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Review

The Role of CSF Transthyretin in Human Alzheimer's Disease: Offense, Defense, or not so Innocent Bystander

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Abstract

Transthyretin (TTR) is secreted by hepatocytes, retinal pigment epithelial cells, pancreatic α and β cells, choroid plexus epithelium, and neurons under stress. The choroid plexus product is the main transporter of the thyroid hormone thyroxine (T4) to the brain during early development. TTR is one of three relatively abundant cerebrospinal fluid (CSF) proteins (Apolipoprotein J [ApoJ] (also known as clusterin), Apolipoprotein E [ApoE], and TTR) that interact with Aβ peptides *in vitro*, in some instances inhibiting their aggregation and toxicity. It is now clear that clusterin functions as an extracellular, and perhaps intracellular, chaperone for many misfolded proteins and that variation in its gene (*Clu*) is associated with susceptibility to sporadic Alzheimer's disease (AD). The function of ApoE in AD is not yet completely understood, although the *ApoE4* allele has the strongest genetic association with the development of sporadic late onset AD. Despite *in vitro* and *in vivo* evidence of the interaction between TTR and Aβ, genomewide association studies including large numbers of sporadic Alzheimer's disease patients have failed to show significant association between variation in the *TTR* gene and disease prevalence. Early clinical studies suggested an inverse relationship between CSF TTR levels and AD and the possibility of using the reduced CSF TTR concentration as a biomarker. Later, more extensive analyses indicated that CSF TTR concentrations may be increased in some patients with AD. While the observed changes in TTR may be pathogenetically or biologically interesting because of the inconsistency and lack of specificity, they offered no benefit diagnostically or prognostically either independently or when added to currently employed CSF biomarkers, i.e., decreased Aβ_{1-42} and increased Tau and phospho-Tau. While some clinical data suggest that increases in CSF TTR may occur early in the disease with a significant decrease late in the course, without additional, more granular data, CSF TTR changes are neither consistent nor specific enough to warrant their use as a specific AD biomarker.

Keywords: transthyretin; Alzheimer's disease; choroid plexus; cerebrospinal fluid; biomarkers; Aβ

1. Introduction

Transthyretin (TTR) was first identified in the 1940s as a human serum and cerebrospinal fluid (CSF) protein [1]. Initially classified based on its electrophoretic mobility (prealbumin), it was more precisely described as thyroxine binding prealbumin (TBPA) after it was shown to bind T4 in plasma [2]. The molecule was formally named transthyretin after being found to bind plasma retinol binding protein charged with retinol, hence its official name, **Trans**porter of **Thy**roxine and **Retin**ol binding protein (RBP) [3]. Two laboratories independently identified functional TTR mRNA and protein in both the liver and the choroid plexus in rats and humans, suggesting that CSF TTR was likely to be synthesized locally [4,5]. The observed *TTR* transcriptional responses to inflammation differed between hepatocytes and choroidal plexus epithelial (CPE) cells, indicating that the gene was probably regulated differently in the two organs [6]. In the liver, transcription was regulated by a series of transcription factors (Hepatocyte nuclear factors 1 [HNF1], 3, 4) that were suppressed by the pro-inflammatory cytokines IL-1 and tumor necrosis factor alpha (TNFα), accounting for the long-standing observation that serum TTR, like serum albumin, behaved as a negative acute phase reactant [7]. While CPE *TTR* transcription has not been studied in detail, by inference it appears that it is unaffected by inflammatory cytokines. Recent observations have indicated that neurons also produce TTR under conditions of stress, regulated by the major stress responsive transcription factor HSF1 [8]. Hence, quantitatively precise knowledge of the source(s) of CSF TTR is sparse.

2. Transthyretin and Alzheimer's Disease, the Pre-Aβ Period

A possible association between Alzheimer's disease (AD) and CSF TTR was first addressed in a survey of CSF proteins in three groups of Finnish subjects: patients hospitalized for dementia (n = 10), patients ambulatory but with evidence of cognitive impairment (n = 22), and an age-matched group of individuals without defined intellectual deficits (n = 22). In this relatively crude analysis, immunoglobulin classes, haptoglobin, transferrin, albumin, and TTR (as prealbumin) in serum and CSF were measured nephelometrically. The only significant finding with respect to TTR was a lower mean serum level in the institutionalized AD patients, which appeared to be a function of their nutritional status, since the serum albumin concentra-

tion also trended lower in this group than in the controls or the ambulatory AD cohort [9]. CSF TTR concentrations did not differ significantly among the three clinical groups. In a subsequent publication, the same investigators compared the values in the ambulatory AD group with similar measurements in 29 patients with multi-infarct dementia and did not report any significant differences in CSF or serum prealbumin or albumin levels [10].

In a contemporaneous Norwegian study, CSF TTR concentrations in 24 subjects with dementia of the Alzheimer type, seven with multi-infarct dementia, 14 age- and sex-matched, non-demented individuals with a variety of medical conditions, a younger group with multiple sclerosis (n = 17), a group with amyotrophic lateral sclerosis (n = 6), and a group with post subarachnoid hemorrhage patients (n = 10) were measured. The degree of dementia was determined using the no longer utilized Roth dementia scale and the concentration of TTR in serum and CSF was measured by rocket immunoelectrophoresis [11]. The only patient group that had a significantly different mean CSF TTR concentration was the sub-arachnoid hemorrhage cohort in which it was lower. The authors assumed this to be a function of reduced choroid plexus function. However, they did find a significant inverse correlation between the level of dementia as measured by the Roth score and the total CSF TTR concentration ($p < 0.013$ by Spearman Rank analysis and student's *t*-test), independent of the specific diagnosis [12].

3. Aβ and Alzheimer's Disease

At the time of these analyses, Aβ had not yet been identified as the major component of AD plaques and some investigators considered TTR as a candidate major AD fibril precursor. One study reported positive immunofluorescent staining for TTR in the neurofibrillary tangles and plaques in AD brains [13], a finding not reproduced by others [14]. The identification of what is now called Aβ as being the major fibrillar component in the blood vessels of patients with AD [15], its identification in neuritic plaques [16], and other findings independently obtained in Australia [17] led to the formulation of the "Amyloid Cascade" hypothesis by Hardy and colleagues [18]. This hypothesis was further elaborated on by Hardy and Selkoe [19] and the protein Tau was also found to be a major component of neurofibrillary tangles [20,21]. Subsequent CSF analysis has concentrated on those molecules which appear to play a direct role in AD pathogenesis, i.e., A$\beta_{1–40/42}$, Tau, and phosphorylated Tau, with less consideration given to potential contributors, possible response elements, and innocent bystander molecules such as TTR [22–24].

The demonstration that human CSF could inhibit Aβ aggregation *in vitro* led to studies of the interactions of specific CSF proteins, notably Apolipoprotein E, Apolipoprotein J (clusterin), and transthyretin, with Aβ. Each of these reduced the *in vitro* formation of amyloid fibrils by Aβ [25–28]. The most puzzling of these observations was the inhibition of Aβ fibrillogenesis by TTR, as it was already known to be a human systemic amyloid fibril precursor in familial amyloidotic polyneuropathy, an autosomal dominant disorder of the peripheral nervous system [29], and senile systemic amyloidosis, a sporadic cardiomyopathy found predominantly in elderly males [30]. It was generally assumed that the fibrillar structure common to all amyloids was likely to lead to cross aggregation [31].

4. CSF TTR and AD: The Early Aβ Period

The first published systematic examination of CSF TTR concentrations in AD performed after the *in vitro* demonstration of inhibition of Aβ fibrillogenesis by TTR compared CSF TTR in 40 patients with probable AD (National Institute of Neurologic and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association [NINCDS-ADRDA] criteria) with apparently non-demented elderly controls (51 subjects between 20 and 60 years of age) and 17 younger individuals. There was a gradual increase in CSF TTR concentration with age in the non-demented individuals, but TTR levels were significantly lower in patients with AD (mean age 74.2 years) than in age matched controls (mean age 76.0 years; $p < 0.001$). The authors argued that the decrease in CSF TTR could contribute to the pathogenesis of AD, rather than the converse hypothesis that the TTR concentration was lowered because it was complexed with Aβ and removed from the system [32].

A later report describing CSF TTR concentrations (as determined by radial immunodiffusion) in 49 individuals ranging in age from 27 to 82 years with a variety of neurologic disorders, but none with a history of dementia, stroke, or recent head trauma, found no relationship between *ApoE* genotypes and TTR levels nor with A$\beta_{1–40}$ or $_{1–42}$. Thus, in the absence of dementia, related to AD or any other conditions, there appeared to be a trend toward increasing CSF TTR concentration with age, confirming the previous observation (*vide supra*), but the authors did not think it significant and instead used the TTR concentration as a normalization factor for the measurements of the Aβ and ApoE concentrations in CSF [33].

An examination of CSF obtained by lumbar puncture from 26 patients with AD, eight with vascular dementia and 18 age- and sex-matched controls, was designed to enhance the quantitative sensitivity of the determination of proteins present in low concentrations by removing high abundance serum proteins (Blue Sepharose for albumin, protein G Sepharose for immunoglobulins, and an immunosorbent column with a multi-specific anti human serum protein antibodies). The "cleared" CSF was then separated by micro-reverse high-performance liquid chromatography (HPLC) and the fractions analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Mass spectrometry was per-

formed on some fractions and some samples were analyzed by isoelectric focusing. The results of the isoelectric point and mass spectrometry analyses were not conclusive. When the TTR quantitation was performed by nephelometry with a commercially available TTR-specific antibody, total CSF TTR in AD patients was lower than in controls or patients with vascular dementia. Serum TTR in the three dementia groups was higher than controls, but not significantly so [34].

A post-mortem analysis, in which brain (middle temporal gyrus, middle frontal gyrus, inferior parietal lobule, hippocampal CA1) and ventricular CSF samples were obtained within 24 hours of death, was performed with material obtained from 20 patients with confirmed AD and 10 sex- and age-matched controls without AD or any other neurologic diseases (mean ages 84.4 years and 83.1 years respectively) although eight had cerebrovascular disease (three with multi-infarct dementia). There was no significant difference in CSF ApoE concentrations between the AD and control cohort, but the TTR levels were significantly lower in the AD subjects (p = 0.0094). There appeared to be an inverse relationship between TTR concentration and senile plaque number as determined immunohistochemically (using antihuman β4 amyloid antibodies). The plaque frequency was not correlated with ApoE levels but positively correlated with *ApoE4* allele frequency [35]. In discussing their findings, the authors supported the notion that TTR might sequester Aβ reducing fibrillogenesis, but also posited the idea that TTR synthesis could be reduced by AD related damage to the choroid plexus.

A dementia-focused analysis of CSF samples from 106 elderly (ages 66–74) German individuals with a variety of conditions including AD (n = 23), Creutzfeldt Jakob disease (CJD) (n = 18), dementia with Lewy bodies (DLB) (n = 23), frontotemporal dementia (FTD) (n = 10), normal pressure hydrocephalus (NPH) (n = 13), and 19 non-demented controls used enzyme-linked immunosorbent assays (ELISAs) for Tau, A$\beta_{1–42}$, and A$\beta_{1–40}$, and a nephelometric immunoassay for TTR. Each diagnosis was established clinically, i.e., brain histopathology was not available. TTR was significantly lower in the samples from the AD (p = 0.002) and NPH ($p < 0.001$) subjects. The degree of suppression of TTR appeared to be related to the severity of AD, but the sample size was too small to definitively establish such a relationship. Tau was elevated in AD, CJD, DLB, and FTD but low in NPH. A$\beta_{1–42}$ was lower than in the normal controls in all the groups (790 pg/mL vs 126–483 pg/mL) with the lowest level seen in the NPH cohort. A$\beta_{1–40}$ was highest in the controls (5787 pg/mL) and ranged between 3078 and 4772 pg/mL in the other groups [36].

CSF concentrations of TTR, Cystatin C, β-trace, and α1 anti-trypsin (all previously shown to bind Aβ *in vitro*) were measured immunochemically using commercial antibodies and nephelometry in samples from 35 AD subjects, 18 subjects with frontotemporal dementia, and 29 controls (all female) from Sweden. Albumin and A$\beta_{1–38}$, $_{1–40}$, and $_{1–42}$ were measured in the same samples. TTR was significantly lower in both the AD (p = 0.012) and FTD (p = 0.0002) CSF samples. The concentrations of all the Aβ peptides correlated positively with the TTR concentrations in the AD group with a Spearman coefficient of approximately 0.6 (p = 0.001) [37].

The first serious attempt to broadly examine CSF proteomics in AD utilized micro 2D gel analysis with SYPRO staining and mass spectrometric analysis of eluted proteins in samples obtained from 15 AD patients (diagnosed clinically according to NINCDS-ADRDA criteria with a mean age of 77.2 years) and 12 controls (mean age 67.3 years) participating in a longitudinal geriatric population study in Northern Sweden. The investigators found increases in RBP, TTR (not significant), β_2 microglobulin, and Zn-α-2 glycoprotein, and decreases in ApoE and proapolipoprotein isoforms (ApolA1) in the AD patients relative to the controls. Aβ and Tau peptides were not included in the analysis, hence the precise neuropathologic state of the AD patients was not clear and the interpretation of the TTR results is problematic with respect to earlier findings [38].

In a subsequent study, a modification of this approach was used, in which iso-electric focusing was incorporated into the preparation for 2 dimensional gel electrophoresis (2DGE) in CSF samples to enhance the detection of changes in proteins present in low concentrations, from a small number (n = 7) of AD patients and controls (n = 7). The pre-clearing procedure resulted in the identification of nine proteins that differed significantly in the AD patients from the controls. Only α1-antitrypsin was higher. Kininogen precursor, ApoJ (clusterin), ApoE, α-1β glycoprotein, β – trace (prostaglandin D2 synthase) ApoA1, retinol binding protein, and cell cycle progression protein 8 were all lower in the AD samples. When adding isoelectric focusing (IEF) pre-2DGE, the results were somewhat different with 10 spots lower and five increased at least two-fold. Seven were identified by mass spectrometry. However, not all of these showed twofold differences in the AD subjects ($\alpha_1\beta$ glycoprotein, α_2HS glycoprotein, $\beta_2$microglobulin, transferrin, albumin, and transthyretin). All decreased while α_1 antitrypsin was higher in the AD subjects. This was the first indication that technical differences could significantly alter the results and confuse their interpretation [39].

In a technically more sophisticated manner, CSF samples, obtained within 4 hours of death from patients in whom brain pathology confirmed the presence or absence of AD, were analyzed. The samples were pooled from 43 AD and 43 non-demented subjects and analyzed by 2D gel electrophoresis. Protein spots exhibiting differences in the two pools were recovered and analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. Hemopexin and two pigment epithelium-derived isoforms (PEDFs) were shown to be higher in the AD pool, while ApoA1, Cathepsin D, and TTR were significantly reduced [40]. In two follow-

up studies, the pools from the same AD and elderly non-demented patients were compared with a non-AD dementia group (17 subjects) using an improved 2DGE technique. Twenty-one different proteins showed differences in the comparisons among the pools. TTR was significantly lower in both the non-AD demented and the AD subject pools than in the normal pool, without significant differences between the two dementia groups. An increase in hemopexin was confirmed, but no increase in the PEDFs, ApoA1, or cathepsin D was seen [41]. In a parallel analysis, the same investigators used commercially available ELISAs for ApoA1, α1 acid glycoprotein, haptoglobin, hemopexin, PEDF, TTR, Zn α2 glycoprotein, and Apolipoprotein E as well as ELISAs for A$\beta_{1–42}$, A$\beta_{1–40}$, and phospho-Tau. When the mean values were compared, ApoA1, Z2PG, A1GP, hemopexin, and ApoE were all significantly lower in the AD group relative to the non-demented and non-AD demented controls, as was the measured concentration of A$\beta_{1–42}$. Surprisingly, TTR concentrations were not significantly different nor were the mean levels of A$\beta_{1–40}$. Neither total nor phosphorylated Tau differed from the controls. The discrepancy between the results of the dimensional difference gel electrophoresis (DIGE) and serologic data was puzzling as was similarity of the A$\beta_{1–40}$ and Tau proteins in the two groups given the verification of the diagnosis by neuropathology [42].

In a similar 2DGE analysis of CSF from 30 Korean subjects with either mild cognitive impairment (n = 3) or overt AD (n = 27) evaluated using the clinical dementia rating (CDR) to group the subjects, 350 spots were detected. The investigators found that retinol binding protein decreased with an increase in the CDR. While they also found a decrease in haptoglobin precursor 1 they did not report any results for CSF TTR that could have been responsible for the decrease in RBP, as it is the main carrier of RBP charged with retinol in the plasma and the CSF, thus diminishing the informative value of the study with respect to TTR [43].

5. CSF TTR in AD: Proteomics in the Context of CSF Aβ and Tau

By 2011 it appeared likely that lower mean levels of CSF A$\beta_{1–42}$ and higher concentrations of Tau and phosphorylated Tau could distinguish patients with AD from cognitively normal controls. However, it was also clear that for individual subjects, results might not be definitive, particularly in instances of early AD. To potentially enhance CSF assay sensitivity and specificity, a multi-center discovery study in which CSF samples from 24 individuals with clinically mild AD (CDR 1) were compared with those from 24 cognitively normal controls (CDR 0) using 2D gel electrophoresis. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis of the peptides showed differences between the groups. Prior to the quantitative analysis, each CSF sample was depleted of six highly abundant proteins (albumin, IgG, IgA, haptoglobin, transferrin, and α1-anti-trypsin) by immunoaffinity chromatography. In the discovery phase, 119 peptides, representing 47 different proteins, differed between the samples from the demented and normal cohorts. Eight were increased; the remainder, including TTR, were lower in the AD subjects. In this technically and statistically rigorously validated study, individual peptides from some proteins (including ApoE and prostaglandin H2 D-isomerase/β-trace, ApoJ/clusterin) showed increases while other peptides from the same proteins showed decreases in the same analyses; observations presumably related to artifacts intrinsic to the technology. The abundances of the various peptides appeared to cluster according to CDR and ApoE status. Using ELISAs for six proteins that appeared promising in the initial analysis there were significant differences ($p \leq 0.05$) between CDR 0 and CDR 1 subjects in four proteins YKL-40, NrCAM, chromogranin A and TTR. NrCAM and chromogranin A were both lower in the CDR subjects while YKL-40 and TTR (somewhat surprisingly, in contrast to the discovery study) were higher. The subsequent validation study, using commercially available ELISA kits specific for proteins showing significant or close to significant changes in the discovery phase, was performed in a second independent cohort of 292 individuals with very mild dementia (CDR 0.5). ELISAs for Tau, phospho-Tau, and A$\beta_{1–42}$ were also performed in parallel. As expected, A$\beta_{1–42}$ decreased from CDR 0 to 0.5 to 1.0 while Tau and phosphorylated Tau (p-Tau) showed increases. YKL-40, carnosinase, chromogranin A, and NrCAM showed similar significant differences in the validation group, but the transthyretin differences were not significant in the larger study. The analysis included receiver operating characteristics (ROC) and area under the curve (AUC) determinations of specificity and sensitivity for each of the markers and whether they improved the performance of the A$\beta_{1–42}$, Tau, and p-Tau combinations in discerning CDR 0 to CDR 0.5 and CDR 1.0 transition. The changes in transthyretin, which showed an AUC of 0.6190 (compared with 0.8004 for Tau, 0.7339 for p-Tau, 0.7429 for A$\beta_{1–42}$, and 0.8955 for a combined Tau, YKL-40, and NrCAM assessed by logistic regression) did not enhance the discriminatory capacity of the Aβ/Tau/p-Tau combination. The authors also pointed out that TTR showed unusual 2D electrophoretic patterns in which most of the TTR gel features were decreased in AD, while the total CSF TTR concentration (ELISA) was slightly increased. They further opined that the decreases were in TTR peptides that showed post-translational modification. Nonetheless, in this rigorous analysis, the decreases in total CSF TTR seen by others in pathologically confirmed AD, using predominantly immunologic methods, were not observed in the validation cohort, raising the question of whether CSF TTR concentration is dependent on the stage of AD of the patients from whom the CSF is obtained [44].

To define longitudinal changes in CSF biomarkers defined in a discovery analysis of CSF pools from controls (n = 10), mild cognitive impairment (MCI) (n = 5) sub-

jects and patients with fully expressed AD dementia (n = 45) a 1.2-fold increase in TTR was observed in both MCI and AD pools relative to controls. However, when samples collected from individual members of the pools were longitudinally examined by a multiplex proteomic assay over time and compared with changes in $A\beta_{1-42}$, total Tau, and phospho-Tau, TTR concentrations were not informative, although the details of the TTR concentrations were not discussed [45].

In a very small study, CSF was obtained from four age- and sex-matched cognitively normal controls, four subjects defined as having MCI and four with mild AD. The samples were treated with protease inhibitors and the endogenous peptides filtered, concentrated, desalted in formic acid, dried down, resuspended, and examined by LC-MS/MS analysis. 645 peptides, representing 93 protein precursors were identified. In parallel, aliquots of the same samples were passed through a wheat germ agglutinin (WGA) column with the flow through discarded (and not analyzed) and the adherent proteins eluted, tryptic digested and subject to mass spectrometric analysis. In this study it appeared that the TTR concentrations in the MCI subjects were higher than the controls and those from the AD subjects lower. Surprising in this analysis was the identification of TTR peptides in the WGA eluted fraction, since WGA should selectively bind glycoproteins and TTR is not generally glycosylated, although there have been some reports indicating that under some circumstances this might be true. If these findings, obtained in a unique analytic mode, were correct, they would be consistent with the notion that CSF TTR is increased early in AD pathogenesis and diminished in the severely affected. Alternatively, the results may be an artifact of the preparative methodology [46].

A combined study including clinically diagnosed AD (n = 59) and DLB (n = 13) patients and age matched controls from institutions in Denmark, and Sweden explored CSF TTR levels and their relationship to the presence of depression. Mini-mental state examination showed that the AD and DLB patients were clearly more compromised than the controls. There were no differences in mean CSF TTR concentrations as determined immunochemically (enhanced Mancini method). However, TTR was significantly lower in AD patients treated with cholinesterase inhibitors, a phenomenon not previously reported. It was not stated whether these patients received the anti-cholinesterase drugs because they had more severe disease than those who did not. The investigators concluded from their work and previously published studies that "CSF TTR does not appear to be a robust biomarker for differentiating AD from DLB and controls in all cohorts". They also could not reproduce the results from prior published studies indicating that CSF TTR levels were low in depression [47].

In a study focusing on Lewy body dementia (LBD) with AD defined clinically (and with decreased CSF $A\beta_{1-42}$ and increased total CSF Tau and p-Tau) as a comparator, CSFs from approximately age- and sex-matched controls (n = 15), LBD (n = 10), PD (n = 7) and AD (n = 16) patients were analyzed by quadrupole LC-MS/MS after clearing of high abundance proteins, protease digestion, and high to low pH fractionation. In the discovery phase of the project, 74 peptides from 54 potential marker proteins were evaluated and 27 were found to be statistically significantly increased relative to the controls. Transthyretin was 1.8-fold higher in the AD subjects than in the controls and not increased in either the LBD or Parkinson's Disease (PD) cohorts. The TTR concentrations correlated with the CSF $A\beta_{1-42}$ and p-Tau levels in the AD subjects but not for the other neurodegenerative entities. This is somewhat confusing as CSF $A\beta_{1-42}$ has been shown to decrease and p-Tau to increase with increasing disease severity. Hence, the association of the highest concentrations of TTR with the lowest concentrations of $A\beta$ is consistent with TTR increasing in AD, while the observation of the highest concentrations of TTR with the lowest concentrations of p-Tau is not, suggesting either that elevated CSF TTR occurs early, when $A\beta$ is reduced and decreases as CSF Tau increases. Alternatively, the figure may just reflect an error in the way the data are plotted. The authors argue that the increase in TTR (also seen in the study cited above) may differ from studies showing a decrease in CSF TTR in AD because post-translational oxidative modifications (particularly of the free -SH) in CSF TTR may make quantitation using ELISAs unreliable [48]. This seems unlikely as several studies using non-immunologic assays have reported reductions in CSF TTR in AD subjects relative to controls. For example, in a small CSF proteomics analysis (6 AD, 6 N) focused on ApoE isoforms, the major TTR 2DIGE peptide (spot # 1554) was twice as high in the normal subjects as in the AD patients, independent of ApoE isoform status (i.e., 3/3 or 4/4). The observation was not pursued [49].

Two studies examined the oxidation status of TTR Cys10 in CSF from AD subjects to determine if it had any relationship to pathogenesis. In the first, CSF samples from 39 patients identified clinically as having probable AD (CDR 1–1.5) were compared with those from 27 cognitively normal individuals matched for sex, although not necessarily age. Using two different mass spectrometry protocols to quantitate -Cys-Cys and -Cys-Gly, they found the conjugated forms to be significantly less abundant in the AD cohort with individual AUCs of 0.893 and 0.866, making them reasonably efficient in sorting out CSF from AD and non-AD subjects. Unfortunately, the study did not report total TTR concentrations immunologically in order to determine if they were higher or lower than normal; the modifications detected reflected changes in total CSF TTR [50].

In a later study, using rocket immunoelectrophoresis to measure total TTR and immunoaffinity isolation and mass spectrometry to specifically examine S-cysteinylation, S-cysteinylglycinylation, and S-glutathionylation in clinically defined age- and sex-

matched AD patients (n = 37), patients with mild cognitive impairment (MCI) (n = 17), patients with normal pressure hydrocephalus (n = 15), and healthy controls (n = 7), the AD and MCI patients had a significantly higher fraction of oxidatively modified CSF TTR, a clear difference from the findings in the earlier analysis. Mean total TTR was about the same in the AD and NPH patients, being somewhat higher than in the MCI and normal control cohorts. Parallel studies of the same samples using standard ELISAs for $A\beta_{1-42}$, Tau, and p-Tau showed the expected lowest levels of $A\beta$ in the AD cohort, somewhat higher levels in the MCI patients, and the highest concentrations in the controls. Somewhat surprising were the very low $A\beta$ values in the NPH group. Tau and p-Tau concentrations were similar with a gradient from AD through to MCI, then to NPH, and lowest in the controls. The authors pointed out that the data in the earlier paper was obtained on crude desiccated CSF examined by MALDI-TOF and that these technical differences could account for the discrepant findings [51]. In any case, in the absence of further data, the relevance of the observations remains unclear, except to say that these measurements do not support the use of the various oxidized forms of CSF TTR as robust biomarkers for AD pathology.

In an analysis of a different post-translational modification of TTR, carbonylated CSF proteins in a small number of AD subjects by 2D gel (oxy-blotting) and mass spectrometry, TTR was among the most abundant carbonylated proteins, but it was significantly decreased in patients with probable AD relative to controls, suggesting that reduced epitope exposure was not responsible. However, the study did not measure total TTR concentration immunologically [52].

6. The Proteomics Era

A broad review of proteomic analysis of CSF across the entire spectrum of intrinsic and extrinsic neurologic diseases failed to show any specific changes in TTR or its peptides in any of the studies analyzed. Hence, this exercise was not informative for our analysis [53]. A metanalysis, reviewing publications in PubMed from 2012 to 2017 found 28 papers reporting proteomic analyses of CSF that included AD as one of the diagnostic groups, emphasized the changes in technology that had been introduced during this period and cited several examples of potential biomarkers. Only one study of the 28 found significant changes in TTR [54]. A later metanalysis identified 14 studies that reported a difference between CSF TTR concentrations in AD patients and controls. The only proteins in which there were more studies showing differences were ApoE and nerve growth factor induced peptide VGF. TTR (and AponerE) were found to be increased and decreased in different proteomic studies, prompting the authors to comment, "suggesting that they cannot be considered as reliable CSF biomarkers of AD. While it is likely that heterogeneity in response direction may reflect irrelevant physiological or environmental factors, it is also interesting to speculate that the heterogeneity reflects unknown endophenotypes in AD or provides an indication of the profound general protein dysregulation that occurs during AD progression". They also noted that RBP4 was reduced in four of five studies and that it circulates bound to TTR and it was unchanged in preclinical AD [55].

Reviewing AD biomarkers in 2021, the authors noted that "it is now established that in AD, CSF $A\beta_{1-42}$ is reduced to about 50% or normal but that the ratio of $A\beta_{42/40}$ is more accurate in identifying AD pathology". In addition, there is high concordance between CSF $A\beta$ and brain positron emission tomography (PET) scanning for $A\beta$, which, in turn shows agreement with AD pathology at autopsy. In conjunction with the decrease in $A\beta$ there is an increase in total Tau (t-Tau) and phosphorylated Tau (p-Tau) in the CSF. While the review mentions several other CSF constituents that may be potentially helpful in sorting out patients with AD from those with other neurodegenerative disorders, it does not mention TTR, suggesting that in their view, CSF TTR determinations are not helpful in AD diagnosis or follow up [56]. A subsequent analysis from the same group did not include TTR among candidate biomarkers that might reflect changes in the AD process which they conceive as evidence of synaptic pathology, activated glial responses or characteristic of other dementia associated neurologic disorders, e.g., PD, LBD or TDP-43 mediated neurodegeneration [57].

7. Summary and Discussion

Across a broad range of studies, it appears that CSF TTR determinations are not consistent within AD populations nor specific enough with respect to other neurodegenerative disorders to be clinically useful in determining the diagnosis or prognosis of AD or response to therapy. Nonetheless, given the frequency of the observations regarding TTR in AD patients, it is worth considering their relevance with respect to the pathogenesis of neurodegeneration and why they were considered in the first place.

There are three possible technical explanations as to why TTR has appeared to fail as an AD biomarker. The assay/measurement itself may have technical issues. TTR ELISAs have been around for a long time and have been reasonably reliable in looking at serum TTR concentrations, particularly of the wildtype molecule. Although it has been noted that the concentrations of some mutant TTRs are not accurately measured by all commercially available antibodies. It was suggested in some of the proteomic studies that post translational modifications of TTR, particularly at cys10, could have changed the reactivity with the antibodies, although the Poulsen study suggests this is not the case [51].

There are clearly differences when the same samples or sample pools are assayed by both proteomic and immunochemical techniques. Several of the proteomic anal-

yses discuss the variation in the stoichiometry of the TTR peptides in 2DGE or mass spectrometry; the former is perhaps related to post-translational change while the latter may reflect variation in the cleavage of the protein either in the preliminary proteolytic step or during the mass spectrometric analysis itself. It is also possible that in some of the studies that used pre-clearing of highly abundant proteins from the CSF samples, the TTR may have been non-specifically bound to the presumably specifically cleared protein species. In no instance was it stated that the CSF was assayed before and after pre-clearance. There is no good reason why TTR should be specifically affected by the pre-clearance; nonetheless, it would have been reassuring to see analyses of the total and the pre-cleared CSF as well as the proteins included in the clearance fraction. None of the studies examined the CSF for TTR–Aβ complexes. Such complexes have been extracted from the brains of mice carrying transgenes encoding both human TTR and human AβPP (the Aβ precursor), and in some human AD brains [58]. It is possible that TTR in such complexes would not be accurately assessed in the immunologic assay and the molecule might not be completely cleaved in the mass spectral analyses. In neither of those instances was CSF TTR analyzed.

An additional potential source of the inconsistency may be related to the clinical staging of the patient populations studied. Various clinical staging systems have been used to assess the patients whose CSF was being assayed. It is likely that the clinical classification systems are relatively crude measures of the pathologic and biochemical changes taking place intra-cerebrally. Patients within a given clinical class may also show considerable pathologic variation, although this has not been examined in detail. The use of PET scan diagnostics and the reasonably well correlated levels of CSF Aβ, Tau, and phospho-Tau make this a bit better, but there was still some inconsistency when CSF TTR was assayed in populations who were also subject to those assays. In the studies that examined CSF obtained shortly after death and that had undergone anatomic evaluations of the extent of disease, i.e., disease staging was unequivocal, CSF TTR concentrations were quite low.

It is our conclusion, despite our reservations and the inconsistencies in the studies summarized above, that CSF TTR may be increased early in the evolution of AD but that in full blown, anatomically recognized AD, CSF TTR concentration is lower than normal. In the absence of definitive data supporting that conclusion, the potential dynamics of CSF TTR concentration related to the rate of pathogenesis of AD makes TTR an unreliable marker for either prognosis, diagnosis, or response to therapy. It would have been interesting if some of the recent studies showing plaque clearance by PET scanning had included serial determinations of CSF TTR to assess whether there was a systematic change related to the reduction in Aβ plaques.

Given the well documented observations that TTR inhibits Aβ fibrillogenesis and cytotoxicity *in vitro* [25,59–62], and appears to have protease activity for which Aβ may be a substrate [63], its capacity to suppress the AD phenotype in murine models of human AD [64,65] and its apparent behavior as a neuronal stress responder both *in vivo* and in tissue culture [8], it is disappointing that CSF TTR has not thus far been useful as a biomarker for monitoring AD. The variation in results among investigative groups is currently not readily explainable. Whether greater knowledge of the biology of the disease or even better technology will change this is presently unclear.

Author Contributions

JNB wrote the manuscript.

Ethics Approval and Consent to Participate

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Conflict of Interest

The author is a member of the Scientific Advisory Board of Protego Biopharma, but has no conflict of interest with respect to the subject of this paper.

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