

Plasma neurofilament light chain: an early biomarker for hereditary ATTR amyloid polyneuropathy

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ABSTRACT

Transthyretin amyloidosis due to V30M mutation (ATTR-V30M) is the most frequent hereditary ATTR amyloidosis. Besides neurophysiological measures, there are no biomarkers to detect preclinical disease or monitor disease progression. CSF or plasma Neurofilament light chain (pNfL) have recently been considered sensitive biomarkers to quantitate neuro-axonal damage in several disorders of the peripheral and central nervous system.

Objective: Characterize plasma NfL levels in a series of untreated ATTR-V30M patients stratified by clinical severity using a cross-sectional retrospective study design.

Methods: 60 ATTR-V30M patients and 16 controls from 2 independent cohorts were analysed for pNfL by single molecule array (SIMOA) technique. Disease severity was assessed with Polyneuropathy Disability Score.

Results: pNfL is elevated in ATTR-V30M patients as a function of disease severity in both cohorts. Moreover, pNfL discriminates asymptomatic mutation carriers from early symptomatic patients (AUC=0.97;p<0.001) with high sensitivity (92.3%) and specificity (93.8%). pNfL elevation (>66.9 pg/mL) also discriminates patients with sensory neuropathy from patients with motor neuropathy (AUC=0.91;p<0.01) with a sensitivity of 61.5% and a specificity of 92.3%.

Conclusion: pNfL is an easily accessible biomarker to establish ATTR-V30M disease conversion and to monitor disease progression. pNfL could be used as efficacy measure of disease-oriented therapies in clinical and pre-clinical trials.

Keywords: ATTR, biomarker, Neurofilament Light-chain, plasma, ,
Transthyretin

Abbreviations

ATTR-V30M: transthyretin amyloid protein due to V30M mutation; AUC: area under the curve; CNS: central nervous system; FAP: familial amyloid polyneuropathy; NfL: neurofilament light chain; pNfL: plasma neurofilament light chain; PND - Polyneuropathy Disability Score; PNS: peripheral nervous system; ROC: Receiver Operating Characteristic

Introduction

Transthyretin amyloidosis due to V30M mutation (ATTR-V30M), also known as Familial Amyloid Polyneuropathy (FAP), is the most frequent hereditary transthyretin amyloidosis. The most frequent clinical phenotype at disease onset is a combination of sensory and dysautonomic symptoms due to an axonal small fiber polyneuropathy [1,2]. Later in the course of the disease motor neuropathy and progressive disability emerge. ATTR-V30M amyloidosis is an highly disabling disease that leads to death within 7-12 years after onset if left untreated [3]. Currently, patients are stratified according to clinical scales that are subjective and entail a high degree of inter-rater and inter-test variability [4-6]. So, disease biomarkers are needed to allow better patient stratification and treatment monitoring.

Currently, disease modifying therapies are increasingly used in routine clinical practice and novel therapeutic approaches are in development [5]. Therefore, the need for reliable disease biomarkers is gaining further interest, both for clinical trials and individual patients, to identify disease in pre-symptomatic or very early disease stages. At these early stages either the patients have no symptoms or signs or they have symptoms that cannot be easily assessed by objective measurements [4,6]. Neurophysiology testing is helpful in current practice, but is time consuming, operator dependent, requires patient cooperation and is at best uncomfortable [6]. For all these reasons, a biomarker or a set of biomarkers that could detect disease conversion and that could monitor disease progression would certainly open new perspectives in the implementation of earlier therapeutic intervention.

Neurofilament light chain (NfL) is one of the neural cytoskeleton proteins with important roles in axonal and dendritic branching and growth [7]. This protein can be measured in the blood of patients and has been shown to be elevated in neurological diseases that involve neuronal damage of the central nervous system (CNS) while now more data is emerging for peripheral nervous system (PNS) diseases [8-12]. Having this in mind, we aimed to characterize the relation of pNfL levels and standard disease features in untreated hereditary ATTR-V30M patients at different stages of clinical disease evolution. Our results suggest that pNfL may represent a reliable and sensitive marker of disease onset and progression in ATTR-V30M amyloidosis.

Material and methods

Study participants

ATTR-V30M mutation carriers and non-carriers were evaluated at our clinical and genetic counselling centres in Portugal (Cohort #1) and a validation cohort in Italy (Cohort #2). Patients included in the study had a neurological evaluation performed by a neurologist at the time of blood collection that was further validated by one of the study neurologists (IC, AC and LM). Patients under any disease modifying treatments were excluded from the study. In all cases disease severity was determined using Polyneuropathy Disability Score (PND), that stratifies patient disability into 6 stages: PND 0 no impairment; PND I sensory disturbances but preserved walking capacity; PND II impaired walking capacity but ability to walk without a stick or crutches; PND IIIa walking only with the help of one stick or crutch and IIIb walking with the help of two sticks or crutches and PND IV confined to a wheelchair or bedridden [13]. Patients were classified retrospectively based on the clinical records at the time of blood collection. Disease duration was calculated based on patient information on the onset of the first symptoms attributable to the disease. Healthy controls included in the study were available from Portugal and were either healthy relatives of patients attending the clinic (non-carriers of the TTR V30M mutation) or healthy volunteers from the same country region. Cohorts #1 and #2 were evaluated independently. Local ethical committees approved the study: Cohort #1 (Hospital de Santa Maria #401/18 and University of Porto #36/CEUP/2017);); for cohort #2 written informed consent was obtained for using biological

samples and clinical data for research purposes, according to the local Institutional review board guidelines.

Blood samples

Blood collection was performed in each centre, into EDTA tubes. The blood was centrifuged at 2000g at room temperature and plasma stored at -80°C. All the samples had one additional freeze-thaw cycle before the analysis. The samples were all analysed in Basel, Switzerland.

NfL measurement:

Plasma NfL measurements were performed using a highly sensitive single-molecule array assay (Simoa) using the capture monoclonal antibody 47:3 and the biotinylated detection antibody 2:1 (UmanDiagnostics AB) [14]. The samples were measured in duplicate on a Simoa HD-1 platform (Quanterix) using a two-step neat assay. Plasma samples were measured at 1:4 (Tris-buffered saline, 0.1% Tween 20, 1% non-fat milk powder, HeteroBlock (300µg/mL; Omega Biologicals). Batch-prepared calibrators (bovine lyophilized NfL) ranging from 0 to 10,000 pg/mL were stored at -80 °C (Uman Diagnostics AB). All samples were measured blinded. For plasma, the mean intra-assay coefficient of variation of duplicate determinations for concentration was 7.4%. The inter-assay coefficients of variation for plasma was 8.7% (mean concentration 38.7 pg/mL).

Statistical analysis

Normal distribution of quantitative data was assessed with the Shapiro-Wilk test. Non-normally distributed variables were logarithmic-transformed and compared using ANOVA. Bonferroni's Post-Hoc tests were used to compare the different groups. To evaluate if pNfL levels varied with disease stage, a trend test derived from an ANOVA analysis was calculated. Potential confounders (age and sex) were used as covariates in the analysis of variance. Multiple linear regression analysis was used to assess pNfL according to age, sex and disease stage. Mann-Whitney test was used to compare ages and disease duration between groups. Receiver operating characteristic (ROC) curves were drawn by plotting the true-positive fraction (sensitivity) against the false-positive fraction (100% – specificity) for varying cutoff values in both cohorts. The area under the curve was calculated. Youden index was used to calculate the cut-off that best discriminates different disease stages. Plasma NfL levels and different disease stages were analyzed using Spearman correlation coefficients. In all cases, statistical significance was set at $P < 0.05$. GraphPad Prism version 6 was used to generate the graphics and Statistical analysis was performed using IBM SPSS Statistics version 25.

Data availability

Data used in preparation of the figures and tables will be shared in anonymized format by request of a qualified investigator to the corresponding author for purposes of replicating procedures and results.

Results

ATTR-V30M patients

We included in the study 60 genetically confirmed ATTRV30M patients, out of which 44 were symptomatic and 16 asymptomatic carriers. We included 16 healthy controls. Three patients from the cohort #1 were excluded due to insufficient clinical data at the time of blood collection and 5 patients from cohort #2 were not included because they were under disease modifying treatment with tafamidis.

Clinical and demographic characteristics of the patients are shown in Table 1. Disease severity was stratified using the Polyneuropathy Disability Score (PND) score that includes the neurological deficits and the functional status of the patients. This classification was done retrospectively by a blinded rater, based on clinical records. Patients from cohort #1 are younger than cohort #2 (Mann-Whitney test $p < 0.001$) but disease duration in equivalent disease stages was not different between the two cohorts (Mann-Whitney test $p > 0.05$). In cohort #1, age of disease onset was not different between patients with a PND score of I and \geq II (Mann-Whitney test $p > 0.05$). However, age at blood collection ($p < 0.05$) and disease duration (Mann-Whitney test $p < 0.001$) were significantly higher in patients with a PND score \geq II compared to patients with a PND score of I. Control patients did not differ in the age at collection. In cohort #2 age of disease onset and age at blood collection was not different between PND I, PND II and III and PND IV, but disease duration ($p < 0.05$) was significantly

higher in patients with PND score of IV compared to patients with a PND score of I.

NfL plasma levels are increased in ATTR-V30M patients

To study the role of NfL as a biomarker of ATTR amyloidosis we measured pNfL levels in ATTR-V30M patients at different disease stages and compared them with normal controls. In cohort #1, pNfL was higher in symptomatic ATTR-V30M patients ($PND \geq I$) compared to controls. (Figure 1.) The increase of NfL in blood of patients was 4.8 (PND I) to 15.4-fold ($PND \geq II$) higher than in asymptomatic carriers. In both cohorts we found the increase of pNfL across the different disease stages followed a significant linear trend ($F_{2,39} = 66.1$ ($p < 0.001$) and $F_{2,15} = 5.2$ ($p < 0.05$) for cohort #1 and #2, respectively) even after adjusting for age ($F_{3,38} = 49.8$ ($p < 0.001$) and $F_{3,14} = 3.8$ ($p < 0.05$) for cohort #1 and #2, respectively). (Figure 1 and Figure e-1). Consistently, in both cohorts we observed that pNfL increase was mostly due to disease stage than to patients age (partial R^2 for Disease stage is 0.687 and for age is 0.106). On the other hand, we did not find any association between sex and pNfL levels. To evaluate if the TTR V30M carrier status had an influence in NfL levels we compared the control group that included non-carrier relatives with asymptomatic TTR V30M mutation carriers and found no difference (Figure 1).

NfL plasma levels correlated with disease severity

We found a significant correlation between disease severity and NfL plasma levels in cohort #1 ($r_s = 0.88$; $p < 0.001$) and in cohort #2 ($r_s = 0.60$; $p < 0.01$) (Figure 2 and Figure e-2). Similarly, NfL plasma levels correlated with disease duration

in cohort #1 ($r_s=0.76$; $p<0.001$) while in cohort #2 the correlation did not reach statistical significance. (Figure 2. and Figure e-2)

NfL plasma levels discriminate between disease stages

ROC curve analysis was used to compare pNfL capacity to discriminate different disease stages and the asymptomatic to symptomatic transition (Figure 3). In cohort #1 the area under the curve (AUC) comparing asymptomatic (PND 0) to symptomatic ATTR-V30M patients (PND \geq I) was 0.99 ($p<0.001$) and the pNfL concentration of 10.6 pg/mL discriminated these patients with a sensitivity of 96.2% and a specificity of 93.8%. When looking into specific disease stages we found that pNfL also distinguished with similar accuracy asymptomatic (PND 0) from early stage ATTR-V30M patients (PND I), with an AUC of 0.97 ($p<0.001$), with a sensitivity of 92.3% and a specificity of 93.8% for the NfL cut-off value of 10.6 pg/mL. In addition, when comparing early symptomatic patients (PND I) with patients presenting symptoms and signs of motor dysfunction (PND \geq II) we found a AUC of 0.90 ($p<0.001$) and the pNfL concentration of 66.9 pg/mL discriminated these patients with a sensitivity of 61.5% and a specificity of 92.3%. As for cohort #2 in early symptomatic patients (PND I) versus PND \geq II we found a AUC of 0.86 ($p<0.05$) and the pNfL concentration of 75.7 pg/mL discriminated these patients with a sensitivity of 84.6% and a specificity of 80.0% (Figure e-3).

Discussion

In the present study, we measured pNfL in two independent ATTR-V30M patient cohorts and found that it is elevated in symptomatic ATTR-V30M patients when compared to non-carriers or healthy population controls. In addition, we showed that this increase is already detectable in very early disease stages with isolated sensory or disautonomic symptoms or signs (PND I). Moreover, when we compared pNfL levels of asymptomatic carriers with early stage ATTR-V30M patients we found that pNfL concentration of 10.6 pg/mL had a sensitivity of 92% with a specificity of 94% to distinguish these 2 groups of patients. Given the complexity of validating ATTR-V30M diagnosis in asymptomatic carriers our results constitute a major step forward in hereditary ATTR-V30M diagnosis [4,6,15]. In fact, to confirm the diagnosis of ATTR-V30M in asymptomatic carriers it is recommended and necessary that they undergo systematic and regular monitoring to detect early signs of ATTR-V30M and at least two related symptoms and positive biopsy findings for amyloid deposits. This is a difficult process that relies on patient sensitive or dysautonomic complaints and therefore comprises a high level of subjectivity [16]. Hence, pNfL holds a solid potential to become part of ATTR-V30M diagnostic work-up and, consequently in the admission of patients for disease-oriented therapies.

When we compared NfL plasma levels of ATTR-V30M patients at different disease stages we found, in the two cohorts, that NfL levels correlated significantly with disease associated disability.

Consistently, NfL levels exhibited a positive and significant linear trend as disease evolved from minor disability (PND I) to major disability (PND III and

IV). This finding was independent of patient`s age at collection. As reported in previous studies we observed an age dependent increase of NfL in patients and controls [12]. Still, our study demonstrates that the effect of disease stage on plasma NfL largely outstands age related plasma NfL increase. We did not find any association between sex and pNfL which is in line with available data from different disease patients and corresponding controls [17-19]. Therefore, our study further strengthens the need for age-adjusted cut-off points to best value NfL CSF and plasma levels in individual patients.

The potential of pNfL to distinguish between early and advanced patients exhibiting motor dysfunction ($PND \geq II$) has also been documented but with a sensitivity of 62%. NfL did not differentiate among patients with different degrees of motor disability (PND score between II and IV). This may be attributable to the low sample size or to the fact that these patients are older and may be affected by concomitant pathologies leading to neuronal damage. Nevertheless, pNfL may still provide valuable contribution to document ATTR-V30M disease progression at the individual patient level.

Elevation of pNfL has been observed in several neurological conditions that usually involve the CNS, such as amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Alzheimer`s disease (AD) and Parkinsonism [8-12, 19]. In diseases involving the peripheral nervous system (PNS) like Charcot Marie Tooth neuropathy (CMT), and chronic inflammatory demyelinating polyneuropathy (CIDP) plasma NfL has recently been shown to be elevated as well [9,20]. As CNS-based pathology is unlikely in these cases, these observations suggest that the damage to peripheral neurons can lead to relevant release of NfL into

the plasma [21]. Therefore, in ATTR-V30M patients, the early non-myelinated sensitive and autonomic small fiber neuropathy is probably underlying the initial increase in pNfL. In alternative, subclinical axonal neuropathy may contribute to the increase of pNfL levels in early disease stages. Later in the course of the disease progression, CNS involvement may play a role and partially justify the higher and more variable NfL levels in patients with longer disease duration [22, 23]. Further studies are needed to clarify the relative contribution of PNS and CNS in NfL dynamics of ATTR-V30M patients.

Our study holds some limitations, like its retrospective nature and the cross-sectional design that limits inferences regarding biomarker validity for disease progression. Still, the fact that we evaluated two independent patient cohorts with the same mutation (V30M) and obtained coherent findings strengthens the potential of NfL as a ATTR-V30M disease progression biomarker.

The fact that pNfL increases in many neurological conditions limits its role as a diagnostic marker in the population setting. However, in a more homogeneous setting like hereditary ATTR-V30M, NfL is a promising tool for pre-clinical disease stratification and possibly may have a role in predicting disease progression, in line with what has been documented in familial AD patients from the DIAN study [10].

Conclusion

In this study we have shown that pNfL, a minimally invasive biomarker, is a) sensitive to detect peripheral axonal damage in ATTR-V30M amyloidosis, that b) NfL levels correlate with disease severity, and that c) NfL levels can be a valid instrument to distinguish asymptomatic from early stage ATTR-V30M

patients. These findings are of primary relevance in a disease where the diagnosis at very early stages is largely based on subtle symptoms and subjective complaints with normal or nearly normal nerve conduction studies [6]. Recent advances in treatment options have led to a need for more granular patient stratification, particularly when aiming at preclinical interventions. Further studies addressing this point are in progress.

Disclosure of interest

Dr. Obici received speaker honoraria from Pfizer, Alnylam Pharmaceuticals and Akcea.

Dr. Leppert is a former employee of Novartis (until Jan 31st 2019). He has received personal compensation for consulting and speaking at conferences, and travel reimbursement from Quanterix, Orion and Sanofi.

Dr. Kuhle received speaker fees, research support, travel support, and/or served on advisory boards byECTRIMS, Swiss MS Society, Swiss National Research Foundation (320030_160221), University of Basel, Bayer, Biogen, Celgene, Genzyme, Merck, Novartis, Roche, Teva.

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Figures Legends

Figure 1. Neurofilament Light Chain (NfL) plasma levels are elevated in symptomatic ATTR-V30M patients from cohort #1. NfL levels revealed a significant linear trend with disease progression assessed with PND score: $F_{2,39} = 66.1$ ($p < 0.001$). Plasma NfL levels were significantly increased in early symptomatic patients (PND I) compared with asymptomatic carriers (PND 0) and in more severely affected patients ($PND \geq II$) compared to early stage patients (PND I). NfL levels of population controls ($n=16$) and asymptomatic carriers ($n=16$) were undistinguishable. Bonferroni's post hoc test for multiple comparisons were used to compare all ATTR-V30M patient groups. We used a logarithmic (\log_{10}) scale. In $PND \geq II$, full diamonds are patients with a PND score of III. All data are represented as group means \pm SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2. Plasma NfL (pNfL) and disease severity ATTR-V30M patients from cohort #1. (A) Disease severity as assessed by the PND score (0-III) correlated positively and significantly with pNfL levels. (B) Consistently disease duration and pNfL also exhibited a significant positive correlation. r_s = Spearman correlation coefficient.

Figure 3. Receiver operating characteristics (ROC) curves of NfL levels for ATTR-V30M patients from cohort #1, illustrating the sensitivity and the specificity pNfL in differentiating patients at different disease stages. (A) Asymptomatic (PND 0) versus early stage symptomatic (PND I). (B) Early stage symptomatic (PND I) versus patients with motor dysfunction (PND II). (C) Patients with motor dysfunction without walking aids (PND II) versus patients with motor dysfunction in need of walking sticks or crutches. AUC = area under the curve.

Table 1.- ATTR-V30M patient clinical and demographic characteristics (Cohort #1 and #2)

Patient Group	PND Score	Patient(n)	Sex (male : female)	Age of Onset (years)	Age at collection (years)	Disease duration (years)
Control	N/A	16	9:7	N/A	33.5 [26.7-40.3]	N/A
	0	16	7:9	N/A	36.3 [31.4-41.2]	N/A
ATTR-V30M (Cohort #1)	I	13	6:7	41.0 [32.4-49.6]	42.3 [33.9-50.7]	1.4 [0.8-2.1]
	II and III	13	5:8	49.5 [41.9-57.0]	53.5 [45.7-61.4]	4.1 [2.9-5.3]
	0	0	N/A	N/A	N/A	N/A
ATTR-V30M (Cohort #2)	I	5	4:1	55.4 [38.5-72.4]	57.4 [39.6-75.2]	2.0 [0.2-3.8]
	II and III	8	4:4	62.0 [55.6-68.4]	65.9 [60.1-71.7]	3.9 [2.2-5.6]
	IV	5	4:1	63.8 [53.1-74.5]	69.2 [57.5-80.9]	5.4 [3.1-7.7]

Polyneuropathy Disability (PND) score: **0** - no impairment; **I** - sensory disturbances but preserved walking capability; **II** - impaired walking capability but ability to walk without a stick or crutches; **III** - walking only with the help of one stick or crutch (**IIIa**) or walking with the help of two sticks or crutches (**IIIb**); **IV** - confined to a wheelchair or bedridden. Age is shown as mean and 95%CI.