Therapeutic Monoclonal Antibodies to Detect and Halt ATTR Cardiac Amyloidosis and Neuropathy

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Abstract

Transthyretin (prealbumin, TTR) is an abundant serum protein which transports vitamin A and thyroid hormone T4 throughout the body. Although TTR normally forms stable soluble homotetrameric complexes, point mutations and unknown pathological conditions can favour the dissociation of the TTR tetramer into misfolded monomers which can aggregate and accumulate as transthyretin amyloid (ATTR) throughout the body, particularly in the heart and peripheral nerves. Cardiac ATTR deposition leads to cardiomyopathy, heart failure, and death; nerve deposition causes neuropathy. At present, endomyocardial biopsy is the only method for conclusively diagnosing ATTR amyloidosis and no FDA-approved pharmacotherapies exist. The only treatment option is organ transplantation (heart and/or liver) for a subset of eligible patients.

This thesis focuses on the development of conformation-specific polyclonal/monoclonal antibodies which can potentially diagnose/treat both hereditary and wild-type ATTR amyloidosis, through their ability to specifically recognize and bind to disease-associated forms of TTR via a cryptotope (an epitope normally buried and inaccessible in the native protein, but exposed in its altered conformation). These monoclonal antibodies (mAbs) were demonstrated in vitro to specifically bind misfolded TTR, inhibit fibril formation, induce phagocytic clearance of non-native and aggregated TTR, and specifically recognize TTR amyloid in diseased heart tissue. We further investigated the mechanism of mAb-mediated inhibition of fibrillogenesis using immunogold transmission electron microscopy (IGEM). This high resolution imaging technique
confirmed this cryptotope to be an effective antibody target due to its exposure within both pathological TTR misfolding intermediates and end-point insoluble TTR fibrils. In addition to their ability to recognize TTR aggregates \textit{in vitro}, these monoclonal antibodies demonstrated the ability to specifically recognize soluble, misfolded TTR aggregates \textit{in vivo} in the plasma of transgenic ATTR mouse models. These results further support the use of monoclonal antibodies to target pathological protein conformations as potentially effective immunotherapies for ATTR amyloidosis.
I can say with absolute confidence that pursuing a Ph.D. was one of my best life decisions to date. There are a number of individuals who helped make my experience as enjoyable as it was.

I am grateful for my family – particularly my parents, Lydia M. and Steve N. Galant – for their encouragement and constant support of my studies, no matter where on this planet my research interests have taken me.

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1 Transthyretin amyloidosis: An under-recognized neuropathy and cardiomyopathy


N.J.G. wrote the manuscript with input from P.W., J.N.H., and A.C.

1.1 Abstract
Transthyretin (TTR) amyloidosis (ATTR amyloidosis) is an underdiagnosed and important type of cardiomyopathy and/or polyneuropathy that requires increased awareness within the medical community. Raising awareness among clinicians about this type of neuropathy and lethal form of heart disease is critical for improving earlier diagnosis and the identification of patients for treatment. The following review summarizes current criteria used to diagnose both hereditary and wild-type ATTR (ATTRwt) amyloidosis, tools available to clinicians to improve diagnostic accuracy, available and newly developing therapeutics, as well as a brief biochemical and biophysical background of TTR amyloidogenesis.

1.2 Abbreviations used in this chapter
ASO, antisense nucleotide; ATTR amyloidosis, transthyretin amyloidosis; ATTRwt, wild-type ATTR; CAA, cerebral amyloid angiopathy; CMRI, cardiac MRI; CSF, cerebral spinal fluid; CTS, carpal tunnel syndrome; ECG, electrocardiography; ECHO, echocardiography; EGCG, epigallcatechin-3-gallate; MRN, magnetic resonance neurography; NSAID, non-steroidal anti-inflammatory drug; NT-proBNP, pro-brain natriuretic peptide; Pcdh10, protocadherin-10; PET, positron emission tomography; SAP, serum amyloid P component; TTR, transthyretin; TUDCA, tauroursodeoxycholic acid; wt, wild-type.

1.3 Introduction
There is a growing awareness of the prevalence of protein-misfolding diseases in today’s aging population. Under certain conditions, proteins that are normally folded into a thermodynamically low-energy state can misfold and aggregate, forming insoluble ‘amyloid’ fibrils. Amyloidosis
describes a group of diseases characterized by the extracellular deposition of these toxic, insoluble, cross β-sheets within various locations in the body, causing the disruption and dysfunction of normal surrounding tissue [1], [2]. The prevalence of these types of diseases in older generations may be the result of microenvironmental changes associated with aging, including changes in intra/extracellular pH, proteasomal functioning and the body’s ability to eliminate aggregates [3]-[5]. Amyloid formation, however, is not restricted to old age and can occur at any decade of life as a result of mutations within the amyloid precursor protein’s primary structure, chaperonopathies or altered protein metabolism [4], [5].

There are more than 30 proteins known to form amyloid in vivo. All demonstrate the characteristic yellow to green birefringence upon exposure to Congo Red dye and polarized light [6]–[8]. The specific amyloid type and the clinical outcomes associated with misfolding are dictated by the nature of the amyloid precursor protein [7], [9]. For nomenclature purposes, each type of amyloid is prefaced with the prefix A followed by a suffix that identifies the fibril precursor protein [7]. For example, transthyretin (TTR) amyloidosis (ATTR amyloidosis) is a systemic disease caused by the deposition of transthyretin amyloid (ATTR) throughout the body. ATTR amyloidosis is a widely underdiagnosed and important disease that can be lethal if ATTR infiltrates the heart, a common site of ATTR deposition. Although originating from one protein, pathogenesis and manifestations in ATTR amyloidosis may vary and make the disease heterogeneous. Its underestimated prevalence is further supported by the fact that many patients are not diagnosed with ATTR amyloidosis until post-mortem examination [10]–[14]. The following review will outline the basic background behind this condition with the aim of helping clinicians understand the biophysics and pathophysiology behind ATTR amyloidosis, the heterogeneous array of signs and symptoms associated with the disease, current diagnostic tools available to clinicians to improve diagnostic accuracy, as well as current and developing treatment interventions available for those patients identified at early enough stages of the disease trajectory.

1.4 Transthyretin-related amyloidosis

Transthyretin (earlier known as prealbumin due to its molecular mass causing it to characteristically migrate at a band prior to albumin on serum electrophoresis) is an abundant, soluble, serum protein that transports both vitamin A (via retinol-binding protein) and thyroxine throughout the body [15], [16]. TTR is also involved in the binding and redistribution of β-amyloid
in the choroid plexus as well as in the retention of T4 in the cerebral spinal fluid (CSF) [15]. TTR concentration is 10-fold higher in plasma (3.6 μM) than in CSF (0.36 μM) [17]. Although the liver is the primary site of synthesis, alternatives sites of production include the choroid plexus, α-cells in the islets of Langerhans, ocular sites that may include retinal pigment epithelia, and in rabbits, the ciliary pigment epithelia [18]–[21]. TTR is a β-strand rich 55 kDa homotetramer that can dissociate into its 127 amino acid monomeric subunits. These monomers can undergo aberrant changes and become amyloidogenic intermediates that can self-associate and eventually form amyloid fibrils that accumulate as amyloid deposits throughout the body, resulting in ATTR amyloidosis (Figure 1.1).

Figure 1.1. Proposed transthyretin (TTR) fibril formation pathway. Natively folded tetrameric TTR first dissociates into its monomeric subunits, which undergo conformational changes to become altered monomeric intermediates. These altered monomeric intermediates self-assemble into stable nuclei or oligomer aggregates, which allows for fibril formation to proceed.

ATTR amyloidosis can be sub-classified as either wild-type (wt) or hereditary. In wild-type ATTR (ATTRwt) amyloidosis, formerly referred to as ‘senile systemic amyloidosis’, systemic amyloid deposition is the result of the misfolding of wt TTR. This is in contrast with hereditary ATTR amyloidosis, where point mutations within the TTR allele cause the deposition of mutant TTR amyloid throughout the body. Regardless of the type of ATTR diagnosis, post-mortem analysis reveals TTR amyloid deposition in almost all organs and tissues of affected individuals [22], [23]. Despite characteristically wide systemic amyloid deposition, both hereditary and ATTRwt amyloidosis can result in cardiac complications, renal complications such as proteinuria due to glomerular involvement and ATTR deposition in the papillae of the renal medulla [24], [25], as well as neuropathy due to deposition of TTR amyloid in the endoneurium and surrounding neuron vasculature [23]. At initial clinical presentation, however, the most common neurological complaint from patients with hereditary ATTR amyloidosis is the loss of sensory function [26], [27]. At early stages of hereditary ATTR amyloidosis, the effects of neurodegeneration are most pronounced in the small myelinated and unmyelinated nerve fibers, whereas thicker fibers and
their respective sensory nerve action potentials are less affected [28]. The sensorimotor effects caused by this neurodegeneration can result in a progressive reduction in pain and temperature sensation inwards from the distal extremities [29]. When larger fibers become involved at later stages of disease, patients are at risk of developing muscle weakness and atrophy, which can be debilitating [29]. Impotence, urinary and GI dysfunction are also common and can indicate autonomic nervous system involvement [30]. There have been additional reports of vocal hemiparesis and dysarthria in patients with hereditary ATTR due to central nervous system involvement [30]. However, in both ATTRwt amyloidosis and several forms of hereditary ATTR amyloidosis, cardiac manifestations strongly predominate.

Carpal tunnel syndrome (CTS), a well-studied median nerve neuropathy, is also a common finding in affected individuals due to the deposition of ATTR in the tenosynovial tissue [31], [32]. CTS is one of the earliest signs of ATTR amyloidosis development, especially in ATTRwt amyloidosis as CTS can precede ATTRwt-related cardiac complications by 6 (± 4.6) years [14]. Clinicians should therefore perform accurate neurological assessment in those patients who present with clinical signs of CTS and prior to referral for surgical relief interventions such as carpal tunnel release. TTR has also been shown to deposit in soft tissues, and there have been reports of ATTR deposits in the ligamentum flavum of the spinal canal, resulting in the development of lumbar spinal stenosis [33], [34]. Additional complications exclusively associated with hereditary ATTR amyloidosis include ocular amyloid deposition resulting in impaired vision due to vitreous deposits, pupillary and lacrimal dysfunction, as well as glaucoma [35]–[37].

ATTR amyloidosis results in systemic amyloid deposition, but has lethal implications when it results in ATTR deposition within the heart. TTR-related cardiac amyloidosis is often a progressive infiltrative cardiomyopathy and the deposition of ATTR amyloid within cardiac tissue varies. Even if progressive infiltrative cardiomyopathy is not present, cardiac amyloid deposits may cause severe conduction disturbances. Because it mimics hypertensive and hypertrophic cardiomyopathies, TTR-related cardiac amyloidosis often goes undiagnosed and both earlier and accurate clinical recognition by physicians are critical [38].
1.5 ATTR amyloidosis: genetics and inheritance patterns

The first report of hereditary ATTR amyloidosis originated in Northern Portugal in 1952, followed by reports from ‘endemic’ areas in Japan (1968) and Sweden (1976) [39]–[41]. The most frequently reported cause of hereditary ATTR amyloidosis is the TTRVal30Met mutant, commonly affecting but not limited to patient populations in Portugal, Japan, Sweden and Brazil [23]; the lack of geographical proximity between these high areas of prevalence raised the question regarding the origin of the allele [42]. Studies have shown that although the mutation is the same in Portugal and Brazil, the mutation affecting Swedish patient populations has occurred independently [43]. In addition, the geographical origin and ethnic background of the patient can have implications for disease onset and initial symptoms for a given mutation [26]. For example, in ATTRVal30Met amyloidosis, patients of Brazilian and Swedish descent are the youngest and oldest patient populations to start developing signs of disease respectively, despite having the same disease-causing mutation [26], [44]. Although the age of onset for hereditary ATTR amyloidosis follows a bimodal distribution across a wide variety of mutations, the median age of onset is generally earlier in patients with hereditary ATTR amyloidosis in comparison with those of ATTRwt amyloidosis: 39.0 (25.9–64.5) compared with 71.4 (60.1–81.6) years of age respectively [45].

Although ATTRwt amyloidosis is the most common form of ATTR amyloidosis in the U.S.A. (43%) with ATTRVal122Ile being the second most prevalent (23%), ATTRVal30Met amyloidosis is the most common of the hereditary forms worldwide (76%) [46]. In addition to the TTRVal30Met and TTRVal122Ile mutants, there have been >100 additional mutations reported worldwide, though their global distribution varies [2]. Hereditary ATTR amyloidosis is an autosomal dominant disease with heterogeneous phenotypic expression. Signs and symptoms of this form of amyloidosis can be multisystemic, ranging from only neuropathic or cardiomyopathic complications to a mixture of both [18]. Although homozygosity does not necessarily result in earlier disease onset, it has been suggested that those patients who are homozygous for mutant TTR alleles (i.e. TTRVal30Met) often develop more severe signs and symptoms due to increased central nervous involvement in addition to having increased ATTR infiltration of the leptomeninges and subarachnoid vasculature in comparison with heterozygotes [47], [48]. These findings conflict with another study which found phenotypic severity, when compared with heterozygotes, to be unaffected by homozygosity [49]. Phenotypic penetrance can vary between
mutations though, and for example in Sweden, many carriers of the TTRVal30Met mutation never develop the disease [50]. Cases of monozygotic twins in which one twin develops the disease and the other does not, have also been described [51]. Compound heterozygotes, in which a patient has both a disease-causing and disease-inhibiting mutation, can also occur. The introduction of an anti-amyloidosis mutation (i.e. TTRThr119Met) in the otherwise ATTR-causing TTR mutated proteins inhibits aggregation by increasing the tetrameric dissociation activation barrier that thermodynamically stabilizes the TTR protein and impairs clinical manifestation of the disease [8].

TTR mutations are associated with varying risk of cardiac involvement [52]. Patient subpopulations of African American descent carrying the TTRVal122Ile mutation are at particular risk for developing cardiac-related ATTR compared to many other groups. This is also true for the TTRVal111Met mutation [53]. There has been some discussion in the literature regarding the TTRVal122Ile allele prevalence within the African American population [54]–[56]; its current estimate of 3–4% would translate to approximately 1.3 million allele carriers who are at an increased risk of developing cardiac amyloidosis in the U.S.A. alone [54]–[56]. This mutant allele has a phenotypic penetrance of 100% that results in deposition of ATTR in the heart, and is one of the major reasons why clinicians must be cautious in managing patients of African American descent who present with early signs of heart failure.

Regardless of the subtype of ATTR amyloidosis, all affected patient populations are at significant risk of disease progression due to misdiagnosis. ATTR amyloidosis is often masked by symptoms and signs that mimic similar, more common diagnoses that have shared symptomatology (such as obesity, diabetes or hypertension). This is compounded by the similarity of these patients’ profiles to those with ATTR amyloidosis, i.e. ethnic, older patient populations. In addition, correct diagnosis can be delayed further as the first cardiac sign of the disease, left ventricular hypertrophy (LVH) is one with an expansive differential diagnosis, with diseases much more prevalent and common than ATTR amyloidosis [57].
1.6 ATTR subtypes and pathology

The cardiac pathophysiology of ATTR amyloidosis is due to the deposition of amyloid in the heart. In Type A amyloidosis, ATTR deposition in cardiac tissue results in the development of a restrictive cardiomyopathy and can have lethal clinical outcomes. For ATTRVal30Met amyloidosis, there are two discrete and well-separated patterns, each with a very different clinical outcome. In Type A ATTR amyloidosis, amyloid appears patchy in the myocardium and these foci tend to coalesce into homogeneous amyloid masses, compressing cardiomyocytes [58]. This form of ATTR amyloidosis is associated with progressive restrictive cardiomyopathy and can lead to severe cardiomegaly. In Type B ATTR amyloidosis, amyloid appears as thinner streaks interstitially and subendocardially [58]. Although quite conspicuous amounts of amyloid can also build up in this type of ATTR amyloidosis, restrictive cardiomyopathy does not develop. However, conduction disturbances are common and are the reason many patients require the insertion of a pacemaker.

Interestingly, Type A and Type B fibrils are different in that Type A mainly contain C-terminal fragments of TTR whereas Type B fibrils only contain full-length TTR molecules. The resulting amyloids differ in that Type A fibrils are short and have weak affinity for Congo Red whereas Type B fibrils are long, slender, and strongly stain with Congo Red. The most important distinction, however, is that Type A fibrils tend to recruit more wtTTR to deposits than Type B fibrils do. Typically, Type A ATTR deposits continue to grow following liver transplantation in contrast to Type B amyloid [59]. In addition, for at least Swedish patient populations with ATTRVal30Met amyloidosis, Type B amyloid is most common in early onset cases whereas Type A is seen in late onset (>50 years of age). Type A ATTR amyloidosis is by far the most common form found for all other TTR mutations and is also the single finding in ATTRwt amyloidosis [60]. The differences between Type A and Type B fibrils make it reasonable to believe that there are (at least) two different ways by which TTR can form fibrils. Previous investigations have shown the importance of the C-terminal segment of TTR in fibrillogenesis, but for Type B amyloid other mechanisms may exist [61]–[63].
1.7 Diagnostics
1.7.1 Imaging
Cardiac MRI (CMRI) can be of great help for ATTR amyloidosis diagnosis. CMRI utilizes the relaxation times of protons within a magnetic field to generate images of tissue based on differential proton density or water content. CMRI signal intensities within amyloid-infiltrated tissue can be enhanced when paired with a contrast agent such as gadolinium and result in diffuse, global and subendocardial patterning [64]. The unique patterning of late gadolinium enhancement in cardiac amyloidosis is useful for ruling out hypertrophic or hypertensive cardiomyopathies and diagnosing cardiac amyloidosis (80% sensitivity; 94% specificity) [64], [65]. Contrast-enhanced CMRI does, however, have limitations in that it cannot distinguish between different subtypes of cardiac amyloidoses and does not permit quantification [66]. Native myocardial T1 mapping, however, is a quantitative imaging modality that relies on the mapping of longitudinal relaxation times for a magnetized proton [67]. Native T1 myocardial mapping has both diagnostic value for ATTR amyloidosis (both hereditary and wt) and allows for tracking of disease progression [68]. In comparison with normal patients or those with hypertrophic cardiomyopathy, a common clinical mimic, patients with ATTR amyloidosis (both hereditary and wt) have increased T1 relaxation times, potentially indicative of the amyloid-induced hydro-effects on ATTR-infiltrated cardiac tissue [68]. Although native T1 myocardial mapping is limited in its ability to differentiate between different types of cardiac amyloidoses, lower T1 times have been reported for ATTR amyloidosis in comparison with AL amyloidosis [68]. There have been additional reports on the diagnostic potential of magnetic resonance neurography (MRN), a type of MRI that can be used for the evaluation of polyneuropathy in ATTR amyloidosis. High-resolution MRN has been used for the detection and quantification of lower limb nerve damage in both symptomatic and presymptomatic gene-carriers of hereditary ATTR-related polyneuropathy [69].

There have been recent advances in the development of alternative imaging strategies that can be used to improve the diagnostic accuracy of cardiac amyloidosis. Imaging studies have demonstrated promise for differentiating and diagnosing cardiac amyloidoses (including ATTR amyloidosis) from hypertensive heart disease using positron emission tomography (PET) with 11C-PIB or 18F-florbetaben labelling [70]–[72]. Technetium-labelled bone scintigraphy, a technique that measures a radiolabelled isotope to characterize disease, is commonly used for the imaging and diagnosis of bone pathologies. Interestingly, this method has been reported as a
technique to specifically identify and characterize TTR-related cardiac amyloidosis, as affected cardiac tissues display an increased uptake of isotopes not seen in other cardiomyopathies, even amyloidogenic ones [73], [74]. This technique has been suggested to provide complete diagnostic information in ATTR amyloidosis potentially making biopsy unnecessary [73]. However, limitations of the technique include the risk of the tracer binding cardiac AL amyloid deposits [75] and its inability to visualize amyloid deposits composed of full-length ATTR (Type B amyloid) [76]; bone markers can visualize amyloid composed of C-terminal TTR fragments (Type A amyloid) possibly due to micro calcifications in the amyloid deposit [77]. There are other amyloid tracers that are less fibril type-specific, such as Pittsburgh compound B (PiB), a radioactive thioflavin-T derivative which in conjunction with PET can recognize both Type A and B amyloid [70]. Nevertheless, while technetium-labelled bone scintigraphy has demonstrated value for characterizing ATTR-related cardiac amyloidosis, whether it can be expanded beyond the heart and used for the visualization of ATTR deposits to characterize ATTR infiltration of the nervous system or other organs would be worth investigation.

ATTR cardiomyopathy leads to decreased diastolic relaxation and consequently results in poor ventricular filling during diastole. ATTR amyloidosis results in the non-specific infiltration of both the left and right ventricles and valves [78]. Although echocardiography (ECHO) is a common ultrasound imaging modality with limited specificity that can help recognize ATTR amyloidosis patients, thickening of the left ventricular free wall or septal thickness in ATTR amyloidosis can mimic hypertrophic or hypertensive cardiomyopathy and result in misdiagnosis [77], [79]. A ‘sparkling’ or ‘granular’ pattern on ECHO can be characteristic of ATTR amyloidosis; however, this pattern cannot differentiate between different forms of cardiac amyloidosis [80]. It is important to note that the diagnostic value of ‘myocardial sparkling’ for cardiac amyloidosis is insufficient for diagnosis when found via harmonic imaging [81]. Although harmonic imaging is a type of ECHO that achieves better visualization of endo- and myo-cardial tissue [82], [83] than conventional fundamental frequency ECHO, evidence of myocardial sparkling in hearts not infiltrated with amyloid remains a common finding of this technique [81].

Strain ECHO imaging, a method of determining the amount of deformation of the myocardial tissue throughout the cardiac cycle, has been demonstrated to be a useful technique to help characterize TTR-related cardiac amyloidosis from hypertrophic cardiomyopathy. Although both
conditions are characterized by impaired atrial reservoirs and contractile function, the impairment is more severe for TTR-related cardiac amyloidosis due to the greater effects of amyloid deposition in comparison with fibrosis [84].

1.7.2 Electrocardiography

Low-voltage QRS complex analysis via electrocardiography (ECG) may be another potential strategy used for the detection of cardiac amyloidosis; however, its clinical usage is limited as the presence of low-voltage QRS complexes in infiltrative cardiomyopathies is inconsistent [78], [85]. This holds true for other classic ECG findings such as the ‘pseudoinfarct’ pattern of Q waves, left atrial enlargement and atrial fibrillation, as well as left bundle branch block (LBBB), as these are not ATTR-specific nor sensitive findings [86]. As such, the absence of low-voltage, restrictive pattern or other classic ECG findings should not be used to rule-out ATTR amyloidosis, especially in those patients with increased left atrial diameter and evidence of increased thickening of the right ventricular free wall, valves and interventricular septum [85]. Because basic cardiac imaging or tracings are of limited value in ATTR amyloidosis diagnosis, tissue biopsy or alternative diagnostic tests are required. For diagnosis of any type of systemic amyloidosis, a tissue biopsy followed by biochemical characterization with immunological methods or mass spectrometry is warranted.

1.7.3 Biopsy

Currently, the diagnostic gold standard for cardiac involvement in ATTR amyloidosis is endomyocardial biopsy, which involves obtaining multiple cardiac biopsy samples via central venous access. Although of high diagnostic value for ATTR amyloidosis, there are multiple risks associated with this invasive procedure, including myocardial perforation, arrhythmia, haemothorax, pneumothorax, pericardial tamponade and death [87], [88]. It is most common, however, to determine diagnosis using a biopsy obtained from alternative sites. Sites most commonly used are subcutaneous abdominal fat tissue and labial salivary glands. In most cases, both these sites provide diagnosis if correctly performed; however, experience in interpretation is crucial.
1.7.4 Biomarkers

Although there are no known diagnostic biomarkers for ATTRwt amyloidosis [18], protocadherin 10 (Pcdh10) expression has been linked to the progression of ATTR peripheral neuropathy. Current work has been focusing on Pcdh10 up-regulation being a potential biomarker of hereditary ATTR progression; however, this potentially promising area of research requires further investigation [89]. Abnormal serum levels of N-terminal fragment of pro-brain natriuretic peptide (NT-proBNP), a cardiac dysfunction biomarker, has also been linked to both hereditary and ATTRwt amyloidosis. Its usage as a biomarker for ATTR amyloidosis, however, is limited since abnormal NT-proBNP levels are also found in patients with immunoglobulin light-chain (AL) cardiac amyloidosis [90].

With the exception of specific centres, clinicians rarely consider ATTR amyloidosis as a cause for their patients’ symptomatology, even when indicated. ATTR amyloidosis should be considered for patients who present with axonal polyneuropathy, especially if they have autonomic involvement [30], or in patients presenting with diastolic heart failure. Given the non-specific symptoms of the disease, and the difficulties in its differential diagnoses, most patients with evidence of heart failure and preserved ejection fraction ideally should be tested for cardiac amyloidosis [18]. Because amyloidosis, especially with cardiac involvement, has poor prognostic outcomes for patients, improved diagnostic accuracy would result in better outcomes for patients due to early detection and more targeted therapeutic interventions [91].

1.8 Therapeutics

1.8.1 Surgically invasive interventions

Depending on the type of ATTR amyloidosis, either hereditary or wt, the main therapeutic intervention for clinical ATTR amyloidosis management is to remove the organ synthesizing amyloid precursor protein and/or affect its rate of TTR synthesis. Accumulated TTR amyloid deposits can have lethal implications, therefore early intervention is required. The gold standard for hereditary ATTR amyloidosis management is orthotopic liver transplantation, a procedure first performed in Sweden for two patients with ATTRVal30Met [92], [93]. Since 1990, this procedure has been associated with positive long-term prognosis for those hereditary ATTR amyloidosis patients who are identified at early enough stages of the disease because it removes the origin of amyloid precursor protein and replaces circulating mutant TTR with normal TTRwt [44].
The long-term survival rates, however, appear to be strongly dependent on the amyloid fibril type (Type A compared with Type B) since those patients with Type A amyloid progress in cardiomyopathy following liver transplantation due to the addition of TTRwt molecules [59], [94]. There are additional factors of importance, such as age at disease onset, patient nutritional status and type of disease-causing TTR mutation [44].

Despite the promising prognosis for these patients, the main concern and long-term complication of liver transplantation in patients with hereditary ATTR amyloidosis is that previously existing hereditary ATTR deposits can seed amyloid growth using the newly circulating normal TTRwt and result in disease progression [46], [95]. Progression of cardiomyopathy in patients who undergo orthotopic liver transplant is therefore not uncommon, especially in those patients with previous cardiac amyloid infiltration or evidence of cardiac involvement, such as posterior wall and/or septal ventricular wall thickening [96]–[98]. Additional concerns for orthotopic liver transplantation include the failure to address additional sites of TTR production, such as the eye, where amyloid deposition can continue along the pupil and increase the risk of post-operative hereditary ATTR patients developing glaucoma [20]. The procedure also neglects to address the issue of continuity between the subarachnoid and endoneural space. The connection between these two contiguous structures can potentially result in the post-operative accumulation of amyloidogenic TTR precursors in the peripheral nervous system [23], [99].

In addition to continued ocular ATTR deposition, failure to address continued post-operative production of variant TTR production by the choroid plexus is also a concern. Even after orthotopic liver transplantation, there have been reports of patients with hereditary ATTR amyloidosis experiencing focal neurologic episodes due to ATTR-related cerebral amyloid angiopathy (CAA) as well as post-mortem evidence of CSF-derived variant ATTR infiltration of the leptomeninges and leptomeningeal vasculature [100]. The risk of developing CNS symptoms attributed to undisrupted variant ATTR production in the CNS varies from 11.3 to 31%, a difference most likely due to the length of post-operative follow-up, with an average onset of 14.6 to 16.8 years from initial ATTR amyloidosis disease onset [100], [101].

An alternative treatment option for patients with severe ATTR cardiac amyloidosis and heart failure is orthotopic heart transplantation, either alone in the case of ATTRwt amyloidosis or in
combination with liver transplantation, for those patients with hereditary ATTR amyloidosis. First performed in England in 1984, heart transplantation for ATTR amyloidosis patients can be controversial due to the limited supply of hearts available compounded by the risk of disease recurrence in the cardiac allograft post-surgery [102], [103]. The variable post-operative survival rates for those patients who do undergo orthotopic heart transplantation vary, with reported 1- and 5-year post-operative survival rates ranging from 60–74.6% and 30–54%, respectively, thereby suggesting this procedure can be used as a last resort for patients with extreme cardiac amyloidosis [103], [104].

1.8.2 Small molecule drugs: TTR stabilizers
Not all patients diagnosed with ATTR amyloidosis, either wt or hereditary, are eligible for surgically invasive interventions such as orthotopic liver and/or heart transplantation. Failure to meet surgical eligibility criteria leaves these patients at risk of premature death, and therefore there is a critical need for the development of alternative, non-invasive treatment strategies. A number of small molecule drugs have been identified as potential pharmacotherapies, but none of these drugs have FDA-approval.

One drug that has gained approval for the treatment of hereditary ATTR in multiple countries, including Japan, Mexico, Argentina and the European Union, is tafamidis (Vyndaqel®) [105]. Tafamidis is a non-NSAID (non-steroidal anti-inflammatory drug) benzoxazole derivative that functions as a TTR stabilizer [106], [107]. TTR contains two pairs of dimer–dimer interfaces that together form two tetrameric hydrophobic-binding pockets normally occupied by thyroxine. Small molecule drugs such as tafamidis have been designed to occupy these T4-binding sites via negative co-operativity, kinetically stabilize the TTR tetramer, and result in the consequent decrease in the rate of tetrameric dissociation rates and fibril formation in vitro [107], [108]. For patients diagnosed with early signs/mild hereditary ATTR amyloidosis, tafamidis can potentially delay neuropathic progression of ATTR amyloidosis for up to 5.5 years; however, a direct control group to assess whether this effect is due to the drug alone or natural disease progression would help clarify this [109]. Although one concern of this drug is that occupation of these hormone-binding sites may have metabolic side effects by affecting thyroxine delivery throughout the body, clinical trials have found minimal evidence of this [110]. This clinical finding supports the paradigm that
thyroxine-binding globulin, rather than TTR, transports approximately 99% of the body’s circulating thyroxine [110], [111].

Other than tafamidis, there have been multiple tetrameric stabilizers that have gained scientific interest due to their reported effects on TTR amyloidogenesis. These include epigallcatechin-3-gallate (EGCG), curcumin, diflunisal, resveratrol, AG10 as well as tolcapone, which has gained more clinical interest and is currently undergoing clinical trials. There have been reports of dietary compounds such as EGCG and curcumin, major components of green tea and turmeric respectively, to have effects of TTR fibrillogenesis. At high concentrations, EGCG is able to stabilize the TTR tetramer and exhibit both fibrillogenesis inhibition and disruption of mature amyloid deposits in transgenic mice [112]–[114]. Transgenic mouse model studies have also shown curcumin (also known as diferuloylmethane) to decrease both ATTR amyloid deposition and ATTR-related cytoxicity, as well as result in amyloid remodelling in tissue [115].

Resveratrol has been shown to induce tetrameric stabilization via the thyroxine-binding site mechanism described above. Resveratrol has also gained recent popularity for its reported benefits for the treatment of Alzheimer’s disease via its ability to stabilize TTR and thereby decrease Aβ levels in transgenic mice [116]. In humans, however, the bioavailability of orally administrated resveratrol is limited to <5% due to rapid intestinal conjugation, limiting its clinical use in treating ATTR amyloidosis [117].

Administration of diflunisal to patients diagnosed with hereditary ATTR amyloidosis results in improvement of autonomic symptoms but its long-term side effects include induction of impaired renal function and thrombocytopenia [118], [119]. Because diflunisal is an NSAID, chronic management of ATTR amyloidosis using this drug is often contraindicated or requires cautious monitoring in both elderly patients and those diagnosed with cardiovascular disease [120].

High-throughput screening has resulted in the development of the TTR kinetic stabilizer, AG10, a small molecule drug that occupies TTR’s T4-binding pocket via negative co-operativity [108], [121]. In comparison with tafamidis, AG10 can significantly both inhibit wt and Val122Ile TTR fibrillogenesis in vitro at substoichometric concentrations and stabilize wt and Val122Ile TTR in human serum [121].
Tolcapone, an anti-Parkinson agent, has been recently repurposed as a potential treatment for ATTR amyloidosis due to its ability to stabilize the TTR tetramer by occupying its T4-binding site, demonstrating higher binding affinity and potency than tafamidis in vitro [122]. Phase I/II clinical trials to evaluate its TTR stabilization effects in patients with ATTR amyloidosis have been ongoing since July 2014.

1.8.3 Small molecule drugs: TTR aggregate disrupters

Drugs that have recently gained more clinical interest for the treatment of ATTR amyloidosis include doxycycline and tauroursodeoxycholic acid (TUDCA). In transgenic mouse models, TUDCA, a hydrophilic biliary acid, has been shown to decrease the deposition of toxic prefibril TTR oligomers and levels of cellular stress biomarkers normally associated with ATTR amyloid deposition, but showed no effect on mature amyloid fibrils [123], [124]. Studies using transgenic mice have also reported additional effects of TUDCA, as well as curcumin, on ATTR amyloidosis progression via modulation of cellular autophagy processes [125]. Doxycycline, a member of the tetracycline family, has exhibited amyloid fibril disruption effects both in vitro and in vivo, but has not demonstrated any effect on toxic pre-fibrillar TTR oligomers [123], [126]. Recent advances include the development of polyglutamate–doxycycline conjugates, which have been reported to have enhanced fibril elimination effects in comparison with parental doxycycline-only controls [127]. Phase I/II clinical trials investigating the effect of combining these latter two treatments (doxycycline and TUDCA) and evaluating their effects on the progression of ATTR cardiac amyloidosis have been underway since June 2013.

1.8.4 Antisense nucleotides and siRNAs

Alternatives to tetramer stabilizing pharmacotherapies are siRNAs and antisense nucleotides (ASOs), which interfere with and silence TTR gene transcription. Hepatic gene silencing of TTR allows for decreased systematic TTR production and decreased progression of ATTR amyloidosis without invasive, surgical intervention. Some of these drugs have had better success at affecting ATTR amyloidosis in vivo than popular TTR stabilizers. TTR specific siRNAs (siTTR) tested in murine models of ATTR amyloidosis have been reported to decrease ATTR amyloid deposition and induce ATTR amyloid regression better than the aforementioned TTR tetramer stabilizer tafamidis [128]. siRNAs with similar mechanisms of action include
patisiran and revusiran [129], [130]. Alnylam Pharmaceuticals had both drugs in Phase III clinical trials until the latter was discontinued in October 2016 due to compromised patient safety; patisiran studies have reportedly been unaffected by this discontinuation. A second-generation antisense oligonucleotide, IONIS–TTRRx, currently in Phase II/III clinical trials, has also been reported to decrease TTR plasma levels by >80% in both monkeys and ATTRIle84Ser transgenic mice. This therapy was also tested in healthy humans, demonstrating that it can decrease wtTTR plasma concentrations in a dose-dependent manner [131]. Phase III clinical trials of IONIS–TTRRx were initially delayed when the U.S. FDA put the trials on hold because of concerns for patient safety due to the development of thrombocytopenia. IONIS Pharmaceuticals has since reported positive results from ongoing trials with IONIS–TTRRx [131].

1.8.5 Immunotherapy

Despite the widespread use of monoclonal antibodies in a range of scientific and medical applications for the past few decades, their use to target pathologically specific conformations of a protein and to treat or prevent protein-misfolding diseases is less common. In immunoglobulin light chain (AL) amyloidosis, monoclonal antibodies that promote the clearance of amyloid in amyloidoma mouse models and that demonstrate positive biomarker responses in clinical trials have been developed [132], [133]. Further monoclonal antibodies for AL amyloidosis treatment are under development [134]. A similar approach is now being investigated for the treatment of ATTR amyloidosis.

Structure-based immunotherapeutic strategies have been recently reported for the potential development of non-surgically invasive treatment interventions for ATTR amyloidosis. Both polyclonal and murine monoclonal antibodies have been developed that target the residues TTR89-97 of human TTR, an epitope or ‘cryptotope’ deeply buried in the TTR tetramer but exposed upon tetrameric dissociation [135], [136]. The concept behind this strategy is that, as mentioned previously, normal TTR remains a tetramer but under conditions that result in misfolding, it can dissociate into its monomeric subunits and expose residues normally inaccessible when in the native tetrameric form. This allows for the specific targeting of the pre-fibrillar and non-native conformations of the TTR protein, but not TTR’s natively folded and non-pathological tetramer. These monoclonal antibodies have successfully demonstrated fibril inhibition, specific ATTR amyloid labelling in TTR amyloid-positive tissue, as well as the ability
to induce phagocytosis by human monocytes upon exposure to non-native TTR in vitro [136]. Murine and humanized monoclonal antibodies that target a cryptotope downstream of that targeted by the TTR89-97 antibodies have been recently reported with similar pre-clinical success in their ability to specifically recognize non-native forms of TTR, inhibit TTR fibrillogenesis in vitro, and induce phagocytosis of TTR fibrils by macrophages [137]. These antibodies target residues TTR115-124 of human TTR and have also been shown to affect ATTR deposition in transgenic rats expressing human ATTRVal30Met [137].

Alternative immunotherapeutic approaches for ATTR amyloidosis include the development of therapeutic anti-serum amyloid P component (SAP) antibodies. SAP is a non-fibrillar plasma glycoprotein found in all types of human amyloid deposits, included ATTR. A phase I trial involving 15 patients with systemic amyloidosis, but not cardiac amyloidosis, studied the effects of CHPHC((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid), a small molecule drug that depletes levels of circulating SAP, followed by a single dose of humanized IgG anti-SAP antibodies [138]. These anti-SAP antibodies target residual SAP in amyloid deposits and have been shown to initiate macrophage-mediated clearance of SAP-infiltrated amyloid in mouse models [139]. This small trial reported reduction in hepatic, renal and lymphatic amyloid load and no serious adverse side effects [138]. Although none of the 15 amyloidosis patients involved had ATTR amyloidosis, expansion of this specific study [139] to include and evaluate the effects of treating patients with ATTR amyloidosis would be of value.

1.9 Biophysics of ATTR

TTR forms a stable orthorhombic crystal structure (P2₁2₁2₁ symmetry), with each of its four subunits consisting of eight β-strands (ABCDEFGH) assembled into a sandwich of four β-stranded anti-parallel sheets (DAGH and CBEF) [140]. Two subunits/monomers can self-associate into a symmetric dimer via F–F' and H–H' strand interactions, and these dimers can in turn associate to form a homotetramer. Tetrameric dissociation is the first step of TTR fibril formation, followed by the partial unfolding of its monomeric subunits, resulting in amyloidogenic precursors with high propensity to aggregate into amyloid [141], [142].

The initiating step of tetrameric dissociation can be initiated by low pH [143], TTR point mutations or age-associated protein modifications. X-ray crystal structures of wtTTR in both low and
physiological pH conditions reveal that low pH results in large conformational changes in the TTR’s E and F strand loop–helix regions, regions involved in the dimer–dimer interfaces. These changes result in large quaternary changes and the consequent unfolding of the tetramer into its amyloidogenic monomers [144]. It is believed that amyloidogenic point mutations in the TTR protein instigate tetrameric dissociation by affecting the tetrameric dissociation kinetics rather than the tetramer’s thermodynamic stability [145], [146]. In addition to point mutations and low pH environments, age-related effects on both tetrameric wt and mutant TTR (TTRVal30Met), either oxidative modifications of sulfur-containing residues (i.e. Met and Cys) or age-associated protein carbonylation, have been reported as potential contributors to TTR amyloidogenicity [147]. These age-associated protein modifications, more specifically carbonylation, also had negative in vitro side effects on the TTR stabilizer resveratrol and its ability to inhibit fibril formation due to carbonylation of residues at the resveratrol’s T4-binding pocket [147]. Whether there is association between accumulation of age-associated protein modifications and efficacy of other TTR stabilizing drugs in aging patient populations remains a relationship to be further explored.

There are multiple reports describing regions of high aggregation propensity within the TTR protein. Some studies report that isolated TTR segments within the thyroxine-binding pocket, TTR10–20 and TTR105–115 (corresponding to strands A and G), undergo amyloid fibril formation in vitro [148] and suggest the effect of TTR stabilizer drugs that target the T4-binding site may be the result of their interaction with the TTR105–115 segment [149]. In silico studies have instead predicted F and H to be responsible for monomeric aggregation and have validated these findings in vitro using peptide inhibitors targeting these regions thereby inhibiting aggregation [61]. Several other reports also show the importance of the C-terminal part of TTR in fibrillogenesis [62], [63]. Although some studies report the C and D strands to be responsible for aggregation [150], others contradict these findings due to the presence of ‘gatekeeping residues’ such as Lys35 [151]. Despite many amyloidogenic mutations reported in the B, C and D strands of TTR (encompassing residues TTR26–57), this region demonstrates poor amyloidogenicity in vitro due to the presence of the Lys35 residue, which decreases TTR’s ability to form fibrils due to its charge and flexible side chain [151]. Heparan sulfate, however, is able to bypass the protective effects of the Lys35 residue and bind to this region (TTR26-57); these findings support heparan sulfate’s reputation as a TTR fibrillogenesis promoter and provide justification for its co-localization with ATTR deposits in cardiomyopathic heart tissue [151], [152].
The mechanism of ATTR amyloid formation is a topic of evolving discussion. Studies have reported amyloid fibrils to contain tight association between F strands and significant solvent protection for multiple residues in both the F and E strands [150], [153]. Others have postulated that if exposure of the natively buried F and H strands at the dimer–dimer interface is required for fibrillogenesis, then tetramer to monomer dissociation is required to allow fibril self-assembly. If true, this theory of TTR amyloidogenesis contradicts the one of amyloid formation via dimer scission [61], [154].

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1.12 Thesis Rationale
ATTR amyloidosis is an under-recognized disease that requires increased awareness among clinicians so that patients are correctly diagnosed and those who are eligible for treatment receive it before it is too late. If left untreated, ATTR amyloidosis can result in debilitating conditions that affect the activities of daily life as well as lead to heart failure and death. There is urgent need for the development of non-surgically invasive treatment interventions for patients who suffer for ATTR amyloidosis, and multiple, novel treatment strategies are currently in development.

1.12.1 Strategic immunotherapeutic targeting using polyclonal antibodies
The present thesis describes in detail the path taken to develop a novel immunotheranostic strategy for combating the current challenges faced by the medical community in ATTR amyloidosis. This strategy was based on the concept of using conformation-specific antibodies which would be designed to specifically target only the upstream misfolding intermediates of the amyloidogenesis pathway and/or amyloid end products. Chapter 2 focuses on the initial development of a
misfolding-specific polyclonal antibody which recognized an epitope that is normally buried deep within the tetrameric TTR complex but is exposed upon tetramer dissociation (i.e. a cryotope). This strategy was employed to specifically target the pre-fibrillar and non-native conformations of the TTR protein, but not TTR’s natively folded and non-pathological tetramer. In the TTR protein, residues #89-97 were identified as exposed on TTR’s dimer and monomer interface but inaccessible when in TTR’s tetrameric form (Figure 2.1). A multi-antigenic peptide (MAP) was synthesized with multiple GGEHAEVVFAGGKG sequences attached to a dendritic core; the underlined peptide indicates the TTR89-97 sequence and the non-underlined residues indicate the Gly-Lys linkers used to increase the molecular weight of the MAP antigen for the enhancement of immune response in the host. Polyclonal antibodies raised to this MAP antigen, termed misTTR, were generated in rabbits, affinity purified, and subject to an array of validation experiments to assess whether this polyclonal antibody was specific for non-tetrameric states of the TTR protein, effective at in vitro inhibition of both mutant and wild-type TTR aggregation, and whether it could specifically recognize ATTR deposits in amyloid-confirmed cardiac tissue (Chapter 2).

1.12.2 Targeting misfolding intermediates with murine monoclonal antibodies

A major disadvantage of polyclonal antibodies is that the antibody source from which the antibodies are obtained is limited to a single animal. Even if a multitude of animals are immunized with the same MAP, each animal will mount a unique immune response and respond differently, resulting in a limited, heterogeneous, and irreproducible source of antibodies. Once the initial animal source is exhausted, following batches obtained from the immunization of subsequent animals will contain batches containing a brand new spectra of antibodies with a wide range of different affinities and specificities to the epitope of interest.

Following the success of developing a rabbit polyclonal antibody (misTTR) which could specifically recognize the misfolded conformations of TTR, murine monoclonal antibodies targeting the same misTTR epitope were developed (Chapter 3). Monoclonal antibodies have multiple advantages over polyclonal antibodies, the main advantage being that hybridoma technology allows for an infinite source of homogeneous antibody production. Monoclonal antibodies raised against the same MAP as the misTTR polyclonal antibody were screened via a variety of biochemical binding assays and techniques (e.g. SPR, immunohistochemistry, monocytic
cellular uptake assays, fibril inhibition assays, western blot analysis) and the best performing antibody was chosen for humanization and subsequent studies.

1.12.3 Characterizing mis-TTR-mAb1 binding sites

Following the biophysical characterization of monoclonal antibodies described in Chapter 3, Chapter 4 aims to explore the possible mechanism by which these antibodies might function when exposed to TTR aggregates in vivo. Using the best performing murine monoclonal antibody from our studies in Chapter 3 (misTTR-mAb1, formerly known as 14G8) and its humanized form (h-misTTR-mAb1), we utilized immunogold electron microscopy (IGEM) to visualize the binding of these antibodies to TTR aggregates and fibers in vitro and in the plasma from ATTRVal30Met transgenic mice. Quantitative methods (i.e. isothermal titration calorimetry and surface plasmon resonance) were used to supplement the IGEM studies and to evaluate both the number and strength of interactions to the misTTR-mAb1 binding sites in TTR aggregates. The chapter concludes by suggesting a possible mechanism by which the misTTR-mAb1 can sequester potentially pathogenic forms of TTR, thereby preventing TTR deposition in the pathogenesis of ATTR disease.

The final chapter of these thesis (Chapter 5) explores the diagnostic potential of these monoclonal antibodies and briefly outlines a proposal for future studies.
2 Substoichiometric inhibition of transthyretin misfolding by immune-targeting sparsely populated misfolding intermediates: a potential diagnostic and therapeutic for TTR amyloidoses


N.J.G, A.B.-T., and R.R. contributed equally to this work. N.J.G, A.B.-T., and R.R. designed the experiments, performed the experiments, made the figures, and wrote the manuscript. P.W., S.S. and P.E.A. performed experiments, made figures, and wrote the manuscript. R.T. and J.T. performed experiments and made figures. P.W. supplied materials, interpreted data and wrote the manuscript. J.N.H. and A.C. designed the experiments, made the figures, and wrote the manuscript.

2.1 Abstract

Wild-type and mutant transthyretin (TTR) can misfold and deposit in the heart, peripheral nerves, and other sites causing amyloid disease. Pharmacological chaperones, tafimidis and diflunisal, inhibit TTR misfolding by stabilizing native tetrameric TTR; however, their minimal effective concentration is in the micromolar range. By immune-targeting sparsely populated TTR misfolding intermediates (i.e. monomers), we achieved fibril inhibition at substoichiometric concentrations. We developed an antibody (misTTR) that targets TTR residues 89–97, an epitope buried in the tetramer but exposed in the monomer. Nanomolar misTTR inhibits fibrillogenesis of misfolded TTR under micromolar concentrations. Pan-specific TTR antibodies do not possess such fibril inhibiting properties. We show that selective targeting of misfolding intermediates is an alternative to native state stabilization and requires substoichiometric concentrations. MisTTR or its derivative may have both diagnostic and therapeutic potential.
2.2 Introduction

Protein misfolding diseases constitute a significant health care burden, especially in terms of their economic impact and associated costs of care [155], [156]. Development of effective therapies for protein misfolding diseases requires an understanding of how proteins change from their natural conformation to misfolded pathological forms. In their natural state, proteins can be either structured or intrinsically disordered to perform their physiological function [157]. For those proteins that adopt a unique three-dimensional structure, which in most cases corresponds to the lowest free energy conformation, mutations or changes in environmental conditions can result in destabilization of this low energy state, and lead to misfolding, aggregation, and/or degradation [158]; this misfolding ultimately results in the development of protein misfolding disease.

Although a number of approved drugs for protein misfolding disease are available, they mainly offer symptomatic relief with few addressing the underlying root cause [159]. Protein misfolding diseases are caused by a multi-step pathway that results in the conversion of native proteins into abnormal conformations that lead to fibril formation and aggregation. The misfolding pathway can in many cases be described by a nucleation and growth model [160] or rather a downhill polymerization reaction model [161]. The native protein must first overcome an energetic barrier to populate transient high-energy intermediates. Conversion of these intermediates to aggregates/fibrils is an energetically favorable process, and pre-formed aggregates can ‘seed’ the conversion of other native proteins into the misfolded form. There are several conceptual strategies available for intervention that target different stages in the protein misfolding pathway (Figure 2.1A).
Figure 2.1. (A) Proposed model for the inhibition of TTR fibrillogenesis by the misTTR antibody. During the fibril formation process, native tetrameric TTR first dissociates into an altered monomeric intermediate. This altered monomeric intermediate self-assembles into stable nuclei, which allows for fibril formation to proceed. Substoichiometric amounts of the misTTR antibody may suppress TTR fibril formation by binding misfolded conformations of the TTR that comprise the critical nuclei, which are likely present at very low concentrations. The misTTR antibody may also act to inhibit TTR fibrillogenesis by binding to the unfolded monomers and/or to the extremities of fibrils, essentially capping the ends of preformed fibrils to prevent fibril growth. (B) Surface representation of native tetrameric TTR (green) with buried misTTR epitope in red. (C) Surface representation of monomeric TTR (green) with exposed epitope in red. Structures were generated using PDB ID 1DVQ in Deep View (Swiss-PDB Viewer 3.7).

Although the native and fibrillar states are the most stable and more populated species under steady-state conditions, the sparsely populated oligomeric intermediates are the ones which have been directly implicated in cytotoxicity (vida infra) [162]. It follows that effective treatments should reduce the toxic oligomeric intermediates. Current strategies in development include native state stabilization, fibril capping, and aggregate sequestration. However, these only indirectly affect the putative toxic oligomeric intermediates, and mostly target species in the misfolding pathway present at high concentrations, namely the native and fibrillar states.

Native state stabilization strategies utilize small molecules called pharmacological chaperones that prevent aggregation by binding to the native protein, which imposes an energetic barrier to the
formation of aggregation-prone misfolding intermediates. Pharmacological chaperones must possess sufficient binding free energy such that they alter the folding equilibrium to de-populate aggregation-prone species on the misfolding pathway. An alternative approach to native-state stabilization that limits aggregation involves capping the ends of growing amyloid fibrils with specific peptides or small molecules [163], [164]. However, capping aggregates results in the build-up of oligomers and small aggregates which can worsen the problem since oligomeric intermediates have often been shown to be more toxic than the final fibrillar end-product [165]. The concentration of toxic oligomeric species can be reduced by accelerating the aggregation process, as shown when small molecules were used to convert toxic Alzheimer amyloid peptide oligomers into less toxic amyloid fibrils [166]. Small-molecule pharmacological chaperones that stabilize the native state remain the strategy with a relatively good track record of development.

Native tetrameric stabilization has been the main approach used to design therapy for a group of protein misfolding diseases caused by the same amyloid-precursor protein, transthyretin. Misfolding of transthyretin (TTR), a human serum transport protein, is the root cause of a group of amyloidoses for which no FDA approved treatment currently exists. TTR is an abundant homotetrameric protein which transports thyroid hormones, T3 and T4, and Vitamin A (in a ternary complex with retinol binding protein) throughout the body [167]. Its primary site of production is the liver, which secretes it into the serum. Secondary sites include the retinal pigment epithelia and the choroid plexus which secretes it into the vitreous humor and cerebrospinal fluid, respectively [9]. Aberrant dissociation of the TTR tetramer into its misfolded monomers leads to formation and deposition of amyloid fibrils at multiple sites throughout the body; these amyloid deposits contain both full-length TTR and proteolytic fragments of TTR [168]. It is believed that the dissociation pathway of the native TTR tetramer is a multi-step process in which the TTR protein cycles through a series of misfolding intermediates before becoming an insoluble amyloid [169]. Although the mechanism of TTR misfolding is uncertain, studies with transgenic mice which over-express TTR suggest that it may be the result of complications either in TTR’s initial folding stages due to defective hepatic chaperones, or during its final stages of clearance as a result of defective proteasomes [170].

There are two main conditions related to the misfolding of the TTR protein: wild-type ATTR amyloidosis (ATTRwt) and hereditary forms of ATTR amyloidosis [171]. ATTRwt is
characterized by wild-type TTR amyloid deposition, while the latter is a heterogeneous group of autosomal dominant diseases caused by mutation in the TTR gene. ATTRwt involves amyloid deposition in the heart that can result in cardiac insufficiency and death, with secondary deposition in the peripheral nervous system and eye [172]. Deposition of wild-type TTR amyloid can also occur in both cartilage and ligaments and is believed to be the cause of other debilitating diseases such as lumbar spinal stenosis [33]. Additional ATTRwt co-morbidities include bilateral carpal tunnel syndrome, which is believed to be one of the first signs of ATTRwt as it precedes ATTRwt diagnosis by 3–5 years [31], [173].

There have been more than 125 different TTR mutations identified in various populations worldwide [2]. The phenotypic expression of these mutations varies but most often results in the development of systemic amyloidosis. A slowly progressing axonal sensory autonomic and motor neuropathy, often called ‘familial amyloidotic polyneuropathy (FAP) or restrictive cardiomyopathy are major consequences [174]. In many cases there is a mixture of these cardinal symptoms.

Current therapeutic strategies used in ATTR amyloidosis involve surgical procedures that either remove amyloid infiltrated tissue (i.e. heart transplants) or decrease the concentration of circulating amyloid-prone TTR protein (i.e. liver transplants) [173]. Two recently developed small molecule agents which have successfully displayed in vitro inhibition of fibril formation are diflunisal and tafamadis (Vyndaqel®). Both drugs are similar in their strategy of tetrameric dissociation inhibition by acting as pharmacological chaperones which directly bind to TTR’s thyroxine binding site at its dimer-dimer interface.

Although diflunisal has been demonstrated to effectively stabilize TTR structure in patients with TTR cardiac amyloidosis, its administration unfortunately compromises glomerular filtration rates and platelet counts [173]. Because diflunisal is an NSAID, administration to elderly patients, especially those with TTR cardiac complications, is contraindicated as a result of its side-effects such as renal failure, peptic ulcer disease, gastritis, and fluid retention [173]. Tafamadis, a benzoxazole derivative, binds to the thyroxine binding pocket, with two molecules of tafamadis per tetramer. While tafamadis has no NSAID-associated side effects, near-stoichiometric concentrations (serum TTR ~3.6 μM) would be required in order to effectively reach binding
saturation and prevent misfolding [107]. Drug binding leads to tetramer stabilization due to changes in the unfolding equilibrium, and the degree of this stabilization is proportionate to the drug binding free energy. The increase in native state stability leads to reduced concentrations of the unfolded state, lower protein degradation rates, and increased half-life of the native protein [175]. Because tafimidis binds TTR relatively weakly, with an apparent dissociation constant in the micromolar range [176], the degree of stabilization is not likely to be large. While tafimidis has been shown to be a clinically effective pharmacological chaperone in slowing disease progression, this strategy is limited by the need for high drug concentrations, the need for saturation binding to prevent misfolding, tighter binding to confer greater stabilization, and competition with endogenous ligands that may interfere with the protein’s normal function. Although the U.S. Food and Drug Administration (FDA) has indicated that the New Drug Application (NDA) for tafimidis has not yet been approved [177], it has received approval for treatment of ATTR amyloidosis in Europe, and has been used to treat TTR-FAP in Mexico, Japan, and Argentina [178].

While small molecule drugs like tafimidis have the advantages of relative ease of synthesis and high oral bioavailability, macromolecular antibody drugs have other advantages. These include their more specific mechanisms of action than conventional chemotherapeutic agents which often result in fewer associated side effects. They trigger complement-mediated processes that scavenge biological materials. Furthermore, humanized monoclonal antibodies, which are the most common protein drugs entering clinical studies, have higher drug approval rates [179] than new small-molecule drugs [180].

We used a structure-based approach to develop a TTR conformation-specific antibody that targets the pre-fibrillar, misfolding intermediates of TTR, but not the natively folded protein. This antibody directly targets the sparsely populated non-native misfolding intermediates and can achieve substoichiometric inhibition of fibril formation at nanomolar concentrations.
2.3 Results and Discussion

2.3.1 Development and validation of misTTR antibody

The use of antibodies, particularly as structural probes, to investigate the misfolding pathway of amyloidogenic and non-amyloidogenic proteins has been applied in the study of ALS [181], prion disease [182] and Alzheimer’s disease [183]. The generation of TTR antibodies have often relied on highly destabilized mutant proteins or randomly generated linear sequences of the molecule [168], [184], [185]. Using a high resolution X-ray crystal structure of TTR, we sought to develop a conformation-specific antibody which could not only be used as a structural probe of TTR misfolding intermediates, but also as a diagnostic tool for the identification of TTR-related amyloidosis patients, and perhaps even as a therapeutic agent.

Examination of the X-ray crystal structure of TTR (PDB code: 3KGU) revealed that residues 89-97 was the longest segment of TTR that was highly buried (>90%) in the native tetrameric fold of the protein (Figure 2.1B). These residues are located in the F-strand that is sequestered at the dimer interface of TTR and inaccessible in the native tetramer. We reasoned that an antibody targeted against this epitope would bind to misfolded TTR conformations with disrupted and exposed dimer interfaces, but not to the native tetramer. This antibody would bind to both monomeric (Figure 2.1C) and non-native oligomeric forms of TTR. Since these misfolded species represent only a fraction of serum TTR, this may result in substoichiometric concentrations of the antibody being sufficient for activity. In our strategy, antibody specificity for the misfolded conformation is designed from the outset rather than screened from large libraries.

To generate such an antibody, we first synthesized a multiple antigenic peptide (MAP) where each branch of the dendritic core contained the sequence ggEHAEEVVFTAagkg; the capitalized sequences represent residues 89–97 of TTR. Gly-Lys linkers were added to the N and C termini so the peptide epitope would resemble an internal sequence as well as to increase the molecular weight of the MAP antigen for an enhanced immune response. Rabbit anti-sera produced from immunization with the MAP antigen were affinity purified and used in all subsequent experiments described herein. We named this antibody, which was designed to specifically target the misfolded conformations of the TTR protein, ‘misTTR’.
To examine the binding specificity of the misTTR antibody, we tested its reactivity with folded tetrameric TTR and monomeric guanidine hydrochloride (GdnHCl) unfolded TTR [186] using indirect and competitive enzyme-linked immunosorbent assay (ELISA). Our antibody reacts preferentially with monomeric or unfolded TTR compared to native folded TTR in an indirect ELISA (Figure 2.2A). We tested the specificity of misTTR in a competition ELISA, where native and monomeric TTR in solution was tested for their ability to compete for binding to unfolded TTR absorbed to the ELISA plate. Native tetrameric TTR in solution was unable to compete (Figure 2.2B). Using previously established conditions to generate the monomeric amyloidogenic intermediate [186], [187] we found that the misTTR antibody was able to bind to this intermediate with an IC$_{50}$ value of 63 ± 20 nM (Figure 2.2B), suggesting that substoichiometric concentrations are sufficient for effective binding. We note that this is not precisely a binding constant because while the misTTR antibody appears to be mono-specific, it is purified from a polyclonal mixture. As a control, the assay was also performed using a commercially available pan-specific anti-TTR antibody (Sigma-Aldrich) that was raised against natively folded TTR protein. As expected, natively folded TTR was able to compete for antibody binding when this antibody was used (Supplementary Figure S2.1). The lack of binding by the misTTR antibody to native TTR supports the concept that the selected epitope is indeed buried within the native fold of the protein as predicted from the crystal structure. This also supports our rational design strategy of targeting an epitope accessible for binding only in the misfolded TTR species, and avoiding interactions with normal, natively folded TTR that is also present at high concentrations in patient serum.
Figure 2.2. (A) Indirect ELISA using monomeric TTR (0.01 mg/mL TTR incubated at 25 °C, pH 3, 44 h), guanidine unfolded TTR (TTR in 6 M guanidine, incubated overnight at 25 °C), folded native TTR and BSA. (B) Competition ELISA using guanidine unfolded TTR as bound antigen, with monomeric and tetrameric TTR as competitors, and misTTR as the antibody. IC₅₀ = 63 ± 20 nM for monomeric TTR’s binding affinity for misTTR. (C) Native and SDS-PAGE analysis was performed on wild-type TTR purified from human plasma (purchased from Sigma-Aldrich). For native PAGE analysis, three pairs of sample lanes were run. The first lane consisted of molecular weight markers while the second lane consisted of 2 µg of wild-type TTR. The three pairs of lanes were excised from the gel. The first excised lane pair was stained with Coomassie Blue dye. The second pair was transferred to PDVF membrane and immunoblotted with misTTR antibody. The third pair was similarly transferred to PDVF membrane but immunoblotted with commercial anti-TTR antibody. The same procedure was repeated for an SDS-PAGE counterpart.
In the native gels, misTTR did not recognize any species, while anti-TTR recognized putative dimers and higher molecular weight oligomers. In SDS gels misTTR only recognized monomers, while anti-TTR recognized monomers and putative dimers.

Our indirect and competition ELISA results comparing the reactivity of misTTR with folded and monomer/unfolded TTR suggest that our antibody has significant conformational specificity for misfolded TTR. To determine the oligomerization states of TTR bound by misTTR antibody we performed native and denaturing Western blots (Figure 2.2C). The pan-specific commercial TTR antibody recognizes native tetrameric TTR, monomeric TTR, and an SDS-resistant TTR dimer in the denaturing western blot (~35 kDa), whereas the misTTR antibody recognizes only monomeric (~15 kDa) TTR without recognizing native tetrameric TTR (Figure 2.2C). The misTTR antibody thus fulfills our design criteria of developing an antibody with very high conformational specificity for misfolded TTR and with reasonable binding strength.

2.3.2 MisTTR antibody recognizes TTR amyloid fibrils formed in vitro and in human tissue

We went on to evaluate the reactivity of the misTTR antibody with TTR amyloid fibrils formed in vitro. To do this, we selected physiologically relevant conditions under which TTR readily misfolds and forms amyloid fibrils (pH 4.5, 37 °C, 3 d) [143]. The aggregates formed under these conditions were able to bind the amyloid specific dye thioflavin-T (ThT), causing an enhancement in the fluorescence emission of the dye as well as a characteristic red shift in its emission spectrum (Supplementary Figure S2.2). The presence of TTR amyloid fibrils under these conditions was also confirmed using negative stain transmission electron microscopy (TEM) (Figure 2.3A). The fibrils observed were found to be ~7 nm in diameter and the morphology consistent with previously published reports of fibrillar structures formed by other amyloidogenic proteins [162], [188]. To determine whether the misTTR antibody would recognize these TTR fibrils formed in vitro, a competition ELISA was performed. TTR fibrils were able to compete with plate-bound GdnHCl-unfolded TTR for binding to misTTR (Figure 2.3B). Thus the misTTR antibody was able to selectively bind not only to monomeric, but to fibrillar TTR as well. Interestingly, addition of antibody during the early stages of fibrillogenesis resulted in reduction of total TTR aggregation, but did not affect the morphology of fibers formed (Supplementary Figure S2.3).
Figure 2.3. (A) Representative TEM image of amyloid fibrils produced from 1 mg/mL TTR. (B) Competition ELISA with misTTR antibody showing selective binding of TTR amyloid fibrils and antigenic peptide.

We then evaluated the reactivity of our anti-misfolded TTR antibodies and isotype controls by immunohistochemical analysis of cardiac tissue from validated cases of ATTR amyloidosis. Cardiac amyloids were identified by staining using the dyes thioflavin-T and Congo red (Figure 2.4C-E) and were confined primarily to the periphery of blood vessels found in these tissue sections. The misfolded TTR antibody selectively labels these Congo red-positive amyloid deposits with marginal labeling of Congo red-negative, non-amyloid TTR, whereas an isotype control antibody shows complete absence of immunoreactivity to the amyloid in the disease tissue (Figure 2.4B).
Figure 2.4. Formalin fixed paraffin embedded (FFPE) cardiac tissue sections from a patient with I84S TTR mutation were labeled using misTTR (dark brown, A), a non-specific isotype control antibody (dark brown, B), or the amyloid specific dyes thioflavin-T or Congo Red (green C, red D, green E).

2.3.3 Misfolding-specific misTTR antibody reduces fibrillogenesis efficiency

In order to determine if misTTR antibody binding has an effect on TTR fibril formation, we evaluated the kinetics of the process using UV/VIS absorbance spectroscopy. A time course of TTR fibril formation revealed what appeared to be a nucleated polymerization mechanism, which is consistent with previously published reports [143], [146]. A lag phase, corresponding to the length of time needed to form the amyloidogenic intermediate, was observed followed by a rapid
growth phase, which began to plateau after 60 h (Figure 2.5A). In the presence of the misTTR antibody, however, a change in the kinetic profile of TTR fibrillogenesis occurred. There is an extended time period, lasting around 30 h, where aggregates do not form, after which rapid aggregation occurs, but the aggregation level reaches only 40% of that seen in the absence of misTTR antibody (Figure 2.5A). Thus it appears that the antibody changes the aggregation mechanism from being a well ordered multi-step process to a less efficient nucleation-mediated process.

Figure 2.5. (A) Time course of TTR fibril formation monitored by turbidity (3.6 μM, 37 °C, pH 4.5). Substoichiometric amounts of misTTR were added at time 0 and after 30 h to determine effect on TTR fibrillization process. (B) Dose dependent inhibition of TTR fibril formation by misTTR after 72 h. ThT emission peak was integrated from 475 nm to 495 nm and plotted versus antibody concentration (IC$_{50}$ = 9.08 ± 0.32 nM).

Although the misTTR antibody was able to prolong the lag phase of TTR fibril formation, we looked to determine if it would have an effect when added to TTR after the fibrillogenesis process had started, namely during the polymerization or growth phase. The addition of substoichiometric amounts of misTTR antibody during the polymerization phase resulted in a slight decrease of the absorbance signal over a span of about 5 h (Figure 2.5A). While this slight decrease could be attributed to a dilution effect, this is highly unlikely due to the volume of antibody added. Since the observed signal decrease was gradual, it is likely that a slight disaggregation of prefibrillar species took place upon addition of the misTTR antibody.
In summary, the turbidity experiments highlight the complex inhibition of TTR fibrillogenesis, which includes a prolonged lag phase and apparent partial aggregation that plateaus at 40% of the level seen in the absence of antibody. To further explore this complexity a different technique was used to investigate the effect of the antibody on TTR fibrillogenesis.

2.3.4 Substoichiometric amounts of the misTTR antibody suppresses TTR fibrillogenesis in vitro

We investigated the concentration dependence of misTTR inhibition using ThT fluorescence, a technique which measures the formation of amyloid fibrils. Physiological concentrations of TTR were left under fibril formation conditions in the presence of various concentrations of the misTTR antibody for 72 h, and ThT was used to assess the extent of fibrillogenesis. Strikingly, a dose dependent inhibition of TTR fibrillogenesis was observed with IC$_{50}$ = 9.08 ± 0.32 nM. Substoichiometric concentrations of the misTTR antibody were sufficient to suppress TTR fibrillogenesis in vitro (Figure 2.5B). To confirm that the observed inhibitory effect is a consequence of the conformational specificity of the misTTR antibody, the experiment was repeated using the commercially available anti-TTR antibody. The pan-specific antibody, which displays no preference for native tetrameric TTR or misfolded conformations, had no effect on TTR fibrillogenesis, as assessed by ThT (Figure 2.5B); it could neither stabilize the native fold of TTR nor prevent the dissociation and self-assembly of TTR into amyloid fibrils under the conditions used. As a third control, a polyclonal antibody directed against the tumor suppressor protein p53 also showed no effect. As such, we propose that the inhibition of TTR fibril formation is not a generic feature of polyclonal antibodies or even TTR antibodies, but likely stems from the design, specificity, and affinity of the misTTR antibody for monomeric, misfolded conformations of TTR present in the fibrillogenesis pathway.

To assess whether the misTTR polyclonal antibody was only effective at inhibiting wild-type TTR protein amyloidosis, the antibody was subject to a pH-induced aggregation experiment using the designed mutant, TTR Y78F, which exhibits misfolding and aggregation formation at a higher pH than wild-type TTR [189]. Results of this experiment showed the antibody to be just as effective at inhibiting mutant TTR aggregation (Supplementary Figure S2.4). These results indicate that the misTTR antibody was not only specific to the non-tetrameric, pathological misfolding
intermediates of the normal TTR amyloidosis pathway, but also effective at inhibiting the aggregation of both wild-type and non-wild type TTR.

While the ThT fluorescence measurements indicate that the misTTR antibody completely inhibits TTR fibrillogenesis, the turbidity experiment revealed a complex inhibition involving a prolonged lag phase with partial aggregation reaching 40% of control. These differences are likely caused by the fact that ThT fluorescence measures amyloid fibril formation, while turbidity measurements gauge all forms of aggregation. The final aggregation products of these experiments may constitute a mixture of amyloid fibrils and nonspecific aggregates. Consequently, our results suggest that while this antibody inhibits TTR fibrillogenesis, it may not inhibit the formation of nonspecific aggregates.

2.3.5 Proposed model of the inhibition of fibrillogenesis by the misfolding-specific misTTR antibody

The proposed mechanism by which misTTR antibody inhibits TTR fibrillogenesis is illustrated in Figure 2.1A. Based on the data presented, we propose that the substoichiometric amounts of the developed antibody may act to suppress TTR fibrillogenesis in one of two ways. The first is as a disrupter of nuclei formation. During the course of fibrillogenesis, several TTR subunits associate to form a core or nucleus before cooperative growth can occur. However, since the association of subunits into a nucleus is an energetically unfavourable process, the lag phase generally persists for a period of time until a stable nucleus is formed. At a molar ratio of ~1:130 (antibody to TTR), misTTR antibody likely binds to small, oligomeric structures that are transiently populated during the lag phase and thereby delay the onset of polymerization. Binding to such structures may disrupt the assembly of stable nuclei needed for fibril growth. A second way by which the misTTR antibody may act to suppress TTR fibrillogenesis is by acting as a cap. At substoichiometric concentrations, misTTR antibody may prevent fibril formation by capping the ends of prefibrillar species and preventing growth.

In a recent publication, Eisenberg and coworkers [61] report the development of two peptide-based inhibitors of TTR aggregation. These peptides were designed using the computational method ZipperDB [190] to locate regions of the TTR sequence that had high fibril formation propensity. These peptide sequences were modified by substituting natural amino acids with N-methyl amino...
acids and by appending a tetra-arginine tail to either the N- or C-terminal. The peptides showed strong inhibition of TTR aggregation at concentrations above 20 μM. The first peptide corresponds to residues 91–96 of TTR. This segment is found right in the middle of our epitope (residues 89-97) (Figure 2.6). This finding may be a coincidence or it may indicate that fibril formation initiation sites consist of buried segments of high fibril formation propensity. The fact that the peptide and the misTTR antibody both inhibited TTR fibril formation suggests that this region of TTR is exposed in misfolding intermediates and perhaps is also exposed on the growing ends of the fibril.

The second peptide inhibitor is derived from residues 119–124 of TTR, which corresponds to the second region of TTR that is buried in the tetramer but exposed in the monomer (Figure 2.6). We had previously examined an epitope peptide that includes this buried area (residues 105–120), but unfortunately that peptide did not elicit an immune response in rabbits and could not be investigated further. Combining our results with those of Eisenberg and coworkers [61], we suggest that fibril initiation sites are particular segments of high fibril propensity that are buried in the native state, but become exposed in early, sparsely populated fibril intermediates, such as monomeric TTR. These sites appear to be ideal targets for both therapeutic antibodies and small molecule drugs.

![Figure 2.6](image_url)

**Figure 2.6.** The solvent excluded surface area (SES) of monomeric and the tetramer subunit form of TTR was calculated using Chimera molecular visualization program [191] using the coordinate file 3KGU. The peptide inhibitors are those developed by Saelices et al. [61]. Note the correlation of the locations of the misTTR epitope and inhibitor peptides with changes in SES associated with monomerization of TTR.
2.4 Conclusion
Using a structure-guided approach, we have designed the misTTR antibody which is able to
distinguish between native and misfolded conformations of TTR. MisTTR is the first antibody to
show inhibition of TTR fibrillogenesis, and more importantly, it achieves aggregation inhibition
at low substoichiometric concentrations ($IC_{50} = 9.08 \pm 0.32$ nM). Our findings suggest that this
antibody has the potential to be used as a diagnostic tool in the identification of those patients with
ATTR amyloidoses. Furthermore, monoclonal versions of the misTTR antibody may have
therapeutic potential for the treatment of FAP, hereditary ATTR, and ATTRwt by acting as
opsonization agents and facilitating clearance of both misfolded TTR and TTR aggregates using
the mononuclear phagocytic system [136]. This work suggests there is value in investigating
whether other oligomeric misfolding diseases involve the exposure of high-aggregation propensity
segments in early stage monomeric misfolding intermediates.

2.5 Methods
2.5.1 Antibody generation
To generate the misTTR antibody, peptide synthesis was carried out using standard
fluorenylmethoxycarbonyl (Fmoc)-based chemistry on a PS3 Automated Solid Phase Peptide
Synthesizer (Protein Technologies Inc). A MAP with the following sequence,
GGEHAEVVFTAGGKG, was synthesized
on an [Fmoc-Lys(Fmoc)]$_4$-Lys$_2$-Lys-$\beta$Ala-Wang resin
(Advanced ChemTech, SM5102) using Fmoc-protected amino acids (Advanced ChemTech,
Applied Biosystems, and Novabiochem). The peptide was subsequently cleaved from the resin
with a mixture consisting of 90% trifluoroacetic acid, 8% anisole, and 2% triisopropylsilane (all
from Sigma-Aldrich) and purified using ether extractions of protecting groups and scavengers;
peptide composition was later verified using amino acid analysis. The MAP was then sent to
Sigma-Genosys for rabbit antiserum production, which followed standard protocol (Sigma-
Genosys) and was in accordance with the Animal Welfare Act (USA).

2.5.2 Antibody purification
To purify the misTTR antibody from rabbit antiserum, a linear peptide with an identical sequence
as the antigen used for antibody production was synthesized onto a non-cleavable TentaGel-SH
resin (Advanced ChemTech). The resin was deprotected, acetylated, and packed into disposable
Columns (Evergreen Scientific). Columns were equilibrated with Phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) before being used for rabbit antiserum purification.

For purification, rabbit antiserum (1 mL) was first pre-cleared by centrifugation (16,000 g), after which an equal volume of saturated ammonium sulfate was added. After one hour incubation at 4 °C, precipitate was recovered by centrifugation and washed several times with 50% saturated ammonium sulfate. The precipitate was then dissolved in PBS-T and added to the affinity purification TentaGel column. The column was subsequently incubated overnight at 4 °C, with end-over-end rotation, to allowing for binding to occur. The antibody-bound column was washed with PBS-T at room temperature until the wash eluent contained little or no protein (A\textsubscript{280} = 0). Antibody fractions (1 mL) were eluted from column using 100 mM citric acid, pH 2.8, into tubes containing 250 μL of 1.5 M Tris and 150 mM NaCl, pH 8.0. The concentration of antibody in each fraction was determined using ultraviolet absorption spectroscopy and an IgG extinction coefficient at 280 nm of 1.35 (mg/mL)\textsuperscript{−1}. The affinity purified antibody was stored at 4 °C and was generally stable for about 1 month.

2.5.3 Protein expression and purification

A pET-21a (+) expression vector carrying TTR-(His)\textsubscript{6} was transformed into Escherichia coli BL21-A1 competent cells (Invitrogen). Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside when an absorbance (A\textsubscript{600}) of ~0.6 was reached. After 12–16 h incubation at 20 °C, cells were harvested by centrifugation at 5000 g. Pelleted cells were re-suspended in Buffer A (50 mM phosphate, 300 mM NaCl, 10 mM imidazole, and 20 mM β-mercaptoethanol, pH 8.0) and lysed by passage through an Emulsiflex-C5 (Avastin) for three cycles at 4 °C. After centrifugation at 27,000 g for 30 min at 4 °C, the supernatant was added to 5 mL of nickel-NTA (nitrilotriacetic acid) agarose slurry (Qiagen), gently mixed for 1 h, and loaded onto a column. The column was washed several times with Buffer B (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, and 20 mM β-mercaptoethanol, pH 8.0) and the fusion protein was eluted using Buffer C (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, and 20 mM β-mercaptoethanol, pH 8.0). The protein solution was dialyzed extensively against 10 mM phosphate, aliquotted, and frozen at −20 °C for later use.
The purity of the protein solution was confirmed by size exclusion chromatography. TTR eluted as a single peak at ~11 mL, representing tetrameric TTR, as shown in Supplementary Figure S2.2. Experiments were performed using recombinant TTR as well as wild-type TTR isolated from human plasma (Sigma-Aldrich). The biophysical properties of both plasma and recombinant TTR were found to be identical.

2.5.4 TTR fibril formation assay
A stock solution of TTR (5 mg/mL) was diluted with 50 mM sodium acetate, pH 4.5, to a final concentration of 0.2 mg/mL. Samples were then incubated at 37 °C for 72 h. The extent of fibril formation was probed by turbidity measurements at 400 nm on a Jasco V-500 UV-visible spectrometer equipped with a temperature control unit. Thioflavin-T (ThT) was also used to assess the extent of fibril formation. In brief, a five-fold molar excess of ThT (Sigma-Aldrich) was added to each sample and left at room temperature for 30 min before measurements were taken. ThT fluorescence was monitored using a Photon Technology International C60 spectrofluorimeter with the excitation and emission slit widths set to 4 nm. Spectra were obtained by scanning the fluorescence emission from 450 nm to 600 nm, with excitation at 442 nm.

2.5.5 Enzyme-linked immunosorbent assay (ELISA)
To examine the ability of the developed antibody to discriminate between native and non-native conformations of the TTR, competition ELISA experiments were performed. For all assays, TTR unfolded in the presence of 6 M guanidine-HCl [186] was used as bound antigen, while folded tetrameric TTR, monomeric TTR [143], and TTR fibrils were used as competitors. In brief, a 96-well plate was coated with 100 ng of GdnHCl unfolded TTR per well and incubated overnight at room temperature (pH 4.5). Each well was washed three times with PBS-T and subsequently blocked with PBS + 1% bovine serum albumin (BSA) w/v (Sigma-Aldrich) for 2 h. Various concentrations of competitor (folded TTR, monomeric TTR, or TTR fibrils) in the presence of 1 μg/mL of affinity purified misTTR or a commercially available anti-TTR antibody (Sigma-Aldrich) were added to wells and incubated for 1 h at room temperature. Wells were washed with PBS-T, and an HRP-conjugated anti-rabbit or anti-goat secondary antibody (1:5000) was added to wells and incubated at room temperature for 2 h. Wells were washed with PBS-T and 100 μL of the chromogenic substrate tetramethylbenzidine (Sigma Aldrich) was added. The
reaction was stopped with 2 M H$_2$SO$_4$, and an absorbance reading at 450 nm of the ELISA plate was taken immediately using a plate reader (SpectraMax M5, Molecular Devices).

All reported IC$_{50}$ values were determined by fitting competition ELISA data to the following equation: $y = \frac{A_{max} - A_{min}}{1 + \left(\frac{x}{IC_{50}}\right)^n} + A_{min}$

### 2.5.6 Western blots

15% native-PAGE and SDS-PAGE gels were run using the protocol as outlined in Sechi and Chait’s publication in *Analytical Chemistry* (1998) [192]. However, 1/3 of the gel was removed and subject to coomassie staining as a visual reference. The remaining 2/3 of the gel were subject to immunoblots, with ½ of the remainder used for immunoblotting with misTTR and the other ½ used for immunoblotting with an anti-TTR rabbit antibody as a control (Sigma-Aldrich, HPA002550). Sample lanes consisted of 2 μg of lyophilized human TTR (Sigma-Aldrich). Nitrocellulose membrane (Pall Life Sciences) was pre-soaked in PBS, pH 7.4 for 5 min and transferred into a PBS solution containing 20% methanol. The blot was blocked overnight at 4 °C with 5% skim milk, washed several times with PBS-T, and incubated at room temperature with affinity purified misTTR or anti-TTR antibody for 1 hour (1:1000). After several washes, the blot was incubated with IRDye® 800CW Goat anti-Rabbit secondary antibody (Li-cor Biosciences, #925-32211) (1:5000) for 1 h, and imaged using LICOR® Western Blot Analysis Software.

### 2.5.7 Electron microscopy

The TTR sample in Figure 2.3A was imaged using a Jeol 1011 microscope operating at 80 kV. Samples were deposited on fresh continuous carbon films prepared from copper rhodium grids (Electron Microscopy Sciences). Grids were charged using a glow discharger for 15 s at 3 mA negative discharge before adding samples. Fibril solutions of 1 mg/mL were adsorbed to grids for 2 min before rinsing with 10 μL ddH$_2$O for 10s. Samples were then blotted using No. 2 Whatman Filter paper and stained with freshly filtered 2% uranyl acetate for 15s.

Supplementary Figure S2.3’s TTR samples (0.2 mg/mL TTR-WT in 140 mM NH$_4$CH$_3$COO, pH 4.5) were incubated with and without 70 nM misTTR antibody for 72 h at 37 °C and 600RPM
agitation. Samples were imaged using a Joel 1200 microscope operating at 80 kV at 1 h, 24 h, and 48 h time points. Samples were deposited on carbon films prepared from copper rhodium grids (Electron Microscopy Sciences). Grids were glow discharged for 4–5 s at 3 mA prior to sample deposition. Samples were allowed 60 s for binding prior to wicking off with No. 2 Whatman Filter paper and then stained with freshly filtered 2% uranyl acetate for 10 s.

2.5.8 Histology and immunohistochemistry

Histochemical and immunohistochemical labeling was performed on 5 μm formalin fixed paraffin embedded sections of cardiac tissue from a patient with the TTR mutation I84S and confirmed ATTR amyloidosis. Congo red and thioflavin-T stains were used to demonstrate the presence of amyloid in the tissue. Congo red stain was performed using an alkaline Congo red solution (T3516-25G; Sigma-Aldrich, St. Louis, MO) following Puchler’s modified protocol and thioflavin-T staining was performed with a filtered solution of 0.015% thioflavin-T (T3516-25G; Sigma-Aldrich, St. Louis, MO) in 50% EtOH.

Immunohistochemistry was conducted on a Leica Bond Rx (Leica Biosystems, Buffalo Grove, IL) autostainer using Leica’s proprietary Bond Polymer Refine Detection kit (DS980). The immunoperoxidase method was the principal detection system. The slides were incubated with 0.5 μg/mL of the misTTR primary antibodies for 1 h followed by incubation in anti-rabbit polymeric HRP-linker secondary antibody conjugates. The staining was visualized with a DAB chromogen, which produced a brown precipitate. The slides were counterstained with hematoxylin, dehydrated in an ascending series of alcohols, cleared in xylenes, and coverslipped with CytoSeal 60.

Negative controls consisted of performing the entire immunohistochemical procedure on adjacent sections of ATTR cardiac sections with a non-immune rabbit IgG isotype control and an omission of the primary antibody. Positive control slides included staining of sections of normal human choroid plexus and normal pancreas.

Slides were viewed with an Olympus BX61 microscope equipped with a 10x/0.40 and a 20x/0.75 UPlanSApo objective. Congo red-stained sections were evaluated under a cross-polarizer and analyzer while thioflavin-T was visualized in the fluorescence using appropriate FITC excitation
and emission filters (Chroma Technology Corp., Bellows Falls, VT). Images were acquired with a Retiga Exi digital camera (QImaging; Surrey, BC) and imported with MetaMorph imaging system (Version 7.6.4.0; Molecular Devices; Sunnyvale, CA). All images were acquired and stored as Olympus Tiff files.

Whole-slides were digitally scanned with a Hamamatsu Nanozoomer 2.0HT slide scanner (Hamamatsu Corporation, Bridgewater, NJ) fitted with an Olympus 20x/0.75 UPlanSApo objective (Olympus).
2.6 Chapter Appendix

Supplementary Figure S2.1. Binding specificity differences of commercial pan-specific TTR antibody (Sigma-Aldrich) (empty circles) and misTTR (filled circles). Competition ELISA using unfolded monomeric TTR as plate-bound antigen with native tetrameric TTR as competitor. While binding of commercial pan-specific TTR antibody (Sigma-Aldrich) to plate-bound unfolded TTR could be inhibited by native tetrameric TTR, binding of misTTR to plate-bound unfolded TTR could not be inhibited by native TTR.

Supplementary Figure S2.2. Fluorescence spectrum of TTR plus thioflavin-T (6-fold molar excess) at pH 4.5 (filled circles), and at pH 7.0 (empty circles). Fluorescence spectrum of TTR in 50 mM Tris, pH 7.0 (filled squares).
Supplementary Figure S2.3. Transmission electron microscopy images of early stage in vitro TTR fibrillogenesis for 0.2 mg/mL TTR-WT (13.6 μM) incubated with (RIGHT column) and without (LEFT column) 70 nM misTTR antibody for 1 h, 24 h, and 48 h. Scale bars = 100 nm.
Supplementary Figure S2.4. *In vitro* pH-induced aggregation profile of 2 mg/mL TTR-Y78F protein with (filled circles) and without (empty circles) 1.4 mM misTTR antibody as measured by right angle light scattering (350 nm) (A) and thioflavin-T fluorescence (B). Both data sets indicate the misTTR polyclonal antibody to be effective at inhibiting the aggregation and amyloid formation of mutant TTR protein across a wide range of pH.
3 Novel conformation-specific monoclonal antibodies against amyloidogenic forms of transthyretin


N.J.G screened the monoclonal antibodies, and designed, performed, and made the figures for the fibrillogenesis studies.

3.1 Abstract

Transthyretin amyloidosis (ATTR amyloidosis) is caused by the misfolding and deposition of the transthyretin (TTR) protein and results in progressive multi-organ dysfunction. TTR epitopes exposed by dissociation and misfolding are targets for immunotherapeutic antibodies. We developed and characterized antibodies that selectively bound to misfolded, non-native conformations of TTR. Antibody clones were generated by immunizing mice with an antigenic peptide comprising a cryptotope within the TTR sequence and screened for specific binding to non-native TTR conformations, suppression of *in vitro* TTR fibrillogenesis, promotion of antibody-dependent phagocytic uptake of mis-folded TTR and specific immunolabeling of ATTR amyloidosis patient-derived tissue.

Four identified monoclonal antibodies were characterized. These antibodies selectively bound the target epitope on monomeric and non-native misfolded forms of TTR and strongly suppressed TTR fibril formation *in vitro*. These antibodies bound fluorescently tagged aggregated TTR, targeting it for phagocytic uptake by macrophage THP-1 cells, and amyloid-positive TTR deposits in heart tissue from patients with ATTR amyloidosis, but did not bind to other types of amyloid deposits or normal tissue. Conformation-specific anti-TTR antibodies selectively bind amyloidogenic but not native TTR. These novel antibodies may be therapeutically useful in preventing deposition and promoting clearance of TTR amyloid and in diagnosing ATTR amyloidosis.
3.2 Introduction

Amyloidoses are progressive diseases characterized by the accumulation of amyloid fibrils originating from a variety of different precursor proteins in multiple organs throughout the body, leading to organ dysfunction and death [193]. The most common hereditary form of this disease is transthyretin amyloidosis (ATTR amyloidosis), caused by the accumulation of the plasma protein transthyretin (TTR) [194]. Hereditary ATTR amyloidosis is an autosomal dominant and incompletely penetrant disease. It is classified by the specific TTR mutation and is associated with cardiac involvement and neuropathic involvement [2], [195]. Hereditary ATTR amyloidosis is caused by the presence of one of more than 100 known point mutations in the TTR gene that produces TTR isoforms having a higher propensity for aggregation and fibril formation than the wild-type protein [174], [196]. A second nonhereditary, sporadic form of ATTR amyloidosis, wild-type ATTR amyloidosis, is associated primarily with cardiac deposition of wild-type TTR in the heart and occasional TTR amyloid in carpal tunnel ligaments [197], [198]. ATTR amyloidoses are age-associated (onset typically at >60 years) and lead to progressive, systemic illness and death, with mean life expectancies of 5–6 years with cardiomyopathy and 9–11 years with non-cardiac pathology [199]. Current therapies include liver transplantation to remove the primary source of circulating, mutant TTR in hereditary ATTR amyloidosis [92] and treatment with agents such as tafamidis meglumine [107], [110] or diflunisal [200] designed to kinetically stabilize the TTR tetramer, delaying its dissociation into potentially amyloidogenic monomers. Several treatments are currently in clinical development that aim to reduce amyloid deposition by lowering de novo TTR production in the liver [129], [201] and thereby prevent TTR fibril formation, deposition and/or enhance clearance [202]. There are no approved treatments that directly clear deposited TTR amyloid to potentially reverse organ dysfunction.

TTR is a 55 kDa homotetrameric protein that transports thyroxin and vitamin A, the latter via an interaction with vitamin-A bound retinol-binding-protein [203]. The TTR tetramer is composed of four 127 amino acid, 14 kDa single-chain monomers with a dimer-of-dimers assembly. However, under conditions that destabilize the TTR tetramer (denaturation with low pH and/or elevated temperature, or the presence of familial point mutations), the tetrameric protein can more readily dissociate, leading to a conformational change of free monomers that favors aggregation and fibril formation [146], [204].
A potential treatment for the diseases characterized by amyloid deposition involves the use of monoclonal antibodies (mAbs) that selectively bind the dissociated monomers, non-native oligomers and/or aggregates to prevent fibril formation, or that bind the amyloid deposits themselves, targeting them for removal by phagocytic mechanisms [132], [205]. Targeting all these pathogenic forms with a single antibody, while sparing the normal tetrameric TTR, is also conceivable. For TTR, such an immunotherapeutic mechanism could supplement the native immune system’s ability to produce antibodies against non-native forms of TTR, thereby delaying or preventing the onset of disease symptoms [206], [207]. In addition, preclinical research demonstrating that active immunization of TTR-V30M transgenic mice (mice expressing human TTR containing a common ATTR amyloidosis-associated mutation) with the aggregation-prone TTR variant TTR-Y78F reduced TTR-V30M deposition and induced the production of non-native TTR antibodies [208]. These findings prompt the idea that conformation-specific antibodies with selectivity for non-native TTR species may be therapeutically beneficial if administered passively.

Previously described TTR epitopes unique to non-native forms of TTR, which distinguish native from misfolded TTR [184], [189], [208]–[212], may be suitable targets for misfolded TTR-specific antibodies. Based on a structural analysis of the TTR tetramer in comparison to the structure of the monomer Galant et al. [135] identified a novel cryptotope, comprising residues 89–97. This epitope is sequestered at the dimer interfaces of the tetrameric protein but is exposed upon tetramer dissociation and subsequent misfolding of the released monomers into amyloidogenic precursors [135]. A polyclonal antibody (pAb) against this cryptotope specifically bound to non-native, misfolded forms of TTR and prevented amyloid formation at substoichiometric levels, suggesting that this epitope might be a critical nucleation site for aggregation and fibril formation [135]. In addition, this pAb selectively immunolabeled TTR amyloid in tissue from patients with ATTR amyloidosis, confirming the non-native structure of TTR present in amyloid as suggested by Gustavsson et al. [209]. The objective of the present study was to produce mAbs specific for this unique epitope, to establish their utility in preventing TTR fibril formation and promoting cellular uptake of aggregated TTR in vitro and to demonstrate binding to TTR deposits in tissue from patients with ATTR amyloidosis. Such mAbs could potentially be used therapeutically to prevent TTR deposition and/or enhance TTR amyloid clearance in patients with ATTR amyloidosis, either alone or in combination with other therapeutic strategies.
3.3 Results

3.3.1 Generation of conformation-specific mis-TTR mAbs

We previously described the production and characterization of a conformation-specific mis-TTR pAb that selectively binds monomeric or misfolded conformations of TTR and inhibits TTR fibril formation in vitro but does not recognize native tetrameric TTR [135]. This pAb was generated using a MAP antigen containing residues 89–97 of TTR chosen on the basis of a structural analysis of tetrameric TTR versus the monomeric, dissociated monomer [184]. Residues 89–97 (EHAEEVVFTA; Figure 3.1A) are located within the F strand of TTR and sequestered at the dimer interface of the tetrameric protein, but they are exposed upon tetramer dissociation and thus constitute a cryptotope (Figure 3.1B). A BLAST search of the protein database did not reveal any other human proteins possessing this sequence [135].

![Diagram](image)

**Figure 3.1.** Generation of conformation-specific mis-TTR mAbs. (A) The amino acid sequence of human TTR highlighting the location of the mis-TTR epitope (residues 89–97, red). (B) Location of the mis-TTR epitope (red), which is inaccessible to mis-TTR mAb binding due to its location within the tetramer (left), but is exposed in the dissociated monomer (right). (C) The primary sandwich ELISA screening assay identified four clones that reacted strongly against pH4-TTR (light gray bars) and with minimal reactivity toward native, tetrameric TTR (dark gray bars).

We used a similar approach to generate mis-TTR mAbs. We synthesized a peptide having the sequence ggEHAEEVVFTAggkg linked to a poly-lysine dendritic core, which generated a MAP immunogen comprising a core of lysine residues with multiple branches linked to the TTR 89–97 peptide. This peptide sequence, ggEHAEEVVFTAggkg, included TTR residues 89–97 (capital
letters) with additional linker residues (lowercase) that were added to increase the solubility of the antigenic peptide and to establish the nine amino acid TTR epitope as an internal sequence.

In addition to this MAP, two other immunogens containing the same nine amino acid TTR epitope were generated by covalently linking the TTR 89–97 peptides (Ac-cggEHAEVVFTA-amide and Ac-EHAEVVFTAggc-amide), via their N- and C-terminal cysteine residues, to keyhole limpet hemocyanin (TTR89–97-N-KLH and TTR89–97-C-KLH). Immunization of mice with these MAP antigens resulted in the production of four unique high-titer hybridoma clones (9D5, 14G8, 6C1 and 5A1) that were chosen based on initial ELISA screening results for strong reactivity toward pH4-TTR with little/no reactivity toward native, tetrameric TTR (Figure 3.1C). The isotypes of these four mouse mAbs were determined as follows: 9D5 and 14G8 (IgG1/κ); 6C1 (IgG2a/κ); 5A1 (IgG2b/κ).

3.3.2 Conformational specificity of mis-TTR mAbs

The conformational specificity of the four mis-TTR mAbs identified in the primary screen was further explored by non-denaturing SDS-PAGE and Western blot analysis. Separation of native TTR by non-denaturing SDS-PAGE and staining with Instant Blue showed the presence of a ∼38 kDa native dimer (ND) with a very small amount of native tetramer (T) (Figure 3.2A). pH4-TTR (F) migrated as primarily a 15 kDa monomer (M) and a ∼37 kDa dimer (D). A close inspection of the dimer bands observed in the native TTR versus pH4-TTR samples showed that the ∼37 kDa pH4-TTR dimer (D) migrated slightly faster than the native TTR dimer (ND), indicating that these dimers were not the same species.
**Figure 3.2.** Characterization of mis-TTR mAbs by Western blot analysis. (A) Native TTR (TTR) and pH4-TTR (F) were separated by non-denaturing SDS-PAGE and stained with instant blue or Western blotted with mis-TTR mAbs. Under these PAGE conditions, native TTR migrated as primarily a ∼38 kDa native dimer (ND) with a minor amount of ∼60 kDa tetramer (T). pH4-TTR migrated as an ∼15 kDa monomer (M) and a ∼32 kDa non-native dimer (D). All four mis-TTR mAbs showed immunoreactivity toward the non-native TTR monomer and dimer, but did not react with native TTR. (B) In contrast, the total TTR mAbs, 8C3 and the Sigma pAb did not discriminate between TTR species. (C) By native-PAGE/Western, native TTR migrated as a tetramer (T) and a higher molecular weight native oligomer (O) while pH4-TTR (F), composed of aggregated forms of TTR, migrated as a high molecular weight smear ranging in size from ∼66–720 kDa. All four mis-TTR mAbs strongly recognized this high molecular weight TTR smear but did not react with the native tetramer or native oligomer. In contrast, the Sigma pAb reacted with all TTR species and did not show any conformational specificity.

Analysis of this non-denaturing SDS-PAGE gel by Western blot using the four mis-TTR mAbs showed that these antibodies identified both the monomer (M) and non-native dimer (D) bands present in the pH4-TTR sample, but did not recognize either the native tetramer (T) or the native dimer (ND) species (**Figure 3.2A**). In contrast, neither 8C3 (a mAb raised against fully denatured TTR) nor the Sigma pAb discriminated between native and non-native TTR species (**Figure 3.2B**).
The specificity of mis-TTR mAbs for non-native conformations of TTR was further investigated by native PAGE/Western blot analysis (Figure 3.2C). Separation of native TTR on a native gel showed the presence of the ∼60 kDa native tetramer (T) and a small amount of a high-molecular weight native oligomer (O) when stained with Instant Blue. Both of these species were identified by the Sigma pAb, but not by any of the mis-TTR mAbs. In contrast, pH4-TTR (F) migrated as a high molecular weight smear ranging from ≈80 to ≈480 kDa in size and showed strong reactivity with all four mis-TTR mAbs, indicating that the pH4-TTR sample was composed largely of non-native TTR aggregates. In contrast, the Sigma pAb did not discriminate between native and non-native TTR species, reacting with both the native tetramer (T) and the native oligomer (O) along with the high molecular weight non-native smear present in the pH4-TTR sample.

The kinetic parameters for each antibody binding to misfolded TTR were determined by SPR analysis using TTR-F87M/L110M. This TTR variant contains a double mutation at amino acid residues 87 and 110 of TTR that disrupts monomer-monomer contacts required for native tetramer assembly [145]. Unlike misfolded, aggregated pH4-TTR, TTR-F87M/L110M yielded a more homogeneous, mostly monomeric preparation of misfolded TTR as determined by gel filtration chromatography (data not shown) that was more suitable for SPR analysis. The kinetic parameters for binding of each of the four mis-TTR mAbs to monomeric TTR-F87M/L110M are shown in Table 3.1. All four mis-TTR mAbs showed nanomolar affinities for this TTR variant. Additionally, similar maximum response values among the four antibodies indicated uniform saturation binding to TTR-F78M/L110M.

**Table 3.1.** Kinetic parameters for binding of mis-TTR antibodies to TTR-F87M/L110M determined by SPR.

<table>
<thead>
<tr>
<th>Mis-TTR mAb</th>
<th>$k_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$K_D$ (M)</th>
<th>$R_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9D5</td>
<td>2.71E + 04</td>
<td>4.93E – 04</td>
<td>1.82E – 08</td>
<td>31.55</td>
</tr>
<tr>
<td>14G8</td>
<td>2.88E + 04</td>
<td>5.36E – 04</td>
<td>1.86E – 08</td>
<td>37.13</td>
</tr>
<tr>
<td>5A1</td>
<td>6.11E + 04</td>
<td>4.69E – 04</td>
<td>7.68E – 09</td>
<td>30.98</td>
</tr>
<tr>
<td>6C1</td>
<td>4.61E + 04</td>
<td>4.15E – 04</td>
<td>9.01E – 09</td>
<td>26.32</td>
</tr>
</tbody>
</table>

$k_a$, association rate constant; $k_d$, dissociation rate constant; $K_D$, equilibrium dissociation constant; $R_{max}$, maximum response.
3.3.3 Inhibition of TTR fibril formation

We evaluated the ability of the mis-TTR mAbs to inhibit TTR fibril formation at substoichiometric ratios, as previously demonstrated with the mis-TTR pAbs [135]. For these studies, TTR-V122I, a TTR variant containing a single V122I amino acid substitution that destabilizes the tetramer, making it more prone to aggregation and fibril formation [213], was exposed to mildly acidic incubation conditions that favored fibril formation yet maintained antibody binding activity. Fibril formation was associated with an increase in ThT fluorescence (Figure 3.3).

![Figure 3.3](image)

**Figure 3.3.** Inhibition of TTR-V122I fibril formation by mis-TTR mAbs. (A) TTR fibril formation (induced by incubation of TTR-V122I at pH 4.8 for 72 h and assessed by end-point thioflavin-T (ThT) fluorescence) was inhibited in the presence of 0–0.2 mg/mL 14G8 mis-TTR mAb (filled circles). Antibody alone (open circles) exhibited very little ThT binding and fluorescence. (B) In contrast to 14G8, the isotype control antibody, EG27/1, did not inhibit TTR fibril formation as demonstrated by no reduction in ThT fluorescence intensity.

Increasing 14G8 mAb concentrations caused a monotonic decrease in ThT fluorescence indicating a substoichiometric inhibition of TTR fibrillation (IC$_{50}$ = 0.028 ± 0.009 mg/mL; n = 3; Figure 3.3A and Table 3.2). The isotype control mAb did not cause inhibition of TTR fibrillation (Figure 3.3B), thus demonstrating the specificity of 14G8 mediated inhibition. The isotype control data were more variable than the 14G8 data, which is likely a result of the stochastic nature of aggregation that produces variability in the size and number of aggregates formed. This variability
decreased in the presence of inhibitory antibodies. Similar observations have been reported with inhibitors of aggregation of the brain protein, TDP-43 [214].

**Table 3.2.** Inhibition of TTR-V122I fibril formation by mis-TTR antibodies (n=3 assays/antibody).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IC₅₀±SD mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>9D5</td>
<td>No inhibition</td>
</tr>
<tr>
<td>14G8</td>
<td>0.028 ± 0.009</td>
</tr>
<tr>
<td>6C1</td>
<td>0.048 ± 0.059</td>
</tr>
<tr>
<td>5A1</td>
<td>0.015 ± 0.02</td>
</tr>
<tr>
<td>EG 27/1</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

Comparable substoichiometric IC₅₀ values determined for 5A1 and 6C1 (Table 3.2) suggested analogous mechanisms of fibril inhibition for each of these mis-TTR mAbs. In contrast, 9D5 unexpectedly failed to inhibit TTR-V122I fibril formation, despite showing similar specificity and affinity for non-native TTR. It remains to be explored whether 9D5 is more sensitive to the assay conditions used (e.g. low pH).

### 3.3.4 Antibody-dependent uptake of TTR by THP-1 cells

To determine whether these mis-TTR-specific antibodies can promote the *in vitro* uptake of non-native TTR by human monocyte phagocytosis, TTR-F87M/L110M was covalently labeled with the pH-sensitive fluorescent dye pHrodo; the pHrodo tag has minimal fluorescence under physiological pH, but fluorescence is enhanced upon engulfment into the low pH environments of endocytic vesicles and thus marks cellular uptake of tagged particles. THP-1 monocytes were added to pHrodo-tagged TTR (native or non-native, TTR-F87M/L110M) after treatment with either 14G8 or the isotype control antibody. Low levels of fluorescence were observed with either antibody incubated with native TTR, and after incubation of the control antibody with non-native TTR. Fluorescence was increased after 14G8 incubation with non-native TTR (Figure 3.4A), suggesting that non-native TTR is not efficiently phagocytized under basal conditions; however, the addition of mis-TTR antibodies specifically elicits phagocytosis of non-native TTR.
Figure 3.4. mis-TTR mAb-dependent phagocytosis. (A) TTR-F87M/L110M or native TTR was covalently labeled with the pH-sensitive fluorescent dye, pHrodo, which shows enhanced fluorescence upon exposure to lowered pH in endocytic vesicles. Uptake of the pHrodo label as measured by relative mean fluorescence intensity (MFI) of THP-1 cells was specific for pHrodo-TTR-F87M/L110 that was treated with 14G8 (gray bars), but not an isotype control antibody (black bars). 14G8 had no effect on the uptake of native pHrodo-TTR. Data are presented as mean ± SEM (n=3). (B) All four anti-mis-TTR antibodies increased uptake of aggregated pHrodo-ATTR V30M in a dose-dependent fashion as measured by pHrodo-positive THP-1 cells. In contrast, no uptake was observed in cells treated with an isotype control IgG and no fluorescence was detected in untreated cells (red circle). (C) Representative 20× objective pHrodo 594 brightfield overlays of THP-1 cells showing uptake of aggregated pHrodo-TTR-V30M in the presence of mis-TTR antibodies (most intensely fluorescent pHrodo-positive THP-1 cells denoted with white arrows).

Dose-dependent phagocytosis of pHrodo-labeled, large aggregated fibrillar particles of TTR was also demonstrated for each of the mis-TTR mAbs (Figures 3.4B and 3.4C). Maximum antibody-dependent uptake was variable for each mis-TTR mAb (6C1 > 9D5 ≈ 14G8 > 5A1), reaching a plateau at mAb concentrations between 5–10 μg/mL. Variable antibody potencies may reflect isotype differences and associated changes in effector function among the four mis-TTR mAbs. Controls, including untreated cells or those treated with an IgG1 isotype control, did not demonstrate detectable or enhanced fluorescence, respectively. These data support the concept that mis-TTR antibodies can elicit clearance of extracellular soluble and insoluble aggregates of non-native TTR by opsonization and subsequent phagocytosis.
3.3.5 Immunohistochemical evaluation of mis-TTR mAbs

We examined whether the mis-TTR mAbs could specifically immunolabel TTR amyloid deposits in cardiac tissue from patients with TTR-I84S mutation. 14G8 and total-TTR immunoreactivities (Figures 3.5A and 3.5B) were overlapping and identified comparable deposits intercalated between the cardiomyocytes and in the vasculature that were not immunoreactive for 6F10, an IgG1 isotype control antibody (Figure 3.5C). These same deposits were also labeled by thioflavin-T (Figure 3.5D) and Congo red (Figures 3.5E and 3.5F), which confirm their amyloid nature. Less intense 14G8 immunoreactivity (Figure 3.5A, arrows) was also found in regions not staining for amyloid, and thus may represent staining of pre-amyloid TTR deposits.

![Figure 3.5](attachment:image.png)

**Figure 3.5.** 14G8 specifically identifies amyloidogenic TTR deposits in human ATTR amyloidosis cardiac tissue. Immunohistochemical labeling of cardiac tissue derived from an ATTR amyloidosis patient harboring the I84S mutation. (A) 14G8 mis-TTR mAb and (B) total-TTR pAb (DAKO) immunoreactivities were overlapping and identified comparable deposits in the myocardium and vasculature that were not immunoreactive for anti-6F10 (C), an isotype control. Lower-intensity immunohistochemical labeling with 14G8 (black arrows, A) was found in regions not staining for amyloid, and thus may represent pre-amyloidogenic TTR deposits. These same deposits were also labeled by thioflavin-T (D) and Congo red (E), which confirmed the amyloid nature of these deposits. Congo red birefringence (F) aligned with the same areas showing the greatest amount of TTR deposits. Scale bar = 500 μm.

Specific and intense 14G8 staining was also observed in heart tissue samples taken from ATTR amyloidosis patients harboring various TTR mutations (V122I, T60A, T49A and I84S) or
wild-type ATTR amyloidosis (Figure 3.6A). Similar specific immunolabeling was also observed with the other three mis-TTR mAbs (9D5, 6C1 and 5A1; Supplementary Figures S3.1–S3.3). The specificity of mis-TTR immunolabeling was further confirmed by a complete absence of reactivity in cardiac tissue derived from healthy individuals without ATTR amyloidosis and from patients with primary AL amyloidosis, another form of amyloidosis with cardiac deposits (Figure 3.6B).

Figure 3.6. 14G8 immunolabels cardiac tissue from patients with hereditary and sporadic ATTR amyloidosis cardiomyopathy. (A) 14G8 strongly immunolabeled TTR deposits in diseased ATTR amyloidosis heart tissue derived from patients with familial (V122I, T60A, T49A, I84S) and wild-type forms of cardiac ATTR amyloidosis. (B) No 14G8 immunolabeling was observed in cardiac tissue from healthy individuals (Normal) or from cardiomyopathy patients with non-related AL amyloidosis (AL). Scale bar = 500 μm.

We examined mis-TTR immunolabeling in the normal liver, pancreatic α-cells and the choroid plexus, organs known to express and secrete TTR. 14G8 specifically labeled the TTR present in pancreatic α-cells and the modified ependymal cells of the choroid plexus, but not the TTR present in normal human liver (Figure 3.7). In contrast, intense immunolabeling was observed in all this tissue using the total anti-TTR antibody (Dako; Figures 3.7D–F). This finding was expected since the TTR present in this tissue is native, tetrameric TTR. The ability of 14G8 and other mis-TTR
mAbs (see Supplementary Figure S3.4) to recognize TTR produced in the pancreas and choroid plexus but not in the liver suggests that some of the TTR produced in the pancreas and choroid plexus may be structurally distinct from that produced in the liver, possibly a result of different cellular or tissue environments.

**Figure 3.7.** Staining of TTR by the 14G8 mis-TTR mAb in normal tissue known to express TTR. 14G8 did not stain the TTR present in normal human liver (A) but did stain intracellular TTR present in normal pancreatic α-cells (B) and choroid plexus (C) from normal donors. Staining of tissue with 14G8 was specific as demonstrated by the lack of staining in adjacent sections using an isotype control antibody (data not shown). This is in contrast to staining of TTR using the Dako antibody which stained total TTR present in all three of these normal tissue types (D–F). Scale bar = 250 μm.

### 3.4 Discussion

We used a rational, structure-based approach to generate mAbs that selectively target a TTR cryptotope, composed of residues 89–97, which is exposed only in monomers and non-native conformations of TTR. ELISA, Western blot and SPR analysis confirmed that mis-TTR mAbs bind with a high affinity to non-native forms of TTR but do not bind to native, tetrameric TTR in which the 89–97 epitope is inaccessible. This feature allows mis-TTR mAbs to target TTR amyloid or TTR species involved in the amyloidogenic process while sparing the function of the native protein in thyroxin and vitamin A transport.
TTR fibrillogenesis is thought to be initiated by the dissociation of the native tetramer into constituent monomeric subunits, followed by a conformational change that initiates a nucleation-dependent polymerization process, similar to that observed for other amyloidogenic proteins [215], [216]. Stable, self-organized nuclei are expanded by the sequential addition of monomeric intermediates to form fibrils. The rate of nucleation-dependent polymerization can be coupled with cellular injury and death [215], and thus it may be an important therapeutic target.

The ability of mis-TTR antibodies to suppress fibrillogenesis *in vitro* at substoichiometric levels suggests that the TTR amyloidogenic species targeted by mis-TTR mAbs, although very impactful, may be at low abundance in relation to the total TTR in solution. Binding to these precursors likely sequesters them from the amyloidogenic pathway, preventing nucleation and further aggregation and fiber formation. Alternatively, the antibodies may present a catalytic activity, where their binding to TTR aggregates could disrupt the tertiary structure, resulting in disaggregation. This phenomenon, described as catalytic disaggregation, was demonstrated previously for anti-amyloid beta protein antibodies [217]. Although studies are currently under way to determine the precise mechanism by which mis-TTR mAbs inhibit TTR aggregation and fibril formation, the mis-TTR epitope we describe here spans the aggregation-prone region within the F-strand of TTR that was recently found to be directly involved in the TTR aggregation process [61]. Thus, it is likely that our mis-TTR mAbs inhibit TTR aggregation and fibril formation by impeding the self-association of the protein as proposed for small non-native peptides specific for this site [61]. That 9D5 failed to inhibit *in vitro* fibrillogenesis was unexpected since this antibody showed binding affinities and selectivity for non-native conformations of TTR similar to the other three mis-TTR mAbs. This may be due to unique biophysical properties of the antibody (i.e. stability, solubility, or propensity to aggregate) that make it more sensitive to the low pH conditions used in this assay. Further studies are needed to more precisely determine why 9D5 was not active in this assay.

In addition to preventing TTR amyloid fibril formation, mis-TTR mAbs might also have utility in clearing previously deposited TTR amyloid present in affected organs through stimulation of antibody-dependent phagocytosis, as reported for antibodies against other amyloidogenic proteins [132], [218], [219]. Evidence supporting that mis-TTR mAbs may provide this therapeutic advantage comes from *in vitro* experiments demonstrating antibody-dependent phagocytic uptake of soluble and insoluble TTR by human monocytes.
Immunolabeling of amyloid deposits in cardiac tissue from patients with confirmed familial and wild-type ATTR amyloidosis, but not from healthy controls or from patients with AL amyloidosis, demonstrates that the mis-TTR epitope is specific to ATTR amyloidosis. The ability of mis-TTR mAbs to bind to this exposed TTR epitope suggests that other amyloid binding proteins such as serum amyloid P [220] or other post-translationally modified proteins [221] that may also be present do not occlude this TTR amyloid-specific epitope.

The lack of immunolabeling of wild-type TTR in the liver, the primary site of TTR synthesis in humans, indicates that this tissue expresses native, tetrameric TTR, which further supports the hypothesis that mis-TTR mAbs do not target native TTR. However, the significance of mis-TTR mAb binding in the α-cells of the pancreas and in cells from the choroid plexus of healthy individuals remains to be determined. It is possible that this staining may indicate the presence of monomeric or other non-tetrameric conformations of TTR resulting from tissue-specific differences in protein folding and secretion efficiency (e.g., tissue-specific protein chaperones or other binding partners impacting ER processing) [222]. Nevertheless, the mis-TTR mAb staining pattern in the pancreas and the choroid plexus most likely represents TTR that is inaccessible to antibody binding using immunotherapeutic approaches.

Reducing non-tetrameric TTR serum levels in combination with inhibiting TTR nucleation and targeting fibrils for phagocytic clearance may attenuate or block TTR aggregation and improve symptoms in patients. While experiments demonstrating the in vivo efficacy of anti-mis-TTR antibodies are beyond the scope of these studies, we reported that another conformational-specific mAb targeting an epitope unique to the misfolded aggregates of the immunoglobulin light chain protein promoted clearance of AL amyloid extracts in a mouse amyloidoma model, likely by engaging phagocytes to clear deposits [132]. Those studies have been translated into clinical trials using similar immunotherapeutic approaches in patients with AL amyloidosis, where we recently reported positive biomarker responses suggestive of improved cardiac and renal function [133]. The potential significance of mis-TTR antibodies as immunotherapeutic agents is also supported by specific antibody-mediated approaches for other diseases characterized by amyloidosis [205] and may be useful in combination with other ATTR amyloidosis treatments, such as liver transplantation and/or agents that stabilize TTR tetramers.
In addition to their potential use as immunotherapeutics, mis-TTR mAbs may prove useful in the diagnosis of ATTR amyloidosis if levels of immunoreactive TTR species can be correlated with the disease. Patient sera, cerebrospinal fluid or tissue biopsy specimens may be assayed with mis-TTR mAbs to improve the detection of ATTR amyloidosis, which is believed to be significantly underdiagnosed [9]. Improvements in the speed and accuracy of diagnosis could provide patients earlier access to treatments, potentially intervening prior to significant deterioration in organ function and likely improving patient prognoses. In accord with suspected underdiagnosis of ATTR amyloidosis and wild-type ATTR amyloidosis, such ante- and post-mortem testing could also improve estimates of the prevalence of this disease and may be beneficial in gauging prognosis and treatment response.

3.5 Conclusion

In this report, we describe the generation of mAbs (mis-TTR mAbs) that are selective for monomeric, misfolded and amyloidogenic forms of TTR. These antibodies inhibit TTR fibrillogenesis and induce antibody-dependent phagocytic uptake of TTR aggregates in vitro. In addition, these antibodies bind specifically to amyloid deposits in cardiac samples derived from patients with several confirmed ATTR mutations, demonstrating the exposure of this epitope in relevant disease tissue. Future studies will assess the pharmacokinetics of mis-TTR mAbs, their penetration and retention in target tissue, and efficacy in animal models, which could determine the diagnostic and therapeutic potential in patients with ATTR amyloidosis.

3.6 Acknowledgements

The authors thank Dr. Merrill Benson for generously providing ATTR amyloidosis heart tissue samples for these studies. Medical editorial assistance was provided by ApotheCom (San Francisco, CA).

3.7 Declaration of interest

NJG and KCH declare no competing financial or personal interests. AC received a research grant from Prothena Therapeutics Limited. All other authors are employees of and own stock options in
Prothena Biosciences Inc. This study was sponsored by Prothena Biosciences Inc, South San Francisco, CA, USA.

3.8 Methods

3.8.1 Recombinant TTR

*Escherichia coli* (BL21-A1) cells were transformed with a pET21a(+) plasmid containing either the TTR insert Met-hTTR-His$_6$ (tetrameric wild-type TTR), the destabilized variants of TTR associated with familial ATTR amyloidosis (TTR-V122I and TTR-V30M) [213] or the monomeric TTR-F87M/L110M [145]. Transformed cells were grown in 2YT broth (100 μg/mL ampicillin). Plasmid expression was induced overnight (20 °C, 1 mM isopropyl β-D-1-thiogalactopyranoside, 0.05% arabinose), and cells were centrifuged (4000 × g, 10 min) and stored at −80 °C. Thawed pellets (10–15 g) were lysed with Buffer A (50 mL 1× phosphate-buffered saline (PBS), 500 mM NaCl, 20 mM imidazole) using an LV-1 high-shear processor (Microfluidics, Inc., Westwood, MA), centrifuged (27 000 × g; 30 min; 4 °C), filtered (0.2 μm PES filter) and purified on a His-Trap HP column (GE Life Sciences, Pittsburgh, PA), washed (10 column volumes of Buffer A) and eluted with Buffer B (1 × PBS, 500 mM NaCl, 500 mM imidazole). TTR fractions were dialyzed against 1 × PBS and stored at −80 °C.

3.8.2 Production of TTR fibrils

Thioflavin-T–positive TTR fibrils were generated by exposure of tetrameric TTR to low pH conditions for extended periods of time to allow for tetramer dissociation and fibril formation. This was accomplished by diluting recombinant TTR to a final concentration of 0.2 mg/mL (3.6 μM) in 50 mM sodium acetate pH 4 and incubating at room temperature for 72 h. Fibril formation was monitored with thioflavin-T fluorescence (5-fold molar excess, 30 min; excitation wavelength, 442 nm; emissions wavelength, 482 nm) using a Paradigm Imager (Paradigm, Houston, TX) and by native PAGE. The resulting fibrillar TTR sample (pH4-TTR) was stored at 4 °C.

Low pH-aggregated TTR-V30M for the cell-based phagocytosis assay was produced as follows: purified TTR-V30M protein was diluted to 2.0 mg/mL in 50 mM sodium phosphate and titrated to pH 2.7 with citric acid. The sample was placed in a Thermomixer (Eppendorf AG) at 37 °C, 500RPM for 2 wks. Aggregated, thioflavin-T–positive protein was collected by centrifugation at
100 000 × g for 1 h, and the resulting clear, gelatinous pellet was washed once with 1 × PBS, then thoroughly resuspended in 1 × PBS to a final concentration of 2.0 mg/mL.

3.8.3 Antibody generation and hybridoma selection

A multiple antigenic peptide (MAP) with the TTR89–97 sequence flanked by glycine linkers (ggEHAEVVFTAgkg) was synthesized on a [Fluorenylmethyloxycarbonyl (Fmoc) Fmoc-Lys (Fmoc)]4-Lys2-Lys-βAla-Wang resin using Fmoc-protected amino acids, as described previously [135]. Additional immunogens were generated by covalently linking TTR89–97 peptides (Ac-cggEHAEVVFTA-amide and Ac-EHAEVVFTAggc-amide) via the N- and C-terminal cysteine residues to keyhole limpet hemocyanin (TTR89–97-N-KLH and TTR89-97-C-KLH). Mice were immunized with the antigenic peptides (RIBI adjuvant weekly or in TiterMax adjuvant monthly), with a final immunogen boost 3–4 d prior to fusion. Homogenized spleens were fused with SP2/0 myeloma cells, plated and screened after 7–10 d. Hybridomas were selected based upon specific binding to non-native pH4-TTR using a sandwich ELISA and goat anti-mouse (IgG1, 2a, 2b, 3 specific)-HRP (1:5000, 0.5% BSA/PBS/TBS-T). After 3 d of growth, clones were counter-screened with native TTR to eliminate non-discriminating clones.

3.8.4 Antibody expression and purification

Hybridoma cells were expanded in shake flasks and seeded into 10–25 L Wave bag cultures (CD hybridoma expression media with Glutamax [Life Technology]). Batch cultures were prepared using a Wave Bioreactor (GE Healthcare, Piscataway, NJ; 37 °C, 7% CO2 under constant agitation) with periodic cell number monitoring. Cultures were harvested when cell viability declined below 50% (5–7 d), clarified through a depth filter (Millistak Pod COHC; Millipore, Billerica, MA), sterile filtered (0.2 μm filter; Optican XL; Millipore), concentrated 10-fold by tangential flow filtration (Pelicon 2PLC 30K, Millipore) and then sterile filtered (0.2 μm filter; PES Filter System; Corning).

Concentrated conditioned media were loaded onto a pre-equilibrated Protein G Sepharose Fast Flow column (GE Life Sciences; 1 × PBS, pH 7.4, Akta Avant FPLC) and washed (5–10 column volumes, 1 × PBS, pH 7.4) until the OD280 reached baseline. Bound antibody was eluted with 2 column volumes of IgG Elution Buffer (Thermo Scientific, Grand Island, NY) and pH neutralized (2 M tris, pH 9.0, 60 μL/mL). Antibody-containing fractions were pooled and dialyzed
overnight (4 °C; 1 × PBS; pH 7.4), sterilized by ultrafiltration (0.2 μm PES filter) and stored at 4 °C. The final protein concentration was determined by bicinchoninic acid using a bovine gamma-globulin standard (Thermo Scientific).

3.8.5 Surface plasmon resonance
Anti-mouse antibodies (anti-mouse kit; GE Healthcare) were immobilized on a CM5 sensor chip per manufacturer’s instructions. Antibodies were captured at levels to ensure binding of 30–50 response units of analyte (TTR F87M/L110M). Three-fold analyte dilutions in running buffer (HEPES-buffered saline, 0.05% P-20, 1 mg/mL BSA) were passed over the captured ligand at a flow rate of 30 μL/min, spanning a concentration range at least 10-fold above K_D to 10-fold below K_D, based on preliminary studies. The reaction duration was optimized to allow for equilibrium to be reached during the association phase with 10% signal decay during dissociation for each analyte concentration. At least one mid-range concentration was run in duplicate. All surface plasmon resonance (SPR) measurements were performed on a Biacore T200 (GE Healthcare).

3.8.6 SDS- and native-PAGE electrophoresis and western blot analysis
3.8.6.1 SDS-PAGE gels
A 0.1–1 μg sample of TTR or pH4-TTR in lithium dodecyl sulfate sample buffer (Life Technologies) was run on a 10% NuPAGE bis-tris gel (MES buffer; 90 V, 105 min) and stained with Instant Blue (Expedeon, San Diego, CA) or transferred to nitrocellulose membranes for Western blot analysis.

3.8.6.2 Native-PAGE tris-glycine gels
A 0.1–1 μg sample of TTR or pH4-TTR in 1× tris-glycine sample buffer (Life Technologies) was run on an 8–16% tris-glycine native gel (120 V, 105 min) and stained or transferred to nitrocellulose membranes.

3.8.6.3 Western blot analysis
Nitrocellulose transfers (iBlot, Life Technologies) were treated with blocking buffer (LI-COR, Lincoln, NE) and incubated in 0.50 μg/mL primary antibody, washed with 1× TBS and placed in
1:20,000 dilution of IRDye 800CW-conjugated goat-anti-mouse secondary antibody (LI-COR), then imaged on an Odyssey CLx infrared imager (LI-COR).

3.8.7 TTR fibril inhibition assay

A stock solution of TTR-V122I (approximately 5 mg/mL) was diluted into buffer with final concentrations of 0.2 mg/mL TTR, 30 mM sodium acetate, 5 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, 0.02% sodium azide, and varying concentrations of mAb at pH 7.2. This sample was dialyzed against the same buffer at pH 4.5 for 3 h at room temperature. Samples were then incubated at 37 °C for 72 h. The extent of fibril formation was probed by thioflavin-T (ThT) (Sigma-Aldrich, St Louis, MO). A five-fold molar excess of ThT was added to each sample and left at room temperature for 30 min before measurements were taken. ThT fluorescence was monitored using a Photon Technology International C60 spectrofluorometer. The photomultiplier gain was varied and excitation and emission slit widths set to 2–4 nm to maximize signal to noise. Fluorescence measurements were made using 430 and 480 nm as excitation and emission wavelengths, respectively.

The ThT fluorescence was normalized with the following formula to enable comparison of fluorescence measurements taken on different days:

\[
\text{Normalized Fluorescence} = \frac{(\text{Observed Fluorescence} - \text{Fluorescence of ThT in Buffer})}{(\text{Fluorescence of ThT in 0.2 mg/mL aggregated V122I TTR} - \text{Fluorescence of ThT in Buffer})}
\]

3.8.8 Phagocytosis assay

3.8.8.1 pHrodo dye-labeling of TTR

A 1 mg/mL sample of TTR-F87M/L110M, native TTR or low-pH aggregated TTR-V30M was amine coupled with pHrodo dye for 15 min at 37 °C with a protein:dye ratio of ≈15:4 according to the manufacturer’s specifications (Thermo Scientific). Excess pHrodo-label was removed by diafiltration in a spin concentrator with a 10 K molecular weight cutoff (Pierce Thermo) and the pHrodo-TTR was resuspended in 1 × PBS.
3.8.8.2 Phagocytosis assay

THP-1 human monocytes were cultured in cell culture media (RPMI, 10% low IgG serum, pen/strep). A 20 μg/mL aliquot of pHrodo-labeled TTR was separately pre-incubated with 40 μg/mL antibody at 37 °C in cell culture media for 30 min prior to the addition of 5E + 04 THP-1 cells in a 1:1 volumetric ratio. After tissue culture incubation (3 h), cells were washed with cell culture media three times, incubated in media for 10 min, then washed twice with and resuspended in FACS buffer (1% FBS in PBS). Red pHrodo fluorescence intensity was detected using Texas Red channel filters. Epifluorescence microscopy was carried out in a similar fashion. After FACS analysis, the remaining cells were transferred to glass chamber slides and imaged by inverted microscopy. Mean fluorescence intensities were automatically calculated by averaging the relative fluorescence intensities of each individual cell.

3.8.9 Histology and imaging

3.8.9.1 Human tissue samples

Fresh frozen and paraffin-processed cardiac tissue with confirmed genetic and/or histopathological diagnoses of hereditary and sporadic ATTR cardiac amyloidosis and amyloid light chain (AL) amyloidosis were generously donated by Dr. Merrill Benson (Indiana University, Indianapolis, IN). Fresh frozen normal control heart, liver and pancreas were procured from Bioreclamation IVT (Baltimore, MD) and a matching paraffin set of tissue was obtained from American MasterTech (Lodi, CA). Paraffin sections of normal human choroid plexus were purchased from Biochain (Newark, CA).

3.8.9.2 Immunohistochemistry

Lightly paraformaldehyde-fixed, slide-mounted cryosections (10 μm) and paraffin sections (5 μm) were immunoperoxidase labeled on the Leica Bond Rx with the polymer detection kit (DS980, Leica Biosystems, Buffalo Grove, IL). Antibody reagents used for immunohistochemistry are listed in Table 3.3. After primary incubation (1 h) and anti-mouse or anti-rabbit polymeric HRP-linker antibody-conjugates incubation, staining was visualized with diaminobenzidine. Labeling specificity was assessed by staining cardiac tissue from normal and AL amyloidosis patients (negative controls). Reagent controls included performing the staining procedure on adjacent
slides with non-immune isotype control (6F10) primary antibodies and omitting the primary antibodies altogether.

<table>
<thead>
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<th>Antibody</th>
<th>Antibody type</th>
<th>Vendor</th>
<th>Catalog number</th>
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<tbody>
<tr>
<td>6F10</td>
<td>Isotype control</td>
<td>Prothena Biosciences</td>
<td>-</td>
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</tr>
<tr>
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<td>Total-TTR</td>
<td>Dako North America</td>
<td>A002</td>
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<tr>
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<td>AL-κ</td>
<td>Dako North America</td>
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<tr>
<td>Lambda light chains</td>
<td>AL-λ</td>
<td>Dako North America</td>
<td>A0193</td>
<td>1:8000</td>
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3.8.9.3 Demonstration of amyloid and typing

Amyloid was labeled using a Congo red kit (American MasterTech) per manufacturer’s instructions and a filtered 0.015% thioflavin-T (T3516–25G; Sigma-Aldrich; 50% EtOH, 10 min) [223]. The specific type of amyloid deposited in the tissue was identified by IHC using a panel of commercial antibodies to TTR (prealbumin), λ light chains, κ light chains, and serum amyloid A (Table 3.3).

3.8.9.4 Image analysis

Slides were imaged with an Olympus BX61 microscope equipped with a 10×/0.40 and a 20×/0.75 UPlanSApo objective (Central Valley, PA). Congo red-stained slides were evaluated between a crossed polarizer and analyzer for the characteristic apple-green birefringence of amyloid deposits while thioflavin-T fluorescence was visualized using appropriate filters. Whole slides were digitally scanned using a Hamamatsu Nanozoomer 2.0HT digital slide scanner (Hamamatsu, Bridgewater, NJ).
3.9 Chapter Appendix

Supplementary Figure S3.1. 9D5 recognizes various human transthyretin mutations. 9D5 immunolabels cardiac tissue from patients with hereditary and sporadic cardiac ATTR amyloidosis. 9D5 strongly immunolabeled TTR deposits in diseased ATTR amyloidosis heart tissue from patients with familial (V122I, T60A, T49A, I84S) and wild-type forms of cardiac ATTR amyloidosis but not normal hearts. Scale bar = 500 nm.
Supplementary Figure S3.2. 6C1 recognizes various human transthyretin mutations. 6C1 immunolabels cardiac tissue from patients with hereditary and sporadic cardiac ATTR amyloidosis. 6C1 strongly immunolabeled TTR deposits in diseased ATTR amyloidosis heart tissue from patients with familial (V122I, T60A, T49A, I84S) and wild-type forms of cardiac ATTR but not healthy tissue. Scale bar = 500 nm.

Supplementary Figure S3.3. 5A1 recognizes various human transthyretin mutations. 5A1 immunolabels cardiac tissue from patients with hereditary and sporadic cardiac ATTR amyloidosis. 5A1 strongly immunolabeled TTR deposits in diseased ATTR amyloidosis heart tissue from patients with familial (V122I, T60A, T49A, I84S) and wild-type forms of cardiac ATTR amyloidosis but not healthy tissue. Scale bar = 500 nm.
Supplementary Figure S3.4. mis-TTR mAbs staining in normal tissues known to express TTR. 9D5, 6C1, and 5A1 did not stain the TTR present in normal human liver (A, D, G) but did stain intracellular TTR present in normal pancreatic a-cells (B, E, H) and in the choroid plexus (C, F, I) from normal donors. Scale bar =250 nm.
4 Antibody-mediated trapping of soluble spherical aggregates and capping of amyloid fibril ends in transthyretin amyloidosis models


N.J.G designed and performed all TEM experiments, as well as wrote the manuscript. ITC experiments were designed by N.J.G. and P.G., and performed by P.G. SPR experiments were performed by J.S., R.B. and C.I.

4.1 Abstract

Transthyretin (TTR) amyloidosis is an under-recognized cardiomyopathy/polyneuropathy for which monoclonal antibodies with therapeutic potential are currently being developed. Using immunogold transmission electron microscopy (IGEM), we demonstrate *in vitro* that a monoclonal antibody (misTTR-mAb1) specifically targeting misfolded forms of transthyretin (mis-TTR) does so by capping fibril ends and sequestering soluble, spherical aggregates. IGEM analysis of plasma derived from mice transgenic for human TTR-V30M also shows the presence of similar spherical aggregates that are immuno-positive for misTTR-mAb1. Furthermore, using isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR), we find that there are two unique classes of binding sites for misTTR-mAb1, which could correspond to amyloid fibrils and soluble spherical aggregates. Taken together, these results indicate that misTTR-mAb1 specifically binds misfolded TTR fibrils and fibril intermediates in solution and in mouse plasma via a two-pronged binding model that specifically targets mis-TTR species (soluble spherical aggregates) and TTR fibril ends of ATTR amyloidosis and suggest a possible mechanism by which misTTR-mAb1 can sequester potentially pathogenic forms of TTR, thereby preventing TTR deposition in the pathogenesis of ATTR disease.
4.2 Introduction

In theory, inhibition of amyloid formation might be achieved through sequestering amyloid fibril intermediates (e.g. the soluble, spherical aggregates) and/or preventing the growth of the final product, the amyloid fibril. One classic example of inhibition of protein polymeric growth is the effect of colchicine on tubulin polymerization. While it has been known since the mid-20th century that colchicine arrests cellular division [224], [225] through protein polymerization inhibition [226], [227], the exact mechanism of mitotic inhibition (i.e. fibril capping) was not well understood nor proposed until decades later [228]. From the early discussions of nucleation-dependent amyloid polymerization in protein misfolding disease [229], therapeutic fibril capping, as exemplified in the area of microtubule and cytoskeletal research, has never been demonstrated in the amyloid field. The other purported harmful aggregate in protein misfolding disease is the soluble oligomer/aggregate [230]. Targeting these misfolded forms of TTR with a conformation-specific antibody might be a mechanism for mitigating or abolishing these pathologic species through immune-clearance.

Therapeutic interventions for protein misfolding diseases are limited and for some types of protein misfolding disease, such as Alzheimer’s disease, are presently non-existent. Although the design of small molecule fibril cappers of amyloidogenic proteins have been attempted and shown to inhibit fibrillation, direct physical fibril capping has not yet been demonstrated [231], [232]. While N- and C- terminal chemical modification (“capping”) of human islet amyloid polypeptide (hIAPP) fragments have shown to exhibit effects on both fibril morphology and aggregation kinetics [233], this type of covalent capping is different from the trans-acting therapeutic capping described here.

Transthyretin (TTR) is a homotetrameric serum transport protein involved in the transport of thyroxine and vitamin A throughout the body. Dissociation of this homotetrameric protein into its monomeric subunits can initiate the formation of pathological spherical aggregates, protofibrils, and other higher order assemblies of TTR that can eventually lead to tissue deposition of amyloid fibrils culminating in organ dysfunction and disease (Figure 4.1A). ATTR amyloidosis can be classified as wild-type ATTR amyloidosis or hereditary ATTR amyloidosis depending on whether the amyloid deposits are derived from wild-type or mutant TTR protein, respectively. Regardless of type, both diseases can result in systemic amyloid deposition and cause significant damage to
the heart and nerves, resulting in cardiomyopathy and peripheral polyneuropathy, respectively [234]. It takes years for the development of ATTR amyloidosis in humans; however, amyloidogenesis can be accelerated \textit{in vitro} via exposure of the TTR protein (either wild-type of mutant) to low pH [143].

\textbf{Figure 4.1.} (A) Schematic illustration of inhibition of the TTR fibrillogenesis pathway by antibodies. Tetrameric TTR can dissociate into its component monomers, which can either unfold or become amyloidogenic intermediates that associate to form oligomer aggregates and/or amyloid fibrils. (B) Representative negative-stain TEM image of TTR fibrils and oligomers grown \textit{in vitro} via acid-induced fibrillogenesis using recombinant TTR-WT, TTR-V30M, and TTR-V122I, and TTR-WT derived from human plasma.

Although immunotherapy has received significant attention in the field of cancer treatment [235], its application has expanded beyond oncology and been proposed as a potential treatment strategy for ATTR amyloidosis [135]–[137]. Polyclonal and murine monoclonal antibodies (mAbs) raised against a TTR cryptotope (TTR89-97), an epitope hidden in the natively folded TTR tetramer but exposed in TTR’s monomeric state, have been previously described [135], [136]. These monoclonal antibodies have demonstrated specific targeting of the non-native, conformations of TTR, \textit{in vitro} inhibition of TTR fibrillogenesis using thioflavin-T assays, and the ability to specifically label ATTR in cardiac tissue obtained from patients with ATTR amyloidosis. The
The present work uses immunogold transmission electron microscopy (IGEM) and isothermal titration calorimetry (ITC) to investigate the underlying mechanism of action of a monoclonal mis-TTR antibody, misTTR-mAb1.

4.3 Results & Discussion

4.3.1 Recognition of TTR aggregates using murine monoclonal antibodies

Acid-induced fibrillogenesis [143] was used to grow TTR aggregates from TTR isolated from human plasma (Sigma) and recombinant TTR (TTR-V30M, TTR-V122I, and TTR-WT). These aggregates were used to investigate the binding site(s) of the misTTR-mAb1, a previously described murine monoclonal antibody (formerly known as 14G8) [136]. The morphology of these in vitro-generated TTR fibers and oligomer aggregates was not origin-dependent (recombinant vs. human origin) nor mutation-dependent (Figure 4.1B). Mature aggregates consisted of fibers which ranged 8-10 nm in diameter and were variable in length (≤260 nm) (Figure 4.1B), as well as small spherical aggregates, which varied between 10-20 nm in diameter and a molecular weight of approximately 500-600 kDa (Supplementary Figure S4.1). TTR oligomers of similar diameter were observed in an atomic force microscopy study of misfolded TTR [236].

IGEM analysis indicated that misTTR-mAb1 bound to both spherical aggregates and fibril ends (Figure 4.2). This specific targeting of fibril ends and spherical aggregates by misTTR-mAb1 was observed in mature human TTR-WT aggregates (Figure 4.2), and recombinant TTR-V122I and TTR-V30M aggregates (Figure 4.2 and Supplementary Figures S4.2 and S4.3). Preferential binding at fibril ends suggest the lack of epitope exposure in fibril stems and provides a mechanism of fibril inhibition by capping growing fibrils. Spherical aggregates were often labelled at the centroid by the misTTR-mAb1 (Figure 4.2). One possible reason as to why all fibril ends and spherical aggregates were not labelled by the antibody could be due to fibril/oligomer orientation upon adherence of these structures to TEM grids, affecting accessibility of the epitopes to the misTTR-mAb1 monoclonal antibody.

Performing the experiment with a murine isotype IgG control revealed no observable binding, thus indicating the specificity of the misTTR-mAb1 monoclonal antibody for fibril ends and spherical aggregates. A pan-specific anti-TTR polyclonal control IgG demonstrated widespread binding to various sites on fibrils and spherical aggregates (Supplementary Figures S4.2 and S4.3).
Figure 4.2. Magnified examples of preferential binding of misTTR-mAb1 to spherical aggregates and fibril ends, and not along fiber stems, as visualized by gold-conjugated anti-mouse secondary antibodies. Negative stain transmission electron microscopy (TEM) images of human wild-type, recombinant TTR-V30M and TTR-V122I aggregates formed in vitro via acid-induced fibrillogenesis and immunolabeled with the murine misTTR-mAb1 monoclonal antibody and 6 nm colloidal gold-conjugated secondary anti-mouse IgG. Bottom panel: Magnified examples of TTR-WT spherical aggregates bound by misTTR-mAb1 primary mAb as visualized by gold-conjugated secondary antibody.
ITC was used to quantify the thermodynamic binding parameters of the murine misTTR-mAb1 monoclonal antibody using recombinant TTR-V30M, TTR-V122I, and TTR-WT aggregate samples. The misTTR-mAb1 monoclonal antibody’s thermodynamic binding profile for each of these TTR aggregate samples fit to a model consisting of two distinct binding sites present in the TTR aggregate samples. The apparent $K_D$ values obtained by the analysis, however, are not accurate because only the total concentration of TTR is known and the concentration of exposed and epitopes and form is unknown (Figure 4.3). Surface plasmon resonance (SPR) analysis of the same monoclonal antibody with Gu-HCl denatured recombinant TTR-WT sensorgram gave the best fit with a global 1:1 fit. ($K_{D,\text{apparent}}=3.2 \text{nM}$) (Supplementary Figure S4.4). A single binding site, as opposed to two, is expected as the SPR was performed on fully denatured TTR-WT as opposed to the low-pH aggregated TTR used to generate spherical aggregates and fibrils. ITC analysis of natively-folded recombinant TTR-WT titrated with misTTR-mAb1 monoclonal antibody did not result in an exothermal binding curve, thus confirming minimal epitope solvent-exposure in the native TTR state. Lack of exothermal binding by ITC was also observed when a murine isotype IgG control was titrated against recombinant TTR-WT aggregate, indicating that the misTTR-mAb1 monoclonal antibody was binding specifically to TTR aggregates (Figure 4.3).
Figure 4.3. (A) Isothermal titration calorimetry (ITC) analysis and two-site binding model dissociation constants for murine monoclonal antibody misTTR-mAb1 binding to acid-induced TTR aggregates formed in vitro using recombinant TTR (TTR-WT, TTR-V30M, TTR-V122I). (B) ITC analysis of misTTR-mAb1 monoclonal antibody titrated against recombinant, non-aggregated TTR-WT. (C) ITC analysis of IgG isotype control titrated against recombinant TTR-WT aggregate.
4.3.2 Recognition of TTR aggregates using humanized monoclonal antibodies

Humanized versions of the murine misTTR-mAb1 (h-misTTR-mAb1) exhibited specific targeting of fibrils and spherical aggregates in pH-induced TTR-WT aggregates (Supplementary Figure S4.5). Controls for the humanized misTTR-mAb1 monoclonal antibody (h-misTTR-mAb1) included cetuximab (Erbitux®), an anti-cancer chimeric mAb, which exhibited no binding, and a pan-specific anti-TTR polyclonal IgG, which exhibited widespread binding similar to the observations seen in the murine misTTR-mAb1 monoclonal antibody studies.

Following positive IGEM staining of pH-induced TTR-WT aggregates, h-misTTR-mAb1 was used for the immunonegative staining of plasma samples derived from V30M-TTR transgenic mice (Figure 4.4). Specific labelling of misfolded TTR aggregates with h-misTTR-mAb1 was observed in V30M-TTR transgenic mouse plasma. The heterogeneous morphology of these gold-labelled, plasma species did not fully resemble the aggregates seen in the pH-induced in vitro samples. The absence of TTR fibers in plasma samples may have been the result of blood sample centrifugation and preparation. Although soluble TTR spherical aggregates have been detected in plasma samples of ATTR amyloidosis patients, they have never been visualized using TEM [237]. Controls for the V30M-TTR mouse plasma experiment included cetuximab (Erbitux®) which did not exhibit labelling, and a pan-specific anti-TTR IgG that recognizes both native and misfolded TTR, which exhibited widespread labelling (Supplementary Figure S4.6). Immunonegative staining of wild-type mouse plasma with the h-misTTR-mAb1 did not result in labelling, further supporting the hypothesis that misTTR-mAb1 is specific for misfolded TTR (Supplementary Figure S4.6).

Both the murine and humanized misTTR-mAb1 monoclonal antibody did not recognize aggregates and fibers of alternative amyloid-forming proteins, such as islet amyloid polypeptide (IAPP), and beta-amyloid proteins 40 (Aβ40) or 42 (Aβ42). This is in contrast to widespread immunogold labelling of IAPP by anti-IAPP polyclonal antibody and of both Aβ40 and Aβ42 aggregates by commercial anti-β-amyloid monoclonal antibody 6E10 (Supplementary Figures S4.7, S4.8, S4.9). This specificity suggests that the misTTR-mAb1 antibody is specific for TTR aggregates and fibrils and does not recognize amyloid fibrils comprised of non-TTR proteins.
**Humanized monoclonal antibody h-misTTR-mAb1:** Specific recognition of misfolded TTR aggregates

**Figure 4.4.** Negative stain transmission electron microscopy (TEM) images of plasma from human V30M-TTR transgenic mice immunolabeled with humanized misTTR-mAb1 (h-misTTR-mAb1). Binding of primary antibodies was visualized by 6nm colloidal gold conjugated secondary anti-human IgG (BLACK solid spheres). H-misTTR-mAb1 did not recognize non-TTR aggregates within the plasma samples (BLUE arrows). Misfolded TTR aggregates were only detected by the misfolding-specific h-misTTR-mAb1 antibody (RED arrows). Panels A-D are technical replicates.

### 4.4 Conclusion

Fibril capping has been historically demonstrated in microtubule and cytoskeletal research; however, this is the first direct physical demonstration of fibril capping in the amyloid field. Although the debate over which amyloid species is most toxic (oligomer/aggregates vs amyloid fibrils) continues to persist in today’s literature [238], our conformationally-specific TTR monoclonal antibody, misTTR-mAb1 can target both the pre-fibrillar spherical aggregates and the ends of insoluble amyloid fibrils derived from full-length TTR. This dual targeting of both soluble, spherical aggregates and fibril ends in vitro and in plasma derived from V30M-TTR transgenic mice bodes well for the therapeutic potential of misTTR-mAb1 for the treatment of ATTR amyloidosis. Binding of misTTR-mAb1 to soluble, spherical aggregates or TTR fibrils (amyloid fibril precursors) in circulation could potentially prevent deposition of TTR amyloid. Furthermore,
binding of misTTR-mAb1 to TTR fibril ends could facilitate antibody-mediated clearance of TTR amyloid deposits in diseased tissue. The targeting of upstream spherical aggregates and capping end-point insoluble amyloid fibrils using antibodies, as demonstrated here for TTR, can be used as an exemplar strategy for designing treatments to combat other protein misfolding diseases.

4.5 Acknowledgements
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4.6 Author contributions
NJG designed the experiments, performed experiments, made the figures, and wrote the manuscript. PG designed and performed experiments, made figures, and wrote the manuscript. JS, RB, and CI performed experiments and made figures. JNH and AC designed the experiments, made the figures, and wrote the manuscript.

4.7 Competing financial interests
JS, RB, and JH are employees of Prothena Biosciences Inc. This work was supported by Prothena Biosciences.

4.8 Methods
4.8.1 In vitro aggregate formation
Concentrated stock samples of human TTR-WT (Sigma #87090-18-4), recombinant TTR-V30M, recombinant TTR-V122I, and recombinant TTR-WT, were filtered with a 0.2 μm filter and stored in 5 mM sodium phosphate buffer (pH 7.4). TTR stock solutions were then diluted with 280 mM ammonium acetate buffer (pH 4.5) and ddH2O to a total volume of 200 μL to obtain 0.2 mg/mL TTR in 140 mM ammonium acetate (pH 4.5). Samples were then agitated for 72 h at 37 °C, 600RPM and stored for a minimum of 21 d in the dark at room temperature before use. Fully
denatured recombinant TTR was prepared by incubating 1.0 mg/ml TTR-WT in 1xPBS containing 6M guanidine hydrochloride for 48 h at 4 °C. IAPP aggregates were prepared using lyophilized full-length IAPP peptide and incubated at 0.2 mg/mL in 20 mM ammonium acetate buffer (pH 6.5) for 1 wk at 37 °C. Amyloid-beta aggregates were prepared using lyophilized Aβ42 peptide (Sigma #107761-42-2) and Aβ40 peptide (Sigma #131438-79-4), dissolved in hexafluoroisopropanol (HFIP) and prepared in 20 mM Na₃PO₄ buffer (pH 7.4) at 50 μM for 72 h at 37 °C, 900RPM. Aggregate formation was confirmed using ThT fluorescence (data not shown).

4.8.2 V30M-TTR transgenic mice
Mice transgenic for human V30M-TTR were licensed to Prothena Biosciences from Kumamoto University [239] and bred using standard procedures. Plasma from aged animals (6 to 12 months) were collected and immediately frozen. Prior to use, circulating levels of human V30M-TTR were assessed by ELISA (data not shown) and plasma samples with human V30M-TTR levels of 200-400 μg/ml were selected for further study.

4.8.3 Immunogold transmission electron microscopy
Carbon-coated 400-mesh standard hexagonal copper grids (Electron Microscopy Sciences G400H-Cu) were glow discharged at 10 mA for 5s. 10 uL aliquots of V30M-TTR or wild-type (CD-1) mouse plasma stored at -80 °C were thawed on ice for 1-5 min and immediately diluted 1:200 in UltraPure DNase/RNase-Free distilled water (Invitrogen #10977015). 2 μL of this diluted solution was pipetted onto each charged grid for immunonegative staining. For in vitro-derived TTR aggregates experiments, aggregated TTR samples (0.2 mg/mL) were diluted 1:10 using UltraPure DNase/RNase-Free distilled water (Invitrogen #10977015) to a concentration of 0.02 mg/mL. The diluted solution was pipetted (2 μL) onto each charged grid. For IAPP, Aβ40, and Aβ42 experiments, aggregated stock samples were directly pipetted (2 uL) onto each grid. Samples remained on grid for 60s and excess liquid was wicked off using filter paper (No. 1 Whatman). Grids were immediately placed sample-side down on a drop (40 μL) of Aurion Blocking Solution for Goat Gold Conjugates (Aurion #905.002) for 15 min. Grids were subsequently washed 4 times (2 min/wash) with incubation buffer (40 μL PBS + 0.1% Aurion BSA-c, pH 7.4). Primary binding included 30 min incubation on drop (40 μL) of 5 μg/mL of mouse or humanized misTTR-mAb1 monoclonal IgG, isotype murine monoclonal IgG,
chimeric IgG cetuximab, anti-IAPP rabbit polyclonal IgG, commercial anti-β-Amyloid mouse monoclonal IgG 6E10 (BioLegend #803001), or commercial pan-specific anti-TTR polyclonal IgG (Sigma Aldrich #HPA002550) diluted with incubation buffer. Grids were washed 6 times with incubation buffer (40 μL, 2 min/wash). Secondary binding included 60 min incubation on drop (40 μL) of 6 nm Colloidal Gold Affinipur Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch #115-195-146), Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch #111-195-144), or 6 nm Colloidal Gold Affinipur Goat Anti-human IgG (H+L) Jackson Immunoresearch #109-195-088, diluted 1:40 in incubation buffer. Grids were then washed with incubation buffer, PBS, and ddH2O. Grids were then negatively stained with 2 μL of freshly filtered (0.2 μm syringe filter) 2% uranyl acetate for ~10s. Excess reagent was removed using No. 1 Whatman filter paper and grids were allowed to air dry. Grids were imaged using a JEOL JEM-1200 electron microscope operating at 80 kV.

4.8.4 Isothermal titration calorimetry

ITC measurements of recombinant TTR-WT, TTR-V122I, and TTR-V30M aggregates were carried out using a Microcal isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA). A 20 μM solution of murine misTTR-mAb1 was titrated into 6.5 μM recombinant TTR sample aggregates (1.445 mL, aggregated for 6-8 wks) at a constant temperature of 25 °C. An initial injection of antibody (2 μL) was followed by 60 injections at 5 μL. Integration of the thermogram yielded a binding isotherm fitted to a two site model. Data was analyzed using ORIGIN 7.0 software.

4.8.5 Surface plasmon resonance

Analysis was performed using a Biacore T200 to compare the binding affinity of murine misTTR-mAb1 to recombinant human TTR denatured in 6 M Guanidine Hydrochloride (Gu-TTR). Anti-Mouse antibody was immobilized on sensor chip CM3 (GE Healthcare Life Sciences) via amine coupling, and murine mAbs (ligand) were captured to a level to ensure a maximum binding of analyte of 50RU (approximately 250RU of ligand binding). Various concentrations of Gu-TTR (ranging from 0.4 nM to 100 nM) were passed over the captured ligand at 50 μL/min in running buffer (HBS + 0.05% P-20, 1 mg/mL BSA) for 300s association time and 900s dissociation time. Regeneration of the chip surface was accomplished by 2 short injections of 10 mM Glycine-HCl at pH 1.7. Data was blank subtracted to both a sensor not containing ligand
and 0 nM analyte concentration. Analysis was performed using a global 1:1 fit with Biacore Evaluation software (v3.0) with bulk refractive index set to zero RU.

### 4.8.6 High-performance liquid chromatography
A 50 µl sample of 0.2 mg/mL aggregated TTR-V30M in ammonium acetate (pH 4.5) was injected at a flow rate of 0.5 mL/min onto a Shodex Protein KW 804 (8x300mm) gel filtration column equilibrated in 140 mM ammonium acetate (pH 4.5). The protein elution was monitored by measuring the absorbance at 280nm.
Supplementary Figure S4.1. Characterization of TTR spherical aggregates. HPLC chromatogram of 0.2 mg/mL TTR-V30M aggregate with a strong oligomer peak indicating an approximate molecular weight of 600-400 kDa. Blue arrowheads indicate approximate retention times of high-molecular weight standards as reference markers. Representative negative stain TEM image of TTR-V30M spherical aggregates, indicated with black arrowheads.
Supplementary Figure S4.2. Negative stain transmission electron microscopy (TEM) images of recombinant TTR-V122I aggregates formed in vitro via acid-induced fibrillogenesis and immunolabeled with the misTTR-mAb1 mouse monoclonal primary antibody and 6nm colloidal gold conjugated secondary anti-mouse IgG. (A) Representative negative-stain TEM image of TTR-V122I aggregates without misTTR-mAb1 nor gold-conjugated secondary antibodies. (B) Representative TEM image of TTR-V122I aggregates that do not bind murine IgG isotype control as visualized by absence of gold-conjugated secondary antibody. (C) Representative TEM image of TTR-V122I aggregates labelled by commercial anti-TTR control primary antibody as visualized by widespread labelling by Au-conjugated secondary antibody. (D) Representative image of monoclonal antibody misTTR-mAb1 preferentially binding spherical aggregates, in addition to fiber ends rather than along fiber stems.
Supplementary Figure S4.3. Negative stain transmission electron microscopy (TEM) images of recombinant TTR-V30M aggregates formed in vitro via acid-induced fibrillogenesis and immunolabeled with the misTTR-mAb1 murine monoclonal primary antibody and 6nm colloidal gold conjugated secondary anti-mouse IgG. (A) Representative TEM image of TTR-V122I aggregates that do not bind murine IgG isotype control as visualized by absence of Au-conjugated secondary antibody. (B) Representative TEM image of TTR-V122I aggregates labelled by commercial anti-TTR control primary antibody as visualized by widespread labelling by Au-conjugated secondary antibody. (C) Representative image of monoclonal antibody misTTR-mAb1 preferentially binding spherical aggregates, in addition to fiber ends rather than along fiber stems.
Supplementary Figure S4.4. Raw Surface plasmon resonance (SPR) data of misTTR-mAb1 murine mAb bound to Guanidine HCl-denatured TTR.
Supplementary Figure S4.5. Negative stain transmission electron microscopy (TEM) images of human wild-type TTR (TTR-WT) aggregates formed in vitro via acid-induced fibrillogensis and immunolabeled with the humanized misTTR-mAb1 monoclonal antibody (h-misTTR-mAb1) and 6 nm colloidal gold conjugated secondary anti-human IgG. (A) Representative TEM image of human TTR-WT aggregates that do not bind chimeric cetuximab monoclonal antibody control as visualized by absence of Au-conjugated secondary antibody. (B) Representative TEM image of V122I-TTR aggregates labelled by commercial anti-TTR control primary antibody as visualized by widespread labelling by Au-conjugated secondary antibody. (C) Representative image of humanized monoclonal antibody misTTR-mAb1 preferentially binding spherical aggregates, in addition to fiber ends rather than along fiber stems.
**Supplementary Figure S4.6.** Negative stain transmission electron microscopy (TEM) images of transgenic human V30M-TTR mouse model plasma immunolabeled with (I) a pan-specific commercial antibody that recognizes both misfolded and native TTR, (II) V30M-TTR mouse plasma with cetuximab (anti-EGFR chimeric antibody), and (III) wild-type mouse plasma with humanized misTTR-mAb1 monoclonal antibody (h-misTTR-mAb1). Binding of primary antibodies was visualized by 6nm colloidal gold conjugated secondary anti-human IgG (BLACK solid spheres). In V30M-TTR mouse plasma, cetuximab did not recognize misfolded or native TTR, whereas the pan-specific anti-TTR control exhibited widespread labelling and could potentially recognize both types of TTR within the sample. h-misTTR-mAb1 did not recognize
native nor misfolded TTR in plasma from a wild-type mouse. Panels A and B in each row are technical replicates.

**Supplementary Figure S4.7.** Negative stain transmission electron microscopy (TEM) images of full-length islet amyloid polypeptide (IAPP) aggregates formed *in vitro* and immunolabeled with primary IgG and 6nm colloidal gold conjugated secondary IgG. (A) Representative negative-stain TEM image of IAPP aggregates without primary nor gold-conjugated secondary antibodies. (B) Representative TEM image of IAPP aggregates labelled by anti-IAPP rabbit polyclonal primary antibody as visualized by widespread labelling by Au-conjugated anti-rabbit secondary antibody. (C) Representative TEM image of IAPP aggregates unrecognized by murine misTTR-mAb1 IgG as visualized by absence of Au-conjugated anti-mouse secondary antibody. (D) Representative TEM image of IAPP aggregates unrecognized by humanized misTTR-mAb1 IgG (h-misTTR-mAb1) as visualized by absence of Au-conjugated anti-human secondary antibody.
Supplementary Figure S4.8. Negative stain transmission electron microscopy (TEM) images of Aβ40 aggregates formed in vitro and immunolabeled with primary IgG and 6nm colloidal gold conjugated secondary IgG. (A) Representative negative-stain TEM image of Aβ40 aggregates without primary nor gold-conjugated secondary antibodies. (B) Representative TEM image of Aβ40 aggregates labelled by 6E10 primary monoclonal antibody as visualized by widespread labelling by Au-conjugated anti-mouse secondary antibody. (C) Representative TEM image of Aβ40 aggregates unrecognized by murine misTTR-mAb1 IgG as visualized by absence of Au-conjugated anti-mouse secondary antibody. (D) Representative TEM image of Aβ40 aggregates unrecognized by humanized misTTR-mAb1 IgG (h-misTTR-mAb1) as visualized by absence of Au-conjugated anti-human secondary antibody.
Supplementary Figure S4.9. Negative stain transmission electron microscopy (TEM) images of Aβ42 aggregates formed in vitro and immunolabeled with primary IgG and 6nm colloidal gold conjugated secondary IgG. (A) Representative negative-stain TEM image of Aβ42 aggregates without primary nor gold-conjugated secondary antibodies. (B) Representative TEM image of Aβ42 aggregates labelled by 6E10 primary monoclonal antibody as visualized by widespread labelling by Au-conjugated anti-mouse secondary antibody. (C) Representative TEM image of Aβ42 aggregates unrecognized by murine misTTR-mAb1 IgG as visualized by absence of Au-conjugated anti-mouse secondary antibody. (D) Representative TEM image of Aβ42 aggregates unrecognized by humanized misTTR-mAb1 IgG (h-misTTR-mAb1) as visualized by absence of Au-conjugated anti-human secondary antibody.
5 Future Directions

5.1 A need for diagnostic tools for ATTR amyloidosis

TTR-related (ATTR) amyloidosis is a progressive, life-threatening disease caused by misfolded transthyretin (TTR) proteins that accumulate as amyloid fibrils in multiple organs, but primarily in the peripheral nerves and heart. The cardiac form of ATTR amyloidosis is an under-recognized cause of heart failure with preserved ejection fraction (HFpEF), a clinical syndrome which accounts for 50% of heart failure cases [240], [241]. With Canadian heart failure healthcare costs projected to rise to $2.8 billion [242] by 2030 and the concurrent incremental rise of HFpEF, it is crucial that efforts are made to develop diagnostic tools which can identify these patients early and in turn prevent their entry into late-stage heart failure.

Chapter 1 emphasized that ATTR amyloidosis is underdiagnosed because of sharing signs and symptoms with other more common cardiac pathologies, and also because there are no diagnostic tools with sufficient specificity and sensitivity to achieve an accurate diagnosis. It is thus critically important to develop a diagnostic blood test to identify patients who might be eligible to receive the appropriate treatment for ATTR amyloidosis. If left untreated, ATTR amyloidosis can result in debilitating conditions which affect the activities of daily life, heart failure, hospitalization, and death.

Although the most common type of ATTR amyloidosis worldwide is hereditary ATTRVal30Met amyloidosis, the most common form in the USA is wild-type ATTR amyloidosis, followed by hereditary ATTRVal122I iso amyloidosis [46]. Importantly, the prevalence of the TTRVal122Iso allele within the African American population is estimated to be 3–4%, translating to approximately 1.3 million African American carriers who are at an increased risk of developing cardiac amyloidosis in the U.S.A. alone [54], [56], [243]. Thus, ATTR amyloidosis is not a rare disease. Regardless of the ATTR amyloidosis subtype, all affected patient populations are at significant risk of misdiagnosis leading to unchecked disease progression and inappropriate treatment.
Misdiagnosis of ATTR amyloidosis can result in the inappropriate management of ATTR patients, resulting in care plans and prescriptions with harmful side-effects and no curative benefit. Despite advances in cardiac imaging, which can improve recognition of cardiac amyloidosis in clinical practice, antemortem diagnosis continues to be made in those patients with advanced/late-stage disease. The need for histological demonstration of amyloid in heart tissue also delays diagnosis as the technique is restricted to referral centers with expertise in the performance of endomyocardial biopsy, an invasive high-risk procedure that patients often refuse. Consequently, many patients who have the disease are not diagnosed until post-mortem [10]–[14] resulting in its underestimated prevalence.

The main objective of this thesis was to develop monoclonal antibodies for the potential treatment of ATTR amyloidosis. The trajectory of this initiative began with the development of conformation-specific polyclonal antibodies which showed specificity for non-tetrameric TTR, immunohistochemical recognition of ATTR-positive cardiac tissue, and \textit{in vitro} fibril inhibition capabilities (\textbf{Chapter 2}). The project then evolved to the production and biophysical screening of monoclonal antibodies raised towards the same TTR cryptotope (\textbf{Chapter 3}) and the eventual investigation of how these antibodies may function when exposed to TTR fibers and aggregates using both \textit{in vitro} and \textit{in vivo} models (\textbf{Chapter 4}). The diagnostic application of these antibodies, however, was not investigated and remains an area of research that requires further exploration.

\section{5.2 \textit{IGEM} and ATTR amyloidosis}

Immunogold electron microscopy (IGEM) is a well-established protein localization technique which involves the use of electron-dense colloidal gold-bound antibodies for the detection of ligands [244]. Quantification of the relative number of gold-labeled ligands within a sample can be achieved using model-based automatic particle-picking algorithms, a method commonly used in cryoEM for finding particles in EM micrographs [245]. Together, these methods allow for the detection, visualization, and relative quantification of the number of immunogold-labelled particles within a sample.

For the present thesis, IGEM was a valuable technique for visualizing the types of binding sites our misfolding-specific monoclonal antibodies (misTTR-mAb1 and h-misTTR-mAb1) target
within in vitro TTR fibers and aggregates (Chapter 4). IGEM was also critical for providing evidence that our ATTR biomarker-specific antibodies were able to detect misfolded TTR in plasma samples from transgenic mice which overexpress the human TTRVal30Met mutation (Figure 4.4). Our antibodies could successfully detect misfolded TTR aggregates in as little as 10 nL of V30M-TTR transgenic mouse plasma, demonstrating the method’s high sensitivity of detection. This was the first time that soluble, spherical aggregates have ever been visualized in biological samples using IGEM. These misfolded TTR aggregates/oligomers in blood could be used as diagnostic biomarkers for ATTR cardiac amyloidosis (i.e. ATTR biomarkers) and together with IGEM, could be used to produce the first laboratory test for diagnosing both wild-type and hereditary ATTR amyloidosis.

Because molecular-level tests such as IGEM can achieve exquisite sensitivity and specificity, using such techniques to develop a diagnostic blood test for ATTR amyloidosis could lead to early diagnosis, improved treatment efficacy, patient safety, and overall clinical outcomes. Most therapies today, including those for cardiomyopathy, are aimed at symptom management rather than targeting the underlying mechanism of disease. This is especially true if the underlying etiology of heart failure is unclear due to lack of diagnostic tools.

We have already successfully demonstrated that our disease-specific antibodies can detect TTR aggregates in blood fractions of ATTR-V30M transgenic mice and the combined specificity and sensitivity of the IGEM technique suggests exceptional promise for use as an early clinical diagnostic tool. IGEM may have sufficient use in the evaluation of blood fractions due to its ability to detect and visualize disease-related ATTR biomarkers down to the single-molecule level. It could be used to push the limits of early detection, track disease progression via ATTR biomarker blood levels, as well as to correlate clinical outcomes in drug therapy trials. Improved diagnostic accuracy with tools such as IGEM would greatly ameliorate the currently poor prognostic outcomes of cardiac amyloidosis patients by providing early detection and more targeted therapeutic interventions.

A diagnostic blood test would help inform clinicians’ clinical management of their patients with heart disease, as well as help them identify those who may be eligible for ATTR clinical trials (e.g. RNAi, immunotherapy) at an earlier stage of disease when therapies may have the greatest
therapeutic benefit. Together with our misfolding-specific monoclonal antibodies, we could address this problem and potentially revolutionize the field of heart failure by developing a simple blood test which could reveal, for the first time, ATTR patients within the HFpEF population.

5.3 ATTR diagnostic biomarkers

Development of a diagnostic assay which can quantify the level of ATTR biomarkers in the plasma of ATTR amyloidosis patients could have significant impact on the field of ATTR amyloidosis. Such an assay could be used to quantify misfolded TTR aggregates/oligomers in blood and differentiate ATTR cardiac amyloidosis from other cardiac amyloidosis pathologies (i.e. AL amyloidosis). In addition, it could be used to investigate any potential correlation between misfolded aggregates and disease progression or be used as a companion diagnostic for monitoring therapeutic effectiveness. With the recent rise in number of ATTR therapies in clinical trials (e.g. RNAi), there is an urgent need for the development of companion diagnostics to evaluate therapeutic effectiveness. In addition, a simple diagnostic blood test that could track disease progression via levels of biomarker (i.e. TTR aggregates) would facilitate the monitoring of post-transplant patients or the efficacy of other ATTR therapies.

Detecting ATTR biomarkers in plasma of ATTR amyloidosis patients requires an immunodiagnostic technique that is both sensitive and specific enough for detection. While total analyte protein, such as natively-folded TTR, may be present at high levels in the plasma of patients with protein misfolding disease, misfolded proteins or aggregates are instead, often present at low concentrations. One may suggest the use of tools such as IGEM or ELISA to detect low-abundance biomarkers, however, such techniques, individually, can have technical disadvantages. ELISAs have limited sensitivity and may not be able to detect low levels of TTR aggregates in human plasma. Alternatively, IGEM may not be able to quantify the absolute concentration of ATTR biomarker within a sample. However, if these two techniques were developed in parallel, one technique’s limitation could be complimented by the strengths of its counterpart. If the ELISA is not sensitive enough to detect the low concentrations of ATTR in the plasma samples, the IGEM method could potentially compensate for this weakness by detecting TTR aggregates at the single molecule level.
One of the most significant impacts that a diagnostic blood test for ATTR amyloidosis could achieve would be the first-ever establishment of the normal range of ATTR aggregates/biomarker in the plasma healthy patients. Plasma samples from ATTR amyloidosis patients could be used to calculate the range of reference values for ATTR amyloidosis. These biomarker levels could be compared with the normal range (as obtained from healthy controls) to establish a reference cut-off value that distinguishes healthy individuals from ATTR patients (both wild-type or hereditary ATTR amyloidosis). Once the range of diagnostic ATTR amyloidosis reference values are determined, any correlation between ATTR biomarker levels and relevant cardiac and laboratory tests data could be explored to investigate whether disease progression and severity can be tracked. If a clinical correlation between cardiovascular function and ATTR biomarker levels exists, this can be used to assist clinical decision-making and management.

In conjunction with our ATTR biomarker-specific antibodies, IGEM could be used to quantify the level of biomarker (i.e. misfolded TTR) within the plasma from patients with ATTR amyloidosis. Because we have already successfully optimized our IGEM conditions for both ATTR aggregates formed in vitro and ATTR mouse plasma aggregates formed in vivo (Figure 4.4), we are confident that these optimized experimental conditions will be transferrable to human plasma.
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“Is Avi coming in today?”

- Natalie J. Galant