AβPP</td>
<td>amyloid beta precursor protein</td>
</tr>
<tr>
<td>ATTR</td>
<td>transthyretin-related amyloidosis</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cys10</td>
<td>the single cysteine at position 10 in transthyretin</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAC</td>
<td>familial amyloidotic cardiomyopathy</td>
</tr>
<tr>
<td>FAP</td>
<td>familial amyloidotic polyneuropathy</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>IAPP</td>
<td>islet amyloid polypeptide</td>
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<tr>
<td>IVIg</td>
<td>intravenous immunoglobulins</td>
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<tr>
<td>k_{ass}</td>
<td>association rate constant</td>
</tr>
<tr>
<td>K_D</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>k_{diss}</td>
<td>dissociation rate constant</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>MMSE</td>
<td>mini-mental state examination</td>
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<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
</tr>
<tr>
<td>NGAL</td>
<td>neutrophil gelatinase-associated lipocalin</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
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<tr>
<td>RAGE</td>
<td>receptor for advanced glycated endproducts</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol binding protein</td>
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<tr>
<td>SAP</td>
<td>serum amyloid P component</td>
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<tr>
<td>ScFv</td>
<td>single-chain variable fragment</td>
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<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>SSA</td>
<td>senile systemic amyloidosis</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TBG</td>
<td>thyroid binding globulin</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavinT</td>
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<tr>
<td>TTR</td>
<td>transthyretin</td>
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ENKEL SAMMANFATTNING

Proteiner

Proteiner bygger upp celler och ansvarar för många av de processer som sker inuti och mellan celler. Exempel på funktioner som proteinerna har är att vara antikroppar och försvara kroppen mot främmande ämnen, att bygga upp kanaler i cellmembranet för att möjliggöra transport av små ämnen in och ut ur cellen och att fungera som enzym i kemiska reaktioner.

Det som gör att proteinerna kan ha så vitt skilda uppgifter är att de alla har en egen form. Formen bestäms till stor del av ordningen på de aminosyror som bygger upp alla proteiner. Efter att aminosyrorna satts i rätt ordning i ribosomerna inuti cellen börjar den långa kedjan av aminosyror att vecka sig till sin specifika form. Ibland går något fel i veckningsprocessen och proteinet får en fel form vilket gör att proteinet antingen tappar sin funktion eller att det får en ny egenskap som den inte borde ha haft. I många fall stoppas proteiner med fel form redan inuti cellen. De felformade proteinerna skickas till proteasomerna inuti cellen och bryts ner så att de inte kan göra någon skada.

Ibland händer det att proteiner med fel form inte bryts ner i proteasomerna utan istället tillåts att existera antingen inom eller utanför cellen. Då kan det hända att proteinet börjar klumpa ihop sig, aggregera, med andra felformade proteiner av samma sort. De här proteinsammansättningarna kan orsaka skada i kroppen och leda till att den människa som drabbas av dem blir sjuk, hon/han har fått en amyloid sjukdom.

Amyloida sjukdomar

Idag känner man till ungefär 27 olika proteiner som kan klumpa ihop sig inuti kroppen och orsaka sjukdom. Det vanligaste exemplet på amyloid sjukdom är Alzheimers sjukdom som faktiskt mer än 36 miljoner människor i världen lider av. Exempel på andra amyloida sjukdomar är Parkinsons sjukdom, Creutzfeldt-Jakobs sjukdom (mänsklig motsvarighet till galna kosjukan) och transthyretin amyloidos som i Sverige är känt som Skellefteåsjukan.

Anledningen till att de här sjukdomarna kallas amyloida sjukdomar är att proteinerna som ligger bakom dem inte bara slumpmässigt klumpar ihop
sig, de bildar istället en väldigt speciell form som kallas amyloid. Amyloid ser likadant ut när man tittar på det i ett mikroskop oavsett vilket protein amyloiden är bildat av. Amyloiden består av långa trådar, fibriller, av det ihopklumpade proteinet. Fibrillerna är långa och olösliga vilket gör att de fastnar i olika vävnader och organ utan att kunna transporteras bort.

Även om amyloiden orsakar stor skada i de kroppsvävnader där de fastnar tror man att det egentligen är mindre och lösliga klumpar av proteinerna som mer akut dödar de omgivande cellerna. Det är de här mindre, lösliga och giftiga proteinsammansättningarna som jag har studerat under min tid som doktorand.

**Giftiga former av amyloida proteiner**

Oavsett om proteinet från början har en ostrukturerad form (som Aβ i Alzheimers sjukdom) eller om proteinet normalt har en fast form som av någon anledning, tex mutationer, ändras och blir mindre stabil och mer aggregeringsbenägen (som transthyretin i Skellefteåsjukan) så startar aggregeringen med att en instabil form av proteinet bildas. Proteinet vill alltid bli så stabilt som möjligt och kommer därför att göra vad det kan för att bli mer stabilt. Ett sätt att bli mer stabiliserat är att aggregera eftersom amyloid, slutsteget i aggregeringsprocessen, är mycket stabilt.

När det gäller Aβ som är det ihopklumpande proteinet i Alzheimers sjukdom börjar det att aggregera antingen när Aβ är muterat och därför instabilare eller när det finns för mycket Aβ i hjärnan. Anledningen till att för mycket Aβ finns kan vara antingen att man bär på mutationer som gör att mer Aβ produceras eller att någongt har hänt som gör att det Aβ som bildas inte förs bort från hjärnan som det ska. När Aβ börjar aggregera bildas små sammansättningar av proteinet där två eller flera Aβ molekyler har fastnat i varandra. Dessa små klumpar vet man påverkar nervceller och synapser både i cellkulturer och i hjärnan på möss och råttor.

**Att hitta igen de giftiga formerna av amyloida proteiner**

Vetskapen om att de små Aβ sammansättningarna gör stor skada på nervceller har gjort att mycket forskning fokuserar dels på hur man ska kunna känna igen de här formerna av Aβ och dels på hur man ska kunna rädda nervcellerna från dessa. Ett sätt är att använda antikroppar. Antikroppar kan användas i laboratorium när man studerar Aβ och som en möjlig behandling för att bota Alzheimerssjuka.
Det stora inom Alzheimersforskningen har varit att försöka hitta antikroppar som endast känner igen de giftiga formerna av Aβ. Dessa antikropp brukar man kalla konformationsspecifika eftersom de bara reagerar med en specifik form av proteinet. Även om det finns sådana antikroppar inom forskningsvärlden så är det ingen som vet hur de egentligen bildas. Det är alltså svårt att påverka om man ska få en konformationsspecifik antikropp eller en ”vanlig” antikropp när man gör egna antikroppar.


Det finns flera olika typer av antikroppar i kroppen, en variant är IgM-antikroppen som har tio olika bindningsarmar. Vi testade vår hypotes på en IgM-antikropp som vi gjort och fann precis som vi misstänkte att den hittade igen giftiga Aβ-former medan den inte hittade igen de ofarliga singelmolekylerna av Aβ. Vi kunde också visa att vår IgM-antikropp kunde rädda odlade nervceller när vi tillsatte den till cellerna tillsammans med giftigt Aβ. Dessutom testade vi IgM som vi utvunnit från plasma från ett antal friska individer och såg att de IgM-antikropparna som fanns i plasma och som reagerade med Aβ också främst kände igen de giftiga formerna av Aβ.

Även om IgM finns naturligt i människokroppen så är det en ganska svår molekyl att arbeta med då den är svår att framställa och ej heller kan förändras på gentisk väg. Detta gör att IgM inte effektivt kan användas som behandlingsform och inte heller kan användas för att mer ingående studera aviditetseffekten. Vi bestämde oss därför för att testa om aviditetseffekten som man får av en antikropp med två bindningsarmar skulle räcka för att hitta de giftiga formerna av Aβ. IgG är en vanlig antikropp i kroppen, den har två bindningsarmar och är också vanlig i olika behandlingsformer/vaccin.

**Giftiga former av transthyretin**


För transthyretin har man visat att det är små transthyretin-sammansättningar som är giftiga för cellerna. Det verkar främst vara monomerer och nativlika tetramerer som är giftiga medan större aggregat, över 20 transthyretinmolekyler tillsammans, är ogiftiga. Kunskapen om varför transthyretin blir giftigt är dock liten. Vi har försökt reda ut olika faktorer som kan påverka giftigheten av transthyretin.

Vi började med att studera huruvida disulfidbryggor är viktiga för att transthyretin ska bli giftigt. Disulfidbryggor är en typ av kovalent binding som kan finns naturligt mellan vissa aminosyror i ett protein. Disulfidbryggornas uppgift är att stabilisera proteinet. När det gäller transthyretin har man visat att disulfidbryggor finns naturligt i amyloiformen av proteinet och man har föreslagit att dessa bindingar
också skulle vara viktiga för giftigheten av proteinet. Vi muterade bort den aminosyra som ansvarar för att bilda disulfidbryggor och kunde visa att transthyretin fortfarande var giftigt för nervceller. Transthyretin behöver alltså inte bilda disulfidbryggor för att vara giftigt.

INTRODUCTION

1. Protein folding and misfolding

1.1 Proteins and structure

Proteins are responsible for essentially all cellular functions within a living organism. The function of a protein is to a great extent dependent on its fold which, in turn, is dependent both on the milieu in which the protein is expressed and folded and on the sequence of amino acids in the polypeptide chain [1].

The building blocks of all proteins are 20 different amino acids. By changing the sequence of amino acids in the polypeptide chain, all different existing proteins can be obtained. Amino acids consist of one amino group, one carboxyl group, one α-carbon, and one side chain. The carboxyl group of one amino acid forms a covalent bond with the amino group of another amino acid resulting in a polypeptide chain. The side chains, determining the chemical properties of the amino acids, are usually divided into four different groups: acidic, basic, polar, and nonpolar.

Proteins have four levels of structure. The primary structure is the linear sequence of amino acids. The secondary structure consists of three main folds: α-helix, β-sheet, and random coil. α-helixes and β-sheets can be formed by any amino acid since they are stabilized by hydrogen bonds between the N-H and the C=O group in the backbone of the polypeptide chain. These secondary structures are arranged in regard to each other into a tertiary structure, a protein domain. The quaternary structure is formed when several protein domains interact to form a hetero- or homooligomeric protein complex.

1.2 Folding

The polypeptide chain begins to fold instantly after ribosomal synthesis. For some proteins, the N-terminal of the protein has attained most of its secondary structure even before the C-terminal is translated [2]. Other proteins fold in the cytosol or in cellular compartments, such as
mitochondria or endoplasmic reticulum (ER), after ribosomal release. The initial folded state is frequently called a molten globule and defines an intermediate state towards forming the final native globular structure. The final folded state might require side chain modifications or the help of chaperons to form [3].

The goal of the protein is to obtain the fold that has the least amount of free energy. Within a chemical reaction, the amount of free energy is defined using Gibb’s law: \( G = H - TS \) where \( G \) is the free energy, \( H \) is the enthalpy, \( T \) is the temperature, and \( S \) is the entropy. Because the absolute value of free energy is hard to define, the amount of free energy is usually described in the terms of free energy change: \( \Delta G = \Delta H - T\Delta S \). A specific reaction is defined by the difference in free energy between the initial and the final state according to \( \Delta G_{\text{total}} = \Delta G_{\text{product}} - \Delta G_{\text{reactant}} \). Reactions with a negative \( \Delta G \) release energy and will happen spontaneously. The size of \( \Delta G \) reveals nothing about the rate of the reaction, which instead is controlled by the height of intervening activation barriers.

![Figure 1. The protein folding funnel.](image)
The unfolded protein starts of at the top of the funnel at a high energy state. Independent of which folding path the protein takes, the result will be the same – the fold with the lowest amount of free energy.
Protein folding can be described as any chemical reaction where the different components of the above given reaction are defined by its fold. Folding of a free peptide chain is frequently a favourable reaction. The complexity and number of different conformations that are possible within even a rather short polypeptide is immense. Yet, the folding frequently occurs within fractions of a second. The current model regarding how this is obtained in nature is that folding of a peptide is initiated on a local level where the number of possible conformations is limited. The folding is subsequently propagated throughout the peptide chain. This model is frequently visualized as a funnel (see fig.1). The polypeptide chain starts of at the top of the funnel with a high amount of free energy. As the polypeptide chain is arranged into a folded state, both the entropy and the enthalpy decreases. The native structure of the protein is typically the fold with the lowest free energy, explaining why the same polypeptide sequence obtains the same fold [4].

1.3 Forces influencing the protein fold

The folding of a protein is mainly constrained by weak forces. Even though they, in comparison to covalent bonds, are weak, they act together to generate a stable protein fold. The forces are hydrogen bonds, ionic bonds, van der Waals attractions, and hydrophobic force. While the first three forces can involve atoms in both the backbone and the side chain of amino acids, the hydrophobic force is determined by the polarity of the side chains. Amino acids with non-polar (hydrophobic) side chains tend to face each other in the internal part of the protein, whereas polar (hydrophilic) amino acids constitute the outside of the protein making hydrogen bonds with the surrounding water. Folding related to hydrophobicity is entropically favourable [5].

In addition, disulphide bridges are commonly found within and between protein domains. Disulphide bridges are covalently linked sulphur-sulphur bonds that form between the sulphur containing side chains of cysteines. Since covalently linked, disulphide bridges have a great impact on protein stability and are particularly important for proteins secreted extracellularly [6].
1.4 Protein misfolding

The protein production machinery in the cell is constantly working. A portion of all formed proteins, in fact as much as 1/3, obtains an incorrect fold and are thus not functioning as they are intended to. Misfolded proteins are not only unnecessary space filling in the already crowded cell (300 mg/ml); they can also be directly dangerous to the cell. Therefore, the cell has developed a system for protein quality control. Proteins exposing large hydrophobic regions, a sign of misfolding, can either be covered with chaperons for possible refolding or marked with ubiquitin for degradation in the proteasome [7]. If refolding and degradation fail, the hydrophobic surface of a misfolded protein can clump together with other misfolded proteins and form aggregates. In some cases, the aggregate formed is amyloid - a specific protein structure associated with several severe human diseases, denoted amyloid diseases.

1.5 Aggregation of amyloidogenic proteins

Why some proteins aggregate into amyloid and cause disease while other proteins do not, is not completely understood. The stability and the \( \beta \)-sheet content of the precursor protein are believed to be important, but some amyloidotic proteins (for example lysozyme) have natively a predominant \( \alpha \)-helical structure [8]. The amyloid formation trigger can be destabilizing mutations, post-translational modifications, or a change in \( \beta \)-sheet content.

The aggregation prone proteins that manage to escape the cell’s quality control mechanisms and form amyloid, take different paths. The most common aggregation pathway \textit{in vitro} is the nucleated-dependent polymerization. Nucleated-dependent polymerization, described for \( \alpha \)-synuclein and huntingtin among others, is initially energetically unfavoured [9, 10]. However, when a certain nucleus has formed, the following steps are favourable and monomers will add on to the nucleus in a template dependent manner, ultimately forming a fibrillar structure. \textit{In vitro}, nucleated polymerization is characterized by a lag-phase (where the nucleus is formed) and a subsequent rapid growth-phase. In nucleated conformational conversion, a variant of nucleated-dependent polymerization, amorphous micelle-like species are frequently formed at first. These initial species can undergo a conformational change into species that functions as nucleus for the continuous downhill aggregation. Both nucleated conformational conversion and nucleated-dependent polymerization have been described for A\( \beta \) aggregation [11]. A third aggregation pathway is downhill polymerization, described for transthyretin
(TTR). This route is not dependent on a nucleus. In the case of TTR, the formation of a partly unfolded monomer enables downhill polymerization since the following oligomer has less free energy than the former (monomer > dimer > trimer > tetramer and so on). Experimentally, downhill polymerization has no lag-phase and cannot be seeded [12].

In the amyloid formation process, several intermediates are formed. Examples of such intermediates are protofibrils, annual protofibrils, soluble oligomers, and globular neurotoxins. If these species are formed on or off the fibrillar pathway is debated and likely dependent on the protein of origin and the intermediate.
2. Amyloid diseases

2.1 General

Today, at least 27 different proteins and peptides are known to form disease causing amyloid in humans (see table 1) [13]. The localisation of amyloid deposits and types of symptoms varies greatly depending on the disease and protein of origin. If the deposits are found in close proximity to the protein production, it is a localised amyloid disease. Many of the neurodegenerative diseases in the brain are of that type, for example Alzheimer’s disease and Parkinson’s disease. Other amyloid diseases are caused by proteins circulating in the blood and therefore depositing at multiple locations. Example of such systemic amyloid diseases are light chain amyloidosis and transthyretin related amyloidosis.

2.2 Amyloid

The proteins and peptides associated with amyloid disease are normally well-behaving and soluble. However, for different reasons they have the capability of aggregating into long insoluble fibrils, denoted amyloid (see fig. 2a). Unbranched and of indefinite length, amyloid fibrils have a similar cross-β-sheet structure independent of the protein of origin [14]. The β-strand core of amyloid fibrils have been detected with X-ray diffraction studies where reflections are seen at 4.7 Å, the distance between β-strands, and at 10 Å, the distance between β-sheets (see fig. 2b) [15, 16]. Several β-sheet rich protofilaments are usually twisted around each other into a mature fibril, about 7 to 14 nm in diameter [17].

![Figure 2. Amyloid fibrils.](image)
a) Atomic force microscopy (AFM) picture of transthyretin fibrils. b) An illustration of the cross-β-sheet structure of amyloid.
To be denoted amyloid, the protein deposition has to bind thioflavin T (ThT) and Congo red. Pre-clinical studies of amyloid usually use ThT as a detection method. When bound to amyloid, ThT changes its fluorescent pattern which conveniently is detected with fluorimetric methods [18]. Congo red is the gold standard for amyloid detection used in clinical diagnosis. Histological sections are stained with Congo red, showing a red-green dichroism under polarized light when bound to amyloid [19].

**Table 1. Human amyloid fibril proteins and their precursor proteins.**
Adapted from Sipe et al. 2010 [13]

<table>
<thead>
<tr>
<th>Amyloid Protein</th>
<th>Precursor Protein</th>
<th>Systemic (S) / localized (L)</th>
<th>Syndrome or involved tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain</td>
<td>S, L</td>
<td>Primary myeloma-associated amyloidosis</td>
</tr>
<tr>
<td>AH</td>
<td>Immunoglobulin heavy chain</td>
<td>S, L</td>
<td>Primary myeloma-associated amyloidosis</td>
</tr>
<tr>
<td>Aβ₂M</td>
<td>β₂-microglobulin</td>
<td>S</td>
<td>Hemodialysis-associated amyloidosis</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin</td>
<td>S</td>
<td>Senile Systemic amyloidosis</td>
</tr>
<tr>
<td>AA</td>
<td>(Apo)serum AA</td>
<td>S</td>
<td>Secondary, reactive</td>
</tr>
<tr>
<td>AAPoAI</td>
<td>Apolipoprotein AI</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AAPoAII</td>
<td>Apolipoprotein AII</td>
<td>S</td>
<td>Aorta, meniscus</td>
</tr>
<tr>
<td>AAPoAIV</td>
<td>Apolipoprotein AIV</td>
<td>S</td>
<td>Sporadic, associated with aging</td>
</tr>
<tr>
<td>AGel</td>
<td>Gelsolin</td>
<td>S</td>
<td>Familial (Finnish)</td>
</tr>
<tr>
<td>ALys</td>
<td>Lysozyme</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>AFib</td>
<td>Fibrinogen α-chain</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystain C</td>
<td>S</td>
<td>Familial</td>
</tr>
<tr>
<td>ABr i</td>
<td>ABriPP</td>
<td>S</td>
<td>Familial dementia, British</td>
</tr>
<tr>
<td>ALECT2</td>
<td>Leukocyte chemotactic factor 2</td>
<td>S</td>
<td>Mainly kidney</td>
</tr>
<tr>
<td>ADan</td>
<td>ADanPP</td>
<td>L</td>
<td>Familial dementia, Danish</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ precursor protein</td>
<td>L</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein</td>
<td>L</td>
<td>Spongiform encephalopathies</td>
</tr>
<tr>
<td>ACa1</td>
<td>(Pro)calcitonin</td>
<td>L</td>
<td>C-cell thyroid tumors</td>
</tr>
<tr>
<td>AIAPP</td>
<td>Islet amyloid polypeptide</td>
<td>L</td>
<td>Islet of Langerhans Insulinomas</td>
</tr>
<tr>
<td>AANF</td>
<td>Atrial natriuretic factor</td>
<td>L</td>
<td>Cardiac atria</td>
</tr>
<tr>
<td>APro</td>
<td>Prolactin</td>
<td>L</td>
<td>Aging pituitary Prolectinomas</td>
</tr>
<tr>
<td>Alias</td>
<td>Insulin</td>
<td>L</td>
<td>Islet of Langerhans Insulinomas</td>
</tr>
<tr>
<td>AMed</td>
<td>Lactadherin</td>
<td>L</td>
<td>Iatrogenic</td>
</tr>
<tr>
<td>Aker</td>
<td>Kerato-epithelin</td>
<td>L</td>
<td>Senile aortic, media</td>
</tr>
<tr>
<td>ALac</td>
<td>Lactoferrin</td>
<td>L</td>
<td>Cornea, familial</td>
</tr>
<tr>
<td>AOaap</td>
<td>Odontogenic ameloblast-associated</td>
<td>L</td>
<td>Odontogenic tumors</td>
</tr>
<tr>
<td>ASem1</td>
<td>Semenogelin I</td>
<td>L</td>
<td>Vesicula seminalis</td>
</tr>
</tbody>
</table>
In vivo, amyloid is associated with several other proteins, the most common being glycosaminoglycans (GAGs), serum amyloid P (SAP), and apolipoprotein E (ApoE) [20, 21]. The exact function of these amyloid-binding structures is not known but both GAGs (particularly heparan sulfate) and SAP are believed to stabilize and prevent proteolysis of amyloid fibrils, thereby affecting amyloid clearance [22]. ApoE is mostly associated to Aβ where it is believed to promote Aβ aggregation by inducing a β-sheet fold, but ApoE has also been found in amyloid deposits derived from serum amyloid A and immunoglobulin light chain, suggesting a wider importance of ApoE for amyloid formation and stability [23].

2.3 Soluble species in amyloid diseases

Although amyloid diseases are defined by the presence of amyloid fibrils, these do not tell the whole story. The last decade, an increasing amount of evidence point to the importance of soluble species, formed on- or off- the aggregation pathway, in disease pathogenesis. Soluble and cytotoxic species have been described, both in vivo and in vitro, for several amyloidogenic proteins such as Aβ, IAPP, α-synuclein, huntingtin, prion protein, and transthyretin (reviewed by Ferreira et al. [24]). There is a wide-range of different soluble oligomers formed even from the same protein, making structural determinations difficult. However, there are indications of similar structural features of soluble oligomers of different protein origin. Interestingly, a polyclonal antibody was shown to recognize soluble oligomers and inhibit oligomer induced cytotoxicity for Aβ, α-synuclein, IAPP, prion protein, lysozyme, insulin, and poly-Q [25].

The mechanisms by which amyloidogenic proteins or peptides exert their cytotoxic effect are not completely understood, but several pathways have been suggested. A massive deposition of amyloid will disrupt the surrounding tissue and abolish cellular functions [26]. Still, most mechanistic suggestions of cytotoxicity in amyloid diseases involve the soluble oligomers. Soluble oligomers have been found to destabilize membranes, induce apoptosis and change cellular functions by interacting with various receptors, affect calcium channels, change lysosomal pathways, induce oxidative stress, and affect the protein quality control system of the cell [27, 28].
3. Aβ and Alzheimer’s disease

3.1 General

Alzheimer’s disease is the most common neurodegenerative disease with about 150,000 affected in Sweden and more than 36 million patients worldwide [29]. The economic cost for society and the human suffer this disease causes is great.

When sporadic, which about 90% of all AD cases are, AD usually initiates after the age of 65 years old. The disease begins with loss of short term memory and progress with loss of cognitive function, personal changes and eventually death. Neuropathologically, a brain from an AD patient can show signs of cortical atrophy, neuronal loss, amyloid angiopathy, amyloid depositions, neuritic plaques and neurofibrillary tangles. Whereas the tangles consist of intracellularly aggregated hyperphosphorylated Tau protein, the main amyloid component is aggregated Aβ-peptide (see fig. 3) [30, 31].

![Figure 3. Neuropathological features of an AD brain.](image)

Human hippocampus from an AD patient was stained with anti-fibrillar antibody (a) or anti-phosphorylated tau antibody (b) to detect neuritic plaques and neurofibrillary tangles. Photo by Anna Gharibyan.

3.2 Aβ

Aβ is a proteolytic fragment derived from the transmembrane protein amyloid beta precursor protein (AβPP) [32]. Although ubiquitously expressed, the function of AβPP is not completely clear but evidence point
AβPP to be important for the nervous system both during development and later in life [33]. AβPP can be proteolytically cleaved by several aspartyl proteases. The production of Aβ begins with β-secretase (also known as BACE-1) that cuts AβPP into two domains: the AβPPsβ and the Aβ-containing C99 domain [34]. Whereas the AβPPsβ is released extracellularly, C99 is continuously cleaved by either α-secretase that disable Aβ or by γ-secretase that cleaves C99 within the membrane to give rise to, depending on cleavage site, an Aβ peptide of 39-43 amino acids long [35, 36]. The Aβ40 variant is the most abundant one in the human brain but it is the Aβ42 variant that is most prone to aggregate [37]. Recent data presents Aβ43 as an amyloidogenic variant, abundant in the brain, present in AD plaque, and with neurotoxic properties similar to Aβ42 [38, 39].

Aβ-peptides are the major component in AD plaques, but the evidence pin-pointing Aβ as one of the key players in AD pathogenesis are several. Familial forms of AD have been genetically linked to carry mutations in the presenelin 1 and 2 genes (encoding for the catalytic site in γ-secretase) or in the AβPP gene either within or flanking the Aβ region [40, 41]. The result of the mutations is an increased Aβ production, an elevated Aβ42/Aβ40 ratio, or the release of more aggregation prone Aβ. Additionally, patients with Down’s syndrome carrying an extra copy of chromosome 21, inevitably develop AD-like neuropathology [42]. The explanation is that the AβPP gene is encoded on chromosome 21, an extra copy of chromosome 21 leads to an overexpression of AβPP and subsequently to an overproduction of Aβ. Transgenic mice overexpressing AβPP have also showed AD-like features both concerning neuropathology and behaviour [43]. Further evidence pointing towards the involvement of Aβ in AD is the cytotoxic effect Aβ has on neurons both in cell culture and in vivo [44].

3.3 Aβ cascade hypothesis

The evidence pointing Aβ to be a key player in AD pathogenesis have been formulated into an Aβ cascade hypothesis that illustrates how an initial increase in Aβ ultimately lead to AD. An altered Aβ metabolism, through increased production, decreased clearance, or a changed ratio between Aβ42/40, initiates the Aβ cascade. An increased Aβ level triggers Aβ oligomerization, forming both soluble cytotoxic Aβ and diffuse Aβ plaque. While the cytotoxic Aβ induces synaptic injury, an inflammatory response is triggered by the Aβ deposits. A downward spiral has begun and continues with more synaptic and neuronal damage and an increased inflammatory response. The result is oxidative stress and an altered ionic homeostasis. Eventually, hyperphosphorylated tau is found oligomerized and aggregated in neurofibrillary tangles, affecting, among other things, the axonal...
transport. The culmination of the cascade is widespread neuronal and synaptic dysfunction, cell death and deficiency in neurotransmitters presenting as dementia in the patient. Autopsy reveals Aβ plaque and neurofibrillary tangles (reviewed in [27, 45, 46]).

3.4 Aβ aggregation and soluble Aβ species

Aβ plaques, frequently seen in AD brains, have historically been considered the dangerous species and the main focus of AD research. Although large amyloid deposits are detrimental to the surrounding tissue, studies have shown a poor correlation between total amyloid burden and clinical symptoms [47]. The discovery of prefibrillar soluble assemblies of Aβ has opened a new window in the AD field and a new target for therapeutic interventions.

The importance of soluble Aβ oligomers in AD pathogenesis has been strengthened since they were first described to be present in human brains. Studies using synthetic Aβ, AβPP expressing cells in culture, mice overexpressing APP, or human CSF and brain tissue have all confirmed soluble Aβ as a major cause of AD pathogenesis. Most importantly, the severity of synaptic loss and cognitive impairment in the brains of AD patients is correlated to the level of soluble Aβ [48, 49].

Combined by their inability to pellet upon high-speed centrifugation, the term soluble Aβ include a wide range of different in vitro and in vivo Aβ species. Depending on study, soluble Aβ has been proposed to form either on- or off- the fibrillar pathway. Recent studies describe Aβ aggregation as a two-path system where one path, based on trimeric Aβ secreted by neurons, terminates in annular Aβ assemblies, and the other path, with dimeric building blocks, ultimately leads to the formation of fibrils (see fig. 4) [50, 51].

The fibrillar pathway initiates when monomeric Aβ acquire a specific fold, favouring dimerization. Subsequently, Aβ dimers readily form protofibrils further evolving into mature fibrils [52]. Aβ dimers have been thoroughly studied and have been identified both in AD brains and in conditioned media from cultured cells overexpressing AβPP [53]. When investigating brain tissues, AD brains could be distinguished from control brains by measuring the content of Aβ dimers [54]. Interestingly, dimers have so far only been found in human brains and AβPP transgenic mice after AD-outbreak when Aβ-plaque also can be identified. That is, dimers do not seem to be an early sign of AD preceding decline in cognitive function [51]. Aβ dimers are
secreted by cultured cells overexpressing AβPP but have only been found secreted by primary neurons at very low amounts, implying that dimers are not naturally secreted by neurons but rather formed extracellularly [51, 55]. However, dimeric Aβ inhibit hippocampal long-term potentiation (LTP) and induce hyperphosphorylation of tau [56, 57]. Protofibrils, formed from aggregated Aβ dimers, are closely related to fibrils [52]. Rich in β-sheet and up to 150 nm in length, they can bind both Congo red and ThT [58]. Protofibrils have mainly been described for synthetic Aβ but their cytotoxicity was confirmed through studies on neuronal cells [59].

The Aβ oligomerization pathway not related to mature fibrils is based on Aβ trimers. Aβ trimers are secreted both by cultured cells and primary neurons, suggesting that they form intracellularly [60, 61]. Trimers are also found early in the AD pathogenesis. Actually, Tg2576 mice show Aβ trimers already at an embryonic stage with a continuous and increasing expression throughout life [60]. In humans, Aβ trimers are identified early in life with the highest detectable levels before full AD but coinciding with mild cognitive impairment [51]. Trimers are more potent than dimers to inhibit LTP and have also been found to alter cognitive function when injected into rats [62, 63]. Trimers can join together and form higher oligomers like hexamers, nonamers, and dodecamers. Aβ dodecamers found in vivo have been denoted Aβ*56 whereas in vitro formed Aβ dodecamers usually are described as Aβ-derived diffusible ligands (ADDLs) [44, 60]. ADDLs are highly toxic in cell culture, impair cognitive function and inhibit LTP [44]. Aβ*56, found in low levels before AD outbreak, does not induce neuronal cell death but still affects neuronal function. Aβ*56 was shown to reversibly induce memory impairment when injected into mice and have also been associated to hyperphosphorylation of tau [51, 60]. The trimeric pathway culminates in the formation of annular Aβ assemblies. Annular Aβ assemblies, especially found in vitro, can be distinguished from the linear protofibrils by atomic force microscopy (AFM), presenting a doughnut-like shape. At least 20 Aβ molecules constitute the annular Aβ that exhibit pore-forming features and are able to permeabilize membranes and induce neuronal cell death [64]. Interestingly, other amyloidogenic proteins like α-synuclein, huntingtin, and islet amyloid polypeptide (IAPP) also show pore-like activity in vitro [65-67]. Recently, annular oligomers were identified in AD brains, assessing annular oligomers as an important species in AD pathogenesis [68].
Figure 4. The non-fibrillar and fibrillar Aβ-aggregation pathways. Monomers and trimers, secreted by neuronal cells, aggregate into cytotoxic oligomers but evade fibril formation. Misfolded monomers, formed of unknown reason, aggregate into dimers, larger aggregates, and ultimately fibrils. Modified from Larson and Lesné, 2012 [51].
With no cure available, AD is a lethal disease treated with drugs that moderates progression and alleviates anxiety. Acetylcholinesterase inhibitors, aiming at increasing the level of the neurotransmitter acetylcholine thereby increasing neuronal signaling, and N-methyl-D-aspartate (NMDA) receptor antagonists, decreasing the overactivation of NMDA receptors and thus reducing the neuronal influx of calcium ions, are presently the only approved AD drugs in Sweden [69].

Searching for AD therapeutics, there are several levels to target Aβ. First, Aβ formation can be decreased by modulating the expression or activity of secretases responsible for Aβ release. By up-regulating α-secretase, AβPP is cleaved in the middle of the Aβ region and no Aβ is being released [70]. Modulation of BACE-1 can lead to a decrease in Aβ levels since BACE-1 have to process AβPP for Aβ to form [71]. Similarly, inhibition of γ-secretase can also decrease Aβ levels. Other γ-secretase modulators can instead shift the Aβ variants formed, decreasing release of the amyloidogenic Aβ42 variant in favour of the more benign Aβ38 variant [72]. Flurizan (tarenflurbil) is an example of a γ-secretase modulator that has been tested in phase III trials. Although promising in preclinical tests, flurizan was unable to improve cognition in AD patients [73]. One problem with secretase modulators is that the secretases are affecting many systems in the body thereby making it inappropriate to change their expression levels or activity in a greater extent.

If Aβ monomers are formed, they can be disarmed by degradation, stabilization, or clearance using enzymes, substances or antibodies. Regardless of method, the goal is to inhibit the formation of cytotoxic soluble Aβ to prevent neuronal alterations and neuronal death. The use of antibodies in AD treatment is further discussed in section 5 Immunotherapy and amyloid diseases.
4. Transthyretin and transthyretin-related amyloidosis

4.1 General

Transthyretin (TTR) is natively a stable and well-functioning protein. Due to its amyloidogenic properties, TTR is able to aggregate and form long insoluble fibrils. Transthyretin-related amyloidosis (ATTR) is linked to the presence of these TTR fibrils in various organs.

4.2 The 3D structure of TTR

TTR is a 55 kDa homotetrameric protein found in plasma at 5 µM and in CSF at 0.5 µM [74, 75]. It has a β-sheet rich structure, where each monomer consists of eight β-strands, organized into two β-sheets (denoted D-A-G-H and C-B-E-F) and one short α-helix [76]. A TTR dimer is formed when the H-strands of two monomers interact in an anti-parallel manner resulting in a continuous β-sheet composed of eight β-strands (see fig. 5a). Hydrophobic contacts enable two TTR dimers to form a TTR tetramer. In the dimer-dimer interface, a hydrophobic channel is formed where small molecules can bind (see fig. 5b).

Figure 5. The 3D structure of TTR.

a) The H-strands of two monomers interact to form the TTR dimer and a continuous β-sheet. b) Two TTR dimers assemble into a tetramer, forming a hydrophobic channel with two hydrophobic pockets in the dimer-dimer interface. (PDB coordinates:1F41 [77]).
4.3 TTR functions

TTR is primarily synthesized by liver hepatocytes but is also to some extent produced by pancreas, choroid plexus, and retinal pigment cells [78-81]. Although variability in results, TTR has also been suggested to be expressed by neuronal cells [82-84]. The main function of TTR is to transport retinol binding protein (RBP) and the thyroid hormone, thyroxine (T4). It is also from these functions that TTR has obtained its name: a transporter of thyroxine and retinol binding protein.

4.3.1 TTR - the transporter

T4, the inactive form of the thyroid hormone, is, in plasma, transported by thyroid binding globulin (TBG), albumin, and TTR. TBG has the highest affinity for T4 ($K_{d,TBG} = 0.1 \text{ nM}$, $K_{d,TTR} = 15 \text{ nM}$, $K_{d,albumin} = 1.5 \text{ µM}$), whereas albumin has the highest plasma concentration ([albumin] = 620 µM, [TTR] = 5 µM, [TBG] = 0.3 µM) [85]. On the basis of this, TTR only binds 10% of plasma T4 and, consequently, only 1% of the T4 binding-sites in plasma-TTR are bound to T4. TTR carries T4 in the two hydrophobic pockets, formed in the dimer-dimer interface. TTR has the ability to bind two T4 per TTR tetramer but due to negative cooperativity, one TTR tetramer only binds one T4 molecule at the time. However, in a high concentration of T4, in vitro, it is possible to see simultaneous binding of T4 to both hydrophobic pockets. In CSF, the importance of TTR as a T4 transporter is more important than in plasma since TTR is the primary carrier of T4 in CSF [86]. However, low CSF-levels of T4 lead to similarly low T4 occupancy of TTR in CSF and in plasma.

Retinol, fat-soluble vitamin A, is secreted from the liver in complex with RBP. Without binding to a bigger protein, the holoRBP (retinol bound RBP) complex is out-filtrated by glomerulus in the kidneys. TTR has originally a low affinity for RBP, but the structural changes occurring in RBP when bound to retinol, increases the affinity of TTR to RBP allowing binding [87]. In an excess of holoRBP, the ratio of holoRBP:TTR will be 2:1. The actual binding ratio in plasma is though 1:1, mostly due to the stoichiometric concentrations of holoRBP and TTR.

4.3.2 TTR in the nervous system

Recent studies suggest that TTR might have other functions than just being a transporter protein. TTR can interact with Aβ, suggesting it to be one of the players in AD. The first indication of TTR as important in AD pathogenesis came in 1982 when neuritic plaques and neurofibrillary tangles in AD brains
were shown to react with an anti-TTR antibody [88]. Since then, many studies have investigated the interaction of Aβ and TTR, both in vivo and in vitro. It is clear that TTR and Aβ can bind to each other and that TTR binding decreases the aggregation of Aβ [89-92]. In vivo studies in mouse models have not given clear results, but there are indications of TTR as a depressor of AD [93-95]. In any case, TTR levels are decreased in CSF from AD patients [96, 97]. If that is due to Aβ:TTR binding or if it is due to a decreased neuronal expression of TTR is still unknown [82].

The mechanism behind the effect of TTR on Aβ, in vivo, is not completely understood. One possibility is that TTR proteolytically cleaves Aβ while another is that TTR sequesters Aβ [90, 98]. Li et al. presents a model of Aβ sequestering by TTR [99]. In this model, TTR is able to prevent the formation of Aβ or change the ratio of Aβ40/42 by interacting with the secretases. Further on, TTR can inhibit Aβ-aggregation by TTR:Aβ binding and promote Aβ degradation. One possibility is also that TTR affects the transportation of Aβ out of CSF.

Another indication of a possible role of TTR in the nervous system is that TTR null mice, showing no effects on fetal development or T4/vitamin A states, demonstrate behavioral deficits. TTR null mice had an increase in noradrenaline in the limbic forebrain, compared to mice expressing TTR, suggesting that TTR affects the noradrenergic system [100].

4.4 TTR in disease

Aggregation of TTR is the reason for several different types of ATTR, one idiopathic and three inherited variants have been described [101, 102].

4.4.1 Idiopathic ATTR

The idiopathic form of ATTR is called senile systemic amyloidosis (SSA), a common disease predominantly predisposed by age. In the elderly, with an age over 80 years, 25% suffer from SSA [103]. The disease is associated with wide-spread low-level deposits of wild-type TTR (TTRwt) in various organs [104]. Cardiac deposits are common, but the gastrointestinal (GI) tract and the tenosynovium can also be affected [105-107]. SSA is commonly benign and without symptoms, but can, predominantly in males, cause cardiomegaly with subsequent congestive heart failure [107]. In addition, SSA often presents as carpal tunnel syndrome [108].
Why TTRwt starts to aggregate and form deposits is not known. A probable cause is that the mechanisms controlling proper folding deteriorate with age. One study showed a decrease in the expression of hepatic chaperones in transgenic mice with TTRwt cardiac deposits compared to transgenic mice without cardiac deposits [109].

4.4.2 Inherited ATTR

With 10,000 cases throughout the world, inherited ATTR is the most common form of familial systemic amyloidosis [110]. Even though the prevalence of FAP is generally low, there are some endemic areas in the world where the prevalence is higher, namely Portugal, Japan, and northern Sweden.

Inherited ATTR is caused by mutated TTR in peripheral nerves and organs. So far, over 100 point mutations have been described in the ttr gene, most of them being pathogenic [111]. The disease is autosomal dominantly inherited. The symptoms, progression, and age of onset of inherited TTR vary vastly. Type of mutation is the greatest cause of deviation, but far from the only factor. Even monozygotic twins can have completely different progression of disease [112, 113].

Being a disease with straggling clinical picture, inherited ATTR have been divided into three different types: familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomyopathy (FAC), and central nervous system-associated amyloidosis (CNSA). The type of inherited ATTR is determined by the TTR mutation and the site of TTR deposits.

FAP

FAP patients show TTR deposits in the endoneurium of peripheral nerves. Unmyelinated and small myelinated nerves are affected initially, but deposits are shown in larger myelinated nerves as the disease progresses [114]. Usually, symptoms start with numbness, spontaneous pain, and impaired thermal sensibility in the lower limbs and continue with similar problems in fingers and arms. The disease often presents autonomic neuropathy, affecting the heart and GI. The consequences are initially orthostatic hypotension, diarrhea, constipation, and vomiting and later on cachexia and death. FAP is commonly also accompanied by renal dysfunction and amyloid deposits in the eye [115, 116]. The average time of survival from diagnosis is ten years [117].
One of the most common TTR mutations in FAP patients is the Val30Met mutation, accounting for 50% of FAP cases throughout the world [118]. In the endemic areas in Sweden and Portugal, Val30Met is almost the only detected mutation. TTR Val30Met is also the most well-studied TTR mutation, and due to that, much of which is known about ATTR is known from studies on TTRVal30Met. Interestingly, the age of onset and penetrance varies significantly between the endemic ATTRVal30Met areas even though the mutation is the same. In Sweden, where it is called Skellefteåsjukan, the average age of onset is 56 years, whereas it in Portugal is 33 years of age [119, 120]. No genetic reason for the difference in age of onset have been found but maternal inheritance is predisposing for both an earlier onset and a higher prevalence [121, 122]. The prevalence of ATTRVal30Met differs between various regions but is highly dependent on age. At 50 years the prevalence is less than 11% in Sweden and 80% in Portugal whereas it at 70 years is 36% in Sweden and 91% in Portugal [122, 123]. ATTR cases are usually heterozygous for mutant TTR, but homozygous carriers do not have a more severe pathological picture than heterozygous, suggesting that TTR is not the only factor in ATTR [124, 125].

The most aggressive form of FAP is the ATTRLeu55Pro variant. Carriers have an average age of onset of 15-20 years of age and present both cardiac and neurological symptoms [126].

**FAC**

FAC is a variant of ATTR predominantly presenting as cardiac deposits. A common FAC mutation is the Val122Ile which 3.9% of the Afro-American population in USA is a carrier of [127]. FAC has a late onset, but opposed to SSA, most FAC patients eventually suffer from heart failure [128].

**CNSA**

CNSA, an uncommon form of ATTR, shows late-onset CNS symptoms (such as dementia, ataxia, and spasticity) and TTR deposits in the leptomeninges. There are ten known CNSA presenting TTR mutations, for example Ala25Thr and Asp18Gly [129, 130]. When investigating the level of mutated TTR in CSF and plasma in CNSA cases, some studies report that mutated TTR is absent in both systems, while other studies report low levels of mutated TTR in CSF [131, 132]. Regardless, the lack of systemic symptoms is due to the absence of mutated TTR in plasma. TTR mutations presenting with CNSA are highly unstable and are therefore probably degraded either intracellularly before secretion or shortly after secretion [133]. In CNS, the locally high concentration of T4 might enable tetramerization of the highly
unstable TTR variants, thereby allowing secretion. After the release of T4, TTR disassembles and aggregates [132].

4.5 TTR aggregation

Despite the natively high tetrameric stability, β-sheet rich TTR is an amyloidogenic protein. The path by which TTR tetramers convert into fibrillar structures is well-studied but still debated. The common view is that the starting-point and rate-limiting step in TTR aggregation is tetrameric dissociation. The resulting monomers can either reassociate into tetramers or undergo a conformational change and partly unfold into non-native amyloidogenic intermediates that upon association eventually form fibrils (see fig. 6). Once the amyloidogenic intermediate has formed, the aggregation is a downhill polymerization reaction where the formed TTR assembly always is more stable than the former oligomer (monomer<dimer<trimer) [134-137]. Several studies have confirmed the need of tetramer dissociation into monomers for TTR aggregation to occur [138-140]. Whether monomers, dimers, or tetramers constitute the building blocks of TTR fibrils is debated. One possibility is that monomers reassociate into partially shifted dimers enabling further aggregation [141, 142]. Computer simulations have confirmed the TTR dimer as the initial start-off point for formation of TTR fibrils [143]. Altered TTR tetramers have also been suggested to be the building blocks of TTR fibrils [144, 145].

The TTR mutants involved in ATTR causes an increase in TTR aggregation due to the destabilizing effects of the mutations. The destabilized TTR has a higher tendency to dissociate into monomers, thus initiating the aggregation process. There are also examples of TTR mutants that have the opposite effect. TTRThr119Met is a very stable form of TTR that can inhibit the destabilizing effect of other mutants. Several cases are described where individuals, heterozygous for TTRThr119Met and a pathogenic TTR variant, have a benign disease in comparison to individuals heterozygous for TTRwt and the pathogenic TTR variant [146, 147].

TTR modifications have been suggested to affect the aggregation tendency of TTR; modifications of the single cysteine at position 10 (Cys10) have been particularly studied. Cysteinylation and glutathionylation of the SH group of Cys10 were shown to increase the amyloidogenic potential of TTR whereas S-sulfonation showed a decreased aggregation propensity [148]. In contrast, a recent publication state that S-sulfonation also increase TTR amyloid formation [149]. These findings, in combination with commonly found
disulphide bridges in TTR amyloid, suggest that Cys10 might be important for the amyloidogenicity and cytotoxicity of TTR.

**Figure 6. Illustration of TTR aggregation.**
The dissociation of TTR tetramers enables TTR monomers to partly unfold. Misfolded monomers will aggregate into oligomeric and fibrillar structures. (PDB:1F41)

### 4.6 Cytotoxic TTR species

Deposits of TTR amyloid in nerves and organs are detrimental to the surrounding tissue. Still, similar to other amyloid diseases, the acute cytotoxic effect seems to be caused by smaller TTR species. The oligomeric composition of these small soluble cytotoxic TTR species and whether TTR fibrils also exert a cytotoxic effect or not is debated in the field.

Investigations of nerve biopsies from ATTR patients have shown that cell death appears before deposition of TTR amyloid, adjacent to TTR aggregates of non-amyloid type [150]. Nerve biopsies have also revealed that TTR deposits co-localize with the receptor for advanced glycated endproducts (RAGE) and an up-regulation of proinflammatory cytokines and nitric oxide [151]. Ligand binding to RAGE commonly activates NFκB which, in turn,
upregulates the transcription of biglycan, neutrophil gelatinase-associated lipocalin (NGAC), matrix metalloproteinase-9 (MMP-9), and RAGE. The result is a positive feed-back cycle leading to chronic inflammation. NFκB and its downstream factors are upregulated in nerves from FAP patients [152]. An interaction of TTR with RAGE causes cellstress, inflammation, caspase activation, and eventually cell death [151]. Extracellular signal-regulated kinase (ERK) is up-regulated in nerves exposed to TTR deposits whereas it was suggested that ERK activation was necessary for aggregated TTR induced cytotoxicity [153].

According to nerve biopsy studies in FAP patients, TTR aggregates and fibrils can exert a cytotoxic effect. However, from the presented studies, it is unclear if the activation of RAGE and ERK can have been caused by soluble TTR instead of or in addition to TTR fibrils. Most in vitro studies point to soluble TTR as the most potent cytotoxic TTR species. The suggested oligomeric composition of the soluble TTR species vary from monomers to larger oligomers with a molecular weight of ~400 kDa [154-157]. Several of these studies also stated that TTR fibrils are inert to neuroblastoma cells [154-156]. However, TTR fibrils as a cytotoxic species cannot be rejected. One study showed that the cytotoxicity of TTR increased with the presence of amyloid fibrils and that TTR amyloid, bound to the cell membrane by electrostatic interactions, caused membrane fluidity [158].

The path by which soluble TTR induce cytotoxicity is not completely clear but several possible pathways have been suggested. First, soluble TTR was shown to interact with RAGE, suggesting that soluble TTR can cause similar cellular responses as TTR deposits [159]. Binding of TTR oligomers (20-40 associated monomers) to the plasma membrane induces Ca²⁺-influx via voltage-gated Ca²⁺-channels. The change in Ca²⁺-homeostasis was not caused by ER-stress, but by an influx of Ca²⁺ over the plasma membrane [156]. ER-stress is though probably involved in TTR-induced cell death. Oligomeric TTR (trimers to ~20mers) was shown to increase the levels of BiP and induce caspase-3, indications of ER-stress and cell death [157].

4.7 ATTR therapies

ATTR is lethal and the treatment possibilities have been limited. Liver transplantation has, historically, been the only available treatment for ATTR, but an intense search for small TTR-stabilizing compounds has led to the recent approval of an ATTR drug.
4.7.1 Liver transplantation

The liver being the main site for synthesizes of TTR, liver transplantation was suggested as treatment for ATTR in the early 1990’s. The first trial showed encouraging results with diminishing levels of mutant plasma TTR, clinical improvement, and a decrease in amyloid burden [160, 161]. Today, liver transplantation is a common treatment for ATTR, increasing the survival and quality of life, but there are disadvantages. Liver transplantation only targets the TTR secreted by hepatic cells, meaning that mutant TTR still is being synthesized by choroid plexus, pancreas, and retinal pigment cells. Thus, patients suffering from CNSA will have no use of liver transplantation. Follow-up studies of liver transplanted ATTR patients have shown that the effect of the treatment is best on ATTRVal30Met patients. Five years after liver transplantation, 80% of ATTRVal30Met patients were alive, to compare with only 50% of ATTR patients with other mutations [162]. Regardless of TTR mutation, studies have shown that the cardiac deposition of TTR continues after liver transplantation despite the absence of mutant TTR [163]. TTR fibrils, isolated from the cardiac tissue of ATTR patients before or after liver transplantation, were characterized for TTR type. It was shown that the level of mutated TTR was higher than the levels of TTRwt before liver transplantation, whereas the ratio was the opposite after liver transplantation [164]. Thus, TTRwt is responsible for the continuous cardiac deposition of TTR after liver transplantation.

4.7.2 Stabilizing drugs

It is well known that pathogenic TTR mutations destabilize the TTR tetramer, thereby increasing the dissociation into monomers and the subsequent aggregation. The dimer-dimer interface, containing the hydrophobic canal, is the weak planar of the TTR tetramer. Substances binding to the hydrophobic pockets and stabilizing the tetrameric structure could potentially decrease tetrameric dissociation. Such a compound was found in diflunisal [165]. Diflunisal could decrease the aggregation rate of TTR and inhibit TTR cytotoxicity in vitro [166, 167]. Phase I studies showed an increased stability of plasma TTR upon oral administration [168] and a phase III study is currently ongoing. Tafamidis is another TTR stabilizer enrolled in a phase III study. The phase III study showed that tafamidis decrease ATTRVal30Met progression and tafamidis was recently approved as an ATTR drug [169].
5. Antibodies and amyloid diseases

5.1 General

As the knowledge of oligomeric species in amyloid diseases have evolved, the interest for antibodies targeting these species has increased. Antibodies targeting specific intermediates in the aggregation process are important for research purposes since they enable the detection of oligomeric species, but antibodies against oligomeric amyloid proteins can also potentially be used for immunotherapy and diagnosis.

5.2 Research and antibodies targeting oligomeric species

The finding of the “oligomer specific” polyclonal antibody, A11, escalated the search for antibodies targeting soluble oligomers in amyloid diseases. A11 could detect oligomeric species formed from several disease-associated amyloidogenic proteins and also inhibit the cytotoxicity induced by such oligomers [25]. A11 is widely used in research to indicate the formation and presence of oligomeric species. However, the polyclonal nature of A11 is occasionally a disadvantage with a batch to batch discrepancy in oligomer recognition.

As a reaction to A11, many monoclonal antibodies denoted “oligomer specific” have been presented, especially against Aβ but also targeting PrPscrapie [170-172]. In addition, two ScFv antibodies have been presented to have similar generic properties as A11 [173].

5.3 Immunotherapy in amyloid diseases

Immunotherapy is defined as “treatment of disease by inducing, enhancing, or suppressing an immune response” [174]. In the case of amyloid diseases, the goal is to enhance the immune response to enable clearance of cytotoxic species or to prevent the formation of such species. Basically, three different approaches can be used: Passive vaccination, active vaccination, or the administration of intravenous immunoglobulin (IVIg). In active vaccination, an antigen is injected together with adjuvants to induce an immunoresponse. That is, the body is triggered to produce antibodies against the injected antigen. Another approach, passive vaccination, uses premade antibodies against the specific target. Antibodies are injected into the patient to hopefully contribute to the clearance of the pathogenic species. IVIg is a
variant of passive immunization where an antibody-containing fraction of plasma from healthy donors is injected into the patient. The basis for this is that humans have natural occurring autoantibodies against many antigens. One reason for getting amyloid disease could be a decrease in these naturally occurring autoantibodies against the specific amyloidogenic protein.

5.3.1 Active vaccination in AD

The first immunotherapy trials in AD were conducted on AD transgenic mice using active vaccination. Fibrillar Aβ42 was injected peripheral and when analyzed, mice showed improved cognition and a decreased amyloid burden [175-177]. Subsequently, active vaccination was essayed in humans. Although some patients showed a decrease in cognitive decline, the study was terminated due to T-cell induced aseptic meningoencephalitis in 6% of the participants [178, 179]. Follow-up studies have shown scarce results, with little or no improvement in cognitive function and survival [180]. The disappointing results of these active vaccination trials suggests that 1) if active vaccination is conducted, the choice of antigen needs to be improved, and 2) passive vaccination might be a safer and more efficient way to target AD.

5.3.2 Passive vaccination in AD

The first passive immunization with anti-Aβ antibodies was executed in an AD mouse model and presented in year 2000 [181]. The results were promising, showing a decrease in AD neuropathology. Subsequent studies could also present that AD mice treated with anti-Aβ antibodies were cured and restored memory [182]. Since then, many passive immunization trials have been performed in AD mouse models, using different types of antibodies. The mechanisms by which administrated antibodies target Aβ are several. By entering the CNS, antibodies can bind both to Aβ plaque and to soluble Aβ species. Antibody binding will facilitate clearance of Aβ through microglia mediated phagocytosis. Soluble Aβ species will, in addition, be averted from continuous aggregation and from interacting with cells and execute a cytotoxic effect [183, 184]. Antibodies remaining in plasma will function as a peripheral sink. By sequestering soluble Aβ in plasma, antibodies change the Aβ equilibrium between plasma and the CNS, with a resulting increase in Aβ plasma levels and a decrease in Aβ CNS levels [185]. Depending on the antibody, one or several of these mechanisms are applicable.

Several passive immunization trials in AD patients have been essayed and several are on-going. The antibody most thoroughly studied is the N-terminal binding Bapineuzumab. Initial tests showed improved mini mental
state examination (MMSE) scores but the results from the phase II clinical trial were somewhat disappointing [186]. Combining all patient groups, no improvement in cognition could be established. However, AD patients that completed all antibody infusions and were not apolipoprotein E4 carriers benefited from the treatment [187]. A phase III study is currently ongoing.

Both mice studies and clinical trials in AD patients have revealed that passive immunization in AD is accompanied with risks of cerebral microhemorrhages [188, 189]. An explanation is that antibodies detect Aβ fibrils in vessels, thereby inducing an inflammation that eventually leads to vessel wall rupture. Another cause could be that macrophages that have engulfed Aβ die when trying to pass the blood brain barrier, thus contributing to amyloid angiopathy [190]. To circumvent microhemorrhages, the used antibodies need to be improved. One option is to modify antibodies so that microglia mediated phagocytosis is decreased; another option is to use optimized antibodies that more efficiently targets the dangerous Aβ species, thus allowing a lower dose.

5.3.3 Vaccination trials in other amyloid diseases

Although most vaccination trials in amyloid diseases concerns AD, immunotherapy is also a potential treatment in other amyloid diseases. Active and passive immunization trails against α-synuclein in mice models for Parkinson’s disease (PD) have showed promising results. Single-chain variable fragments (ScFv) of anti-α-synuclein intrabodies have decreased α-synuclein aggregation and toxicity and improved cell morphology both in PD-mice and in vitro [191, 192]. Intrabodies do not seem to be a necessity for positive effect of immunotherapy in PD-mice since both passive vaccination using a monoclonal anti-α-synuclein antibody and active vaccination have showed similar results as ScFv intrabodies [193, 194].

Immunotherapy studies are also ongoing in animal models of other amyloid diseases, such as Creutzfeldt-Jacob’s disease, Huntington’s disease, and amyothrophic lateral sclerosis.

5.3.4 IVIg’s and amyloid diseases

Naturally occurring autoantibodies against oligomeric and soluble forms of Aβ and α-synuclein are found in IVIg pools from healthy blood donors [195-197]. Although results are contrasting, there are indications of a lower amount of naturally occurring autoantibodies against Aβ in elderly and in AD patients compared to controls [198, 199]. This suggests IVIg administration to be a possible treatment for AD patients. Human trials are
ongoing where AD-patients are treated with IVIg’s and the results are promising with an improvement of MMSE scores in one of the trials [200, 201]. In addition, prion protein (PrP)-induced cytotoxicity was reduced with IVIg and an ATTR patient with CNSA symptoms showed improvement upon IVIg administration [202, 203].

5.4 Antibody binding kinetics

An antibody binds its antigen by noncovalent interactions. Hydrogen bonds and ionic bonds, as well as hydrophobic interactions and van der Waals attractions, can all be responsible for the binding of the antigen epitope to the variable region of the antibody. To enable binding, the comparatively low strength of these non-covalent interactions is compensated for by the number of interactions.

5.4.1 Affinity and avidity

The binding of an antibody to an antigen can be described in terms of affinity. The affinity describes the binding strength between a single antigen-binding site on an antibody and its antigen. A higher affinity indicates a stronger tendency for the antibody to bind its antigen. If the antigen is a polyvalent target and the antibody has several equal binding sites, the binding can be described in terms of avidity. Avidity is the sum of all present affinities. For an avidity-influenced binding to release, all individual bindings in a polyvalent interaction have to dissociate simultaneously.

Four of the five different antibody isotypes (IgG, IgA, IgE, IgD) have two equal binding sites whereas the fifth variant (IgM) has ten to twelve binding sites. If the antigen is a polyvalent antigen with several equal epitopes, the binding between an antibody and the polyvalent antigen can potentially be influenced by the avidity effect.

5.4.2 The association and dissociation constants

The reversible binding between an antibody and its antigen can be described as showed in equation 1 (where Ab=antibody and Ag=antigen).

\[ Ab + Ag \leftrightarrow AbAg \]  

\[ \text{Eq.1} \]

The equilibrium between the free antibody and antigen and the associated complex is determined both by the concentrations of the antibody and the
antigen and by the strength of their interaction. The strength of the antibody:antigen interaction can be described either by the association constant ($K_a$, also called the affinity constant) or by the dissociation constant ($K_D$). $K_D$, the invers of $K_a$, is an equilibrium constant describing at which concentration of antigen, half of the possible binding sites are occupied. $K_D$ can be determined either from the rate constants of the interaction or from the concentrations of free and complexed antibody and antigen. The relationship between $K_D$ and the rate constants is illustrated in equation 2. The two rate constants are the association rate constant ($k_{ass}$), the rate in which the antigen and the antibody associates, and the dissociation rate constant ($k_{diss}$), the rate in which the antigen and the antibody dissociates.

$$K_D = \frac{k_{diss}}{k_{ass}}$$

Eq.2

Equation 3 illustrates the relationship between $K_D$ and the concentration of free and complexed antigen and antibody. Even though the free and complexed concentrations of the interactants can be used to calculate $K_D$, they cannot be used to calculate the rate constants. The rate constants need to be experimentally determined.

$$K_D = \frac{[AbAg]}{[Ab][Ag]}$$

Eq.3

A strong antibody:antigen interaction will give a low $K_D$. The majority of antibodies have a $K_D$ value in the 1-40 nM range but there are cases of antibodies with an affinity in the low pM range. Importantly, $K_D$ gives no information about the turn-over rate. That is, two different interactions with the same $K_D$ can have vastly different $k_{diss}$ and $k_{ass}$.

5.4.3 Surface plasmon resonance

Surface plasmon resonance (SPR) is a convenient method to study binding kinetics between two molecules, for example an antigen and an antibody. SPR can give information about $k_{ass}$ and $k_{diss}$ of an interaction, thus enabling the determination of $K_D$. Even though SPR can measure the interaction between a wide variety of different molecules, SPR is below explained using the interaction between an antibody and an antigen where the antibody has been immobilized.
Using SPR, an antibody is immobilized on the gold coated (commonly gold, but other metals can also be used) side of a glass prism. A light beam is passed through the prism towards the backside of the gold film at a certain resonance angle. Hitting the gold film, the light will both reflect towards a detector and interact with the electron cloud in the gold film. The electron cloud will, due to the light energy, begin to oscillate within the gold film. This oscillating electron cloud, referred to as plasmon, generates an electrical field around the gold film. Any change in the electrical field will affect the resonance angle of the light which is registered by a reflected light detector. When the antigen, in aqueous solution, is allowed to flow over the immobilized molecules, the antibody:antigen interaction will change the electrical field and subsequently the resonance angle registered by the detector.

The $k_{\text{ass}}$ is determined as the antigen is flown over the immobilized antibodies. After finishing antigen injection, an antigen-free buffer solution is flushed over the gold surface. As a consequence, antigen will dissociate from the antibodies and the $k_{\text{diss}}$ can be determined.
AIMS

The general aim of my project was to increase the understanding of cytotoxic species in amyloid diseases. Why do cytotoxic species form and how can their formation and/or cytotoxic effect be inhibited?

More specifically, in the papers included in this thesis, the aims have been:

To create an antibody targeting only cytotoxic and oligomeric species of Aβ.

To investigate how the binding properties of an antibody affects antigen recognition.

To investigate the avidity effect on antibody:oligomer interaction.

To investigate if disulphide bridges affect the cytotoxicity of TTR.

To investigate how thermodynamic and kinetic stability affect TTR cytotoxicity.
RESULTS & DISCUSSION

Independent of amyloidogenic protein or peptide, the aggregation pathway from native structure to amyloid includes many transient species. An intriguing problem is to capture these individual species to pinpoint their characteristics. In the papers presented in this thesis, we have used antibodies to target intermediates in the amyloid aggregation pathway and a cell based assay, occasionally in combination with size exclusion chromatography (SEC), to study the cytotoxic potential of different species in the aggregation cascade.

Antibodies and cytotoxic Aβ oligomers

Targeting the dangerous Aβ-species

Soluble Aβ aggregates of different oligomeric compositions exert a cytotoxic effect in cell assays and induce cognitive decline in mice. Hence, targeting soluble Aβ oligomers is important both for research and therapeutic purposes. Antibodies specifically targeting the soluble Aβ oligomers have been generated and are usually referred to as conformational dependent antibodies. These antibodies, frequently used in AD research, are though difficult to produce; nobody knows their epitope or their exact functions. Working with A11, the gold standard oligomer specific antibody in amyloid disease research, we found it to not always fulfill our needs. Therefore, we decided to produce our own anti-Aβ oligomer specific antibody.

To avoid the difficulties of producing an antibody recognizing a specific oligomeric epitope, we decided to use another approach; we exploited the avidity effect. By definition, an oligomer is polyvalent. That is, it has several equal epitopes. Similarly, an IgM antibody has ten to twelve binding sites with an equal affinity for the specific antigen. By combining a polyvalent antigen and a polyvalent antibody, a potentially strong interaction can occur.

After screening the supernatants of several hundreds of hybridoma clones, we found one antibody with a potential oligomeric specificity and of IgM isotype. The antibody, denoted OMAB, bound to the N-terminal region (aa 1-16) of the Aβ-peptide. Using SPR, we investigated the interaction between OMAB and monomeric Aβ and determined the $T_{1/2}$ to 57 s and the
dissociation constant to 0.5 µM (see fig. 7a). Thus, OMAB bound monomeric Aβ with a low affinity.

Probing OMAB against Aβ species of different sizes in an ELISA revealed that OMAB preferentially bound Aβ species with an oligomeric composition of 10-20 assembled monomers. SPR revealed that the interaction between oligomeric Aβ and OMAB was strong with a non-detectable dissociation (see fig. 7b). Thus, the low-affinity binding between OMAB and monomeric Aβ was compensated for by multivalent interactions in the OMAB-oligomeric Aβ binding.

![Graph a](image1)

**T_{1/2} = 57s**

![Graph b](image2)

**T_{1/2} = non-detectable dissociation**

**Figure 7. OMAB binds oligomers with a high affinity.**

OMAB was immobilized to a CM5 chip. The dissociation rate of OMAB and Aβ monomers (a) or Aβ oligomers (b) was detected using SPR.
Aβ cytotoxicity

To further study the efficiency of OMAB, we wanted to probe the antibody against toxic Aβ in a cell assay. Generating cytotoxic Aβ in vitro is not as straight-forward as it first appears to be. There are many different protocols circulating and the variability in cytotoxicity is great. A protocol working in one lab will not automatically work in another. The prime reason for this, I believe is the batch to batch variation in Aβ quality. Depending on recombinantly expressed or synthesized and the conditions before lyophilization, the peptide will behave completely different.

For reproducibility, it is convenient to choose one Aβ producer and obtain peptide from that manufacturer for all experiments. We work with recombinantly expressed Aβ, monomerized in hexa-fluoro-isopropanol before lyophilization. After concluding that some of the protocols for producing cytotoxic Aβ were not applicable on the type of Aβ we used, we realized that we needed to establish a method on our own. We dissolved Aβ and preincubated it for different time-points before applying it to SH-SY5Y neuroblastoma cells. Aβ was allowed to interact with cells for 48 hours before cell viability was detected using resazurin. We found Aβ to be highly cytotoxic, but interestingly, cytotoxicity decreased with preincubation (see fig. 8a). The best cytotoxic effect was obtained when solubilized Aβ was immediately added to cells. The conclusion is that the highly aggregation prone Aβ42 is forming high molecular weight and non-toxic Aβ oligomers upon incubation. Even though the non-incubated Aβ is cytotoxic, it is likely that the cytotoxicity is exerted by low-molecular weight oligomers formed during the cell incubation rather than by Aβ monomers. A SEC analysis combined with cytotoxicity measurements confirmed that hypothesis. An Aβ mixture, containing Aβ species of different oligomeric composition, was gelfiltrated and fractions collected throughout the SEC were subsequently probed for cytotoxic potential. The viability analysis revealed that also Aβ species of 20-40 assembled monomers were cytotoxic (see fig.8b). The cytotoxicity exerted by these oligomers is in analogy to other studies showing in vitro cytotoxicity from Aβ oligomers with a molecular weight of about 90 kDa [204].
Figure 8. Cytotoxic effect of Aβ.

a) Aβ (10 µM) was preincubated for different time points and thereafter probed for cytotoxic potential. b) An Aβ mixture was separated by SEC (—) and eluted fractions tested for cytotoxic effect (--) in a SH-SY5Y cell assay.

OMAB as an inhibitor of cytotoxic Aβ

After establishing an Aβ cytotoxicity assay, we were able to test the potential of OMAB as an inhibitor of Aβ induced cytotoxicity. Aβ and OMAB were added to SH-SY5Y cells in different ratios and allowed to incubate for 48 hours before cell viability analysis. The results were clear; OMAB could inhibit Aβ induced cytotoxicity in a substoichiometric ratio. The neuroblastoma cells were completely rescued at a ratio of 1:100 OMAB:Aβ (see fig. 9).
Figure 9. OMAB can rescue SH-SY5Y cells from Aβ-induced cytotoxicity.
Aβ42 was added to SH-Sy5Y with or without OMAB. Cell viability was detected after 48 hours using resazurin. # = p < 0.001 compared to control cells and * = p < 0.001 compared to cells treated with only Aβ.

One factor that complicates the interpretation of the effective OMAB:Aβ ratio is the uncertainty in how many Aβ species that can bind to the same OMAB. In the experiment discussed above, we have calculated one Aβ species per OMAB. Since each OMAB has ten binding sites, there is a possibility, although unlikely, that each OMAB binds ten Aβ species. If each OMAB binds ten Aβ species, the effective ratio OMAB:Aβ is 1:10 instead of 1:100. However, 1:10 is still a substoichiometric ratio and indicative of that OMAB specifically targets the cytotoxic Aβ species.

Avidity binding to Aβ by the IgG isotype

OMAB had the qualities that we aimed for and is very useful in research, but being of IgM isotype, OMAB has some drawbacks. IgM is a large molecule, difficult to purify, not amenable for genetic modulation, and rarely used therapeutically. The IgG isotype is preferable since it is easy to express and purify, amenable for recombinant modifications, and usually the isotype chosen for antibody-based therapeutics. With OMAB, we could establish that avidity binding is effective when targeting oligomers. However, it was unclear how many OMAB binding sites that were needed for the avid binding to be strong enough to specifically target Aβ oligomers. We decided to investigate if the bivalent IgG was able to bind to Aβ oligomers with an avidity effect. Therefore, we sequenced the CDR regions of OMAB and
transplanted them onto a murine IgG1 backbone. The resulting IgG antibody was denoted AC. A dot-blot analysis of fractions from SEC Aβ revealed that AC, similar to OMAB, specifically targeted oligomers. Using a competition assay, both OMAB and AC were shown to target oligomers in a ten times excess of monomeric Aβ (described below, see fig. 10). The results from AC indicated that a bivalent interaction is enough for oligomer specificity.

The passive immunization trials executed in AD mice models and in AD patients have shown promising results but revealed that the antibody dose has to be decreased to avoid vascular inflammation in the brain [188, 189, 205]. To decrease dosing, the antibodies need to be more specific. That is, it is desirable that the antibodies avoid being consumed by monomers to instead enable binding to the cytotoxic oligomers. The possibility to exploit the avidity effect of IgG when targeting oligomers triggered us to explore how binding kinetics of antibodies can be modified to improve oligomer specificity. Based on the OMAB CDR regions, two additional antibodies, EF and DF, were produced on an IgG backbone. The antibodies were probed against Aβ monomers and oligomers using dot-blot, ELISA, and SPR. The K_D value for monomeric Aβ differed from 35 µM for EF to 37 nM for DF, to compare with 0.5 µM for OMAB. To test the oligomer specificity of the antibodies, a competition ELISA was executed. To avoid inducing avidity binding, antibodies were allowed to bind monomeric Aβ in solution before probed against Aβ oligomers coated to an ELISA plate. Antibodies not bound to monomeric Aβ were able to bind to oligomeric Aβ in the ELISA plate, thus detectable with a secondary antibody. As seen in figure 10, AC and OMAB could detect oligomers in a solution of ten times more monomers than antibodies but were partly inhibited if the surplus of free monomer was 100 times or more. EF, with a very low affinity for monomeric Aβ, had an unaffected binding to oligomers even at a surplus of 1000 times more monomers than antibodies. The efficiency of EF clearly demonstrates the importance of a low monovalent affinity and a fast off rate to obtain oligomer specificity via the avidity effect. The ability of DF to bind to oligomers in the presence of monomers is comparable to the ability of OMAB and AC. From the k_{ass} and K_D values we expected DF to have a lower binding to oligomers than AC and OMAB. One reason for the oligomer recognition of DF could be that DF binds oligomers easier and with a higher k_{ass} than OMAB and AC. We intend to investigate this by immobilizing OMAB, AC and DF to the same CM5 chip and detect binding to Aβ oligomers.

To investigate the oligomeric affinity of EF, we mixed EF and oligomers at different ratios and determined the oligomeric concentration where half of the antibodies were bound to oligomers by measuring free antibodies in an ELISA. K_D was determined to 23 nM. That is, due to the avidity effect the
binding strength between EF and Aβ increased more than 1000 times when probing oligomers instead of monomers.

Figure 10. EF detects oligomers in a 1000 times excess of free peptide.
Antibodies and monomeric Aβ1-10 was mixed and added to an ELISA plate coated with oligomeric Aβ. Antibodies bound to oligomers were detected with an anti-mouse antibody.

Specificity of EF

Specificity, important for selectivity in antigen recognition, should be discriminated from affinity. A low affinity is not necessarily associated with a low specificity. In general, the binding strength between a receptor and a ligand is the sum of enthalpic and entropic factors. In biological systems, the entropic contribution is dominated by exclusion of water through association of hydrophobic surfaces. This frequently requires less specificity as compared to the enthalpic forces which are represented by formation of new bonds and requires a specific architecture [206]. Consequently it is the relative contribution of enthalpic and entropic forces that determines the specificity.

To determine that the low affinity of EF did not affect the specificity of the antibody, we tested the specificity of EF in a cell assay and on hippocampus slides. First, we investigated if EF could pinpoint the cytotoxic species in presence of a vast excess of other proteins. We mixed EF with Aβ oligomers and added the mixture to neuroblastoma cells with or without the addition of non-toxic Aβ1-10. EF rescued SH-SY5Y cells from Aβ induced cytotoxicity.
even in presence of a 100 times excess of monomeric Aβ1-10 (see fig. 11). Thus, the low monomeric affinity of EF allows the antibody to omit binding to monomeric Aβ species while the avidity effect enables EF to target and disarm the oligomeric and cytotoxic Aβ species.

**Figure 11. EF inhibits Aβ induced cytotoxicity.**
Aβ1-42 was added to SH-Sy5Y cells in the presence or absence of EF (ratio EF:oligomers 1:5). Aβ1-10 was added in a ratio of 100:1 (Aβ1-10:Aβ oligomers) to show that EF specifically detects the oligomers in a mixed solution.

Second, we elucidated the specificity of EF by essaying the antibody on human AD hippocampus slides. EF could not detect dense amyloid plaque but reacted with intracellular Aβ. This could be compared to the staining of a polyclonal anti-Aβ antibody detecting plaque, extracellular tissue space, and weakly neuronal cells. Once again, EF was shown to bind specifically to oligomeric, and likely, neurotoxic species indicating the efficiency of an avid binding.

**The avidity effect as a general approach to target oligomeric species in amyloid diseases**

An avid binding was proven to efficiently target oligomeric species of Aβ. We hypothesized that the same technique would work on oligomers from other types of amyloid diseases as well. Parkinson’s disease is a localized amyloid disease showing intracellular Lewy bodies, α-synuclein deposits, in substantia nigra of the midbrain. Similar to Aβ, α-synuclein can form soluble oligomers that are cytotoxic to neurons.
To generate α-synuclein monoclonal antibodies, we immunized mice with α-synuclein, produced hybridomas, and screened cell media for α-synuclein reactive antibodies. Analogous to the Aβ antibodies, the binding characteristics of α-synuclein antibodies were investigated by ELISA and SPR. Two antibodies, αSyn2.4 and αSyn51, showed fast $k_{\text{diss}}$ and high $K_D$ values when probed against monomeric α-synuclein whereas a third antibody, αSynM, showed a slow $k_{\text{diss}}$ and a low $K_D$ value towards monomeric α-synuclein. These three antibodies were tested for selectivity in a competition ELISA. Antibodies were preincubated with their respective monomeric counterpart and thereafter probed against oligomeric α-synuclein coated onto an ELISA plate. Antibodies bound to the oligomers were detected by a secondary antibody. As hypothesized, αSyn51 and αSyn2.4 were able to bind oligomeric α-synuclein even in a 1000 times excess of free peptide, whereas αSynM failed to detect oligomers already at a 10 times excess of free peptide (see fig. 12).

![Graph showing the binding of α-Syn2.4 and α-Syn51 to oligomers](image)

**Figure 12. α-Syn2.4 and α-Syn51 detect oligomers in an excess of free peptide**

Antibodies and monomeric corresponding peptides were mixed and added to an ELISA plate coated with oligomeric αSyn. Antibodies bound to oligomers were detected with an anti-mouse antibody.

From the α-synuclein data we can conclude that our technique of producing oligomer specific antibodies works independent of target peptide. By modulating antibodies to having a low affinity and a high off-rate for the monomeric variant, a strong avidity binding with a high specificity can be obtained for the oligomeric counterpart. However, it is important to note that as long as the epitope is exposed in the oligomeric assembly, avidity binding antibodies will detect the oligomeric species. It is thus, using this technique, impossible to distinguish between different oligomers.
TTR-induced cytotoxicity

When discussing amyloidogenic proteins and cytotoxicity, the general idea is that the native protein has to change its native fold and oligomerize to induce cytotoxicity. Concerning TTR, the picture is somewhat different. TTR cytotoxicity has been described for fibrils (although refuted by others), protofibrils, large soluble oligomers, and surprisingly also for monomers. Working with amyloid diseases and cytotoxicity, we decided to further investigate the confusing picture of TTR induced cell death.

Establishing a TTR cytotoxicity assay

Our first obstacle was to establish a robust cytotoxicity assay where different oligomeric assemblies and variants of TTR could be tested. In analogy with another study, we added freshly prepared TTR to our neuroblastoma cell line, SH-SY5Y cells [154]. In contradiction to previous publications, we found both the TTRwt and the disease-associated TTRVal30Met to be toxic in our cell assay (see fig. 13) [154, 156]. Toxic TTRwt was not surprising considering the involvement of TTRwt in SSA, still, we needed to confirm that the observed cell death was TTR induced [104]. TTRThr119Met is a stable TTR variant that is known to protect individuals carrying the TTRVal30Met mutation from developing FAP [207, 208]. We tested the cytotoxic potential of TTRThr119Met in our cell assay and found it to be non-toxic to SH-SY5Y cells (see fig. 13). To further confirm that the observed cytotoxicity for TTRwt and TTRVal30Met was TTR induced, we incubated the TTR variants with a small compound, diflunisal, known to stabilize TTR. Diflunisal could rescue cells, establishing the TTR specificity of the observed cell death. These observations independently pinpointed TTR as mediator of the cytotoxic effect.

Figure 13. Cytotoxic effect of TTR variants. TTRwt, TTRVal30Met, and TTRThr119Met were incubated with SH-SY5Y cells. Cell viability was detected after 72 hours using resazurin. *p<0.05.
**Disulphide bridges are not necessary for TTR to exert a cytotoxic effect**

Each TTR monomer contains one cysteine at position 10. Cys10 have been proposed to be involved in the pathogenesis of SSA where TTRwt causes disease. The distance between the different Cys10 residues in the native tetrameric structure is far too long for disulphide bridges to form. Yet, disulphide bridges are highly abundant in TTR amyloid, even though they are not a prerequisite for amyloid to form [209, 210]. Interestingly, a substitution of Cys10 to a serine could abolish TTR deposition in an ATTR mouse model, suggesting that Cys10 is involved in the pathogenesis of TTR related amyloidosis [211].

We investigated the importance of Cys10 for TTR induced cytotoxicity. TTRwt and TTRVal30Met as well as their Cys10Ser variants were probed against SH-Sy5Y cells. We found no difference in cytotoxicity between TTRwt, TTRVal30Met, and their respective Cys10Ser variant. Thus, we could conclude that disulphide bridges are not a requirement for TTR to induce cytotoxicity. The lack of deposits in the Cys10Ser/Val30Met mouse is probably due to the destabilizing effect in vivo formed Cys10 adducts have on the TTR tetramer [212].

**Oligomeric TTR composition and cytotoxic potential**

In a study by Reixach et al (2004), it is showed that cytotoxic TTR species have a molecular weight below 100 kDa [154]. Sörgjerd et al (2008) presents cold-stored native tetramers (and larger aggregates) as the cytotoxic species [155]. However, in both studies it is unclear if an aggregation of TTR occurs during the incubation on cells. We preincubated TTRwt, TTRThr119Met, and a monomeric TTR variant (TTR-M) at 37°C to enable oligomerization. The incubated TTR samples were separated by SEC to detect oligomeric status of the different TTR variants. TTRwt and TTRThr119Met were unaffected by incubation presented by the elution of a single symmetrical peak at the elution volume corresponding to a TTR tetramer (for TTRwt, see fig. 14a). In agreement with other studies, the oligomerization of TTR-M depended on the protein concentration during incubation [154]. By using a low protein concentration, most of TTR kept its monomeric composition whereas a high protein concentration induced oligomerization. SEC analysis of TTR-M incubated at a high protein concentration showed two peaks of similar intensity; one eluting as a monomer and the other eluting as aggregates (see fig. 14b).
During the SEC analysis, fractions were collected to enable cytotoxicity studies on the different oligomeric assemblies. All TTRThr119Met fractions were non-toxic as suspected whereas the cytotoxicity of TTRwt coincided with a tetrameric elution (see fig. 13a). The TTR-M peak containing oligomers were completely non-toxic to SH-SY5Y cells whereas the TTR-M protein eluting as a monomer had a cytotoxic potential (see fig. 13b). This is in agreement with the results by Reixach et al [154]. TTR being an amyloidogenic protein, we wanted to investigate if the detected cytotoxicity was caused by a possible cytotoxic oligomer formed during the incubation on cells. Therefore, after detection of cell viability, media was collected from affected cells and separated by SEC once again. For TTRwt, no oligomerization had occurred in solution during the time of experiment, indicating that cytotoxicity was indeed induced by tetramers or something smaller. The SEC of TTR-M showed predominately monomers but also a small peak indicative of the presence of aggregates. However, these oligomers eluted at the same volume as the already proven non-toxic aggregates, making it unlikely that these aggregates exerted the cytotoxic...
effect. Thus, our data confirm TTR as cytotoxic without preceding oligomerization.

According to SEC and cytotoxicity analysis we can only confirm that no TTR aggregation occur in solution during the cell experiment. We do not know the composition of TTR species that interact with the cells. It is possible that an oligomerization of TTR occur when TTR binds to or enters the cell. Studies assayed on cytotoxic TTR propose that TTR binds to RAGE, induce ER stress, and/or affect voltage-gated calcium channels [153, 156]. Still, it cannot be excluded that TTR is required to enter the cell to exert cytotoxicity.

**TTR stability and cytotoxicity**

The finding that no preceding aggregation is needed for TTR to exert a cytotoxic effect is intriguing. What is the true cytotoxic species and how does that species form? It is well-known that TTR aggregation begins with tetramer dissociation and that disease-associated mutations in the ttr gene destabilize the tetramer. When establishing the TTR cytotoxicity assay we realized that the common ATTR mutant TTRVal30Met was not more cytotoxic than TTRwt whereas the highly stable TTRThr119Met was non-toxic. This made us question how the thermodynamic and kinetic stability of TTR influence TTR-induced cytotoxicity. In a paper by Sekijima et al (2005), 23 different TTR variants were tested for thermodynamic and kinetic stability and compared to their respective secretion efficiency [133]. We correlated the kinetic and thermodynamic stability determined in that paper to the, by us, determined cytotoxic potential. We probed twelve different TTR variants for cytotoxicity to SH-SY5Y cells and found, not surprisingly, that a high kinetic stability inhibited the cytotoxic effect. However, the kinetic stability could not be correlated to the cytotoxic potential, that is, a TTR mutant with a very low kinetic stability was not more cytotoxic than a TTR variant with a higher kinetic stability. Thus, as long as the TTR variant could dissociate into monomers, it was cytotoxic, indicating that TTR cytotoxicity is not directly dependent on the kinetic stability.

When investigating the correlation between thermodynamic stability and cytotoxicity we found that a low thermodynamic stability attenuated the cytotoxic effect. The five mutants with the lowest thermodynamic stability were all inert in our assay, independent of kinetic stability. Four of the tested TTR variants had similar kinetic stability but vastly different thermodynamic stability, enabling a direct comparison of thermodynamic stability and cytotoxicity without the involvement of kinetics. The variant with the lowest thermodynamic stability, TTRVal30Gly, was non-toxic whereas the other three variants: TTRVal30Met, TTRwt, and TTRArg104His were cytotoxic.
This further strengthens the notion that the thermodynamic stability is important for TTR induced cytotoxicity. Our results imply that, for TTR to exert a cytotoxic potential, an intermediate stability is needed where the kinetic stability should be low enough to allow tetramer dissociation and the thermodynamic stability high enough to permit formation or preservation of the cytotoxic fold.

The importance of thermodynamic stability for cytotoxicity can have several explanations. It is possible that a thermodynamic unstable TTR mutant forms monomers that are too aggregation prone resulting in a low population of the cytotoxic intermediate. An alternative explanation could be that TTR requires a specific fold to induced cytotoxicity, a fold that cannot form if the thermodynamic stability is too low. We have conveyed our results into a model of TTR induced cytotoxicity where the monomers formed through tetramer dissociation can transform into different monomeric species (see fig. 15). The monomers can either partly unfold and form the amyloidogenic monomer associated with amyloid formation or form a cytotoxic monomer. Whether this cytotoxic species obtains a changed oligomeric composition or not when interacting with cells is still unclear. However, we believe that for the TTR-cell interaction to occur, the TTR monomer has to have a specific fold that thermodynamically unstable variants cannot form. The cytotoxicity detected from tetramers and larger TTR oligomers are likely caused by the dissociation of monomers from these species. Another possibility is that the cytotoxic monomeric fold is similar to the native monomeric structure, enabling an incorporation of the cytotoxic species into tetramers or larger oligomers.

Intriguingly, most ATTR patients are heterozygous, carrying one mutated and one wt ttr gene. The resulting TTR tetramers circulating in the body are a mixture of wildtype and mutated monomers. Assuming that the mutated TTR monomers with a low thermodynamic stability are unable to adopt the cytotoxic fold, the cytotoxicity of TTR could actually be caused by TTRwt monomers. To investigate this further it would be interesting to co-express the, in our assay, inert TTR variants TTRThr119Met and TTRVal30Gly. By labeling one of the mutants with a flag, tetramers can be divided according to their monomeric composition (4:0, 3:1, 2:2, 1:3, 0:4) and individually tested for cytotoxic potential [213]. According to our model, the mixed tetramers will be cytotoxic due to that Val30Gly increases the dissociation of the tetramer, thereby releasing cytotoxic Thr119Met monomers.

As a continuation of this study it would also be interesting to clone the kinetically highly stable TTR variants on a TTR-M background. This would enable an investigation of thermodynamic stability versus cytotoxicity
without kinetic stability interfering. According to our model, the thermodynamic unstable TTRTyr69His and TTRPhe64Ser variants would still be inert as monomers whereas the thermodynamically highly stable TTRThr119Met would gain toxicity.

Figure 15. Model of formation of cytotoxic TTR species.
Tetramer dissociation enables monomers to adopt a cytotoxic fold.
CONCLUDING REMARKS

In this study we have pinpointed how oligomer specific antibodies can be generated without the need of a specific conformational fold. By generating antibodies with a low affinity and a high turnover rate for the monomer of a protein or peptide, a strong avid binding can be obtained for the oligomeric counterpart. Antibodies generated in this way are very useful for research when detection of oligomers rather than monomers is needed. If these avidity-binding antibodies also can be used therapeutically remains to be investigated.

To increase the chances of finding a cure for amyloid diseases, the mechanisms behind the diseases need to be understood. We have investigated the cytotoxic potential of TTR and found that disulphide bridges, commonly found within the amyloid form, are not a requirement for TTR to exert a cytotoxic effect. In addition, we have investigated the effect of thermodynamic and kinetic stability on TTR cytotoxicity and found that the thermodynamic stability is more important than previously thought. A too low thermodynamic stability will prevent TTR from exerting a cytotoxic effect. We propose that TTR monomers are the smallest TTR species having a cytotoxic effect and that these monomers have to obtain a specific fold for cell death to occur. We believe that the cytotoxicity of TTR detected from tetramers (and possibly also from larger assemblies) is caused by monomers dissociating from the tetramer rather than by the tetramer (or oligomer) itself. Whether the cytotoxic monomers change their oligomeric composition when interacting with or entering cells is so far unclear. We propose a model where TTR induced cytotoxicity requires an intermediate stability of the TTR molecule. The kinetic stability should be low enough to permit tetramer dissociation and the thermodynamic stability high enough to prevent instant aggregation and to allow formation of the cytotoxic fold.
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