

Leonardi Luca (Orcid ID: 0000-0002-1267-864X)
Luigetti Marco (Orcid ID: 0000-0001-7539-505X)
Fionda Laura (Orcid ID: 0000-0002-8813-2272)
Di Pietro Giuseppe (Orcid ID: 0000-0002-9645-3578)
truini andrea (Orcid ID: 0000-0002-2630-7647)
Galosi Eleonora (Orcid ID: 0000-0002-4464-9982)

Quantitative sensory testing and skin biopsy findings in late-onset ATTRv pre-symptomatic carriers: relationships with predicted time of disease onset (PADO).

Luca Leonardi¹, Rocco Costanzo¹, Francesca Forcina¹, Stefania Morino¹, Giovanni Antonini¹, Marco Salvetti^{1,2}, Marco Luigetti^{4,5}, Angela Romano⁴, Guido Primiano⁴, Valeria Guglielmino⁵, Laura Fionda¹, Matteo Garibaldi¹, Antonio Lauletta¹, Elena Rossini¹, Laura Tufano¹, Marco Ceccanti³, Nicoletta Esposito³, Pietro Falco³, Giuseppe di Pietro³, Andrea Truini³, Eleonora Galosi³

1. Department of Neuroscience, Mental Health and Sensory Organs (NESMOS), Sapienza University of Rome, Rome, Italy.
2. Neuromed Institute IRCCS, Pozzilli (IS)
3. Department of Human Neuroscience, Sapienza University of Rome, Rome, Italy
4. Fondazione Policlinico Universitario A. Gemelli IRCCS. UOC Neurologia
5. Università Cattolica del Sacro Cuore. Sede di Roma. Dipartimento di Neuroscienze

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the [Version of Record](https://doi.org/10.1111/jns.12586). Please cite this article as doi: [10.1111/jns.12586](https://doi.org/10.1111/jns.12586)

This article is protected by copyright. All rights reserved.

ABSTRACT

Introduction: Hereditary transthyretin amyloidosis polyneuropathy (ATTRv-PN) pre-symptomatic carriers often show preclinical abnormalities at small fibre related diagnostic tests. However, no validated biomarker is currently available to use for pre-symptomatic carriers' follow-up, thus helping therapeutic decision making. Our study aimed at assessing nerve conduction study (NCS), quantitative sensory testing (QST), and skin biopsy parameters in a large cohort of late-onset ATTRv pre-symptomatic carriers, and to evaluate whether they correlated with predicted age of disease onset (PADO).

Methods: Late-onset ATTRv pre-symptomatic carriers were consecutively enrolled and underwent NCS, QST, and skin biopsy with intraepidermal nerve fibre density (IENFD) evaluation from a distal and a proximal site. *Douleur Neuropathique-4* (DN4) and Small Fiber Neuropathy-Symptoms Inventory (SFN-SIQ) were used to assess painful and small fibre neuropathy related symptoms. PADO and time-to-PADO (delta-PADO) were estimated for each carrier, and correlations with diagnostic test measures were analysed.

Results: Forty pre-symptomatic ATTRv subjects were enrolled. Twenty carriers (50%) had distal IENFD reduction, with a non-length dependent distribution in 73% of cases. Eleven subjects (27.5%) had cold and/or warm detection threshold (CDT and/or WDT) abnormalities at QST. Delta-PADO positively correlated with sural sensory nerve action potential (SNAP) amplitude ($r=0.416$, $p=0.004$), and z-values of QST parameters like CDT ($r=0.314$, $p=0.028$), WDT ($r=-0.294$, $p=0.034$), and mechanical detection threshold (MDT) ($r=-0.382$, $p=0.012$). Simple linear regression models showed a linear relation between delta-PADO and sural SAP, CDT, and MDT.

Conclusions: Our findings confirm that IENFD reduction and QST abnormalities may occur early in ATTRv pre-symptomatic carriers, often with a non-length dependent pattern. However, only sural SAP amplitude and QST parameters correlated with delta-PADO, suggesting that serial combined QST and NCS evaluation could be useful in ATTRv pre-symptomatic carriers' follow-up.

Keywords: ATTRv, pre-symptomatic carriers, skin biopsy, quantitative sensory testing, small fibre neuropathy, PADO

INTRODUCTION

Hereditary transthyretin amyloidosis polyneuropathy (ATTRv-PN) is a rare multi-system disease with somatic and autonomic peripheral nervous system involvement caused by transthyretin (TTR) mutations [1]. TTR amyloid deposition induces peripheral nerve damage, leading to severe disability and life-threatening complications [2]. In the last decade, the development of new disease modifying agents has significantly improved ATTRv-PN prognosis, thus delaying and preventing disease progression [3]. Therefore, pre-symptomatic follow-up tests able to timely identify the disease onset have become a matter of study and debate, especially for their implications in the early initiation of disease-modifying therapies [4].

Skin biopsy and quantitative sensory testing (QST) are the reference standard tests for small fibre assessment [5] and have been frequently used for morphometric and functional evaluation of small fibre damage in ATTRv-PN patients and carriers [6-13]. Small nerve fibre involvement occurs early in ATTRv-PN [14], with several studies demonstrating small nerve fibre loss and dysfunction in totally pre-symptomatic carriers of amyloidogenic *TTR* mutations [6-14]. However, no biomarker is currently validated to follow up pre-symptomatic carriers and to predict the transition from pre-symptomatic to clinically evident disease, thus helping therapeutic decision making.

In this multicentric prospective study, aimed at investigating potential diagnostic biomarkers to follow up ATTRv pre-symptomatic carriers, we evaluated nerve conduction study (NCS), quantitative sensory testing (QST), and skin biopsy in a cohort of late-onset ATTRv pre-symptomatic carriers with heterogeneous *TTR* amyloidogenic variants, and analysed their relationships with predicted age of disease onset (PADO) [4].

METHODS

Subjects' selection

We enrolled genetically confirmed late-onset ATTRv-PN pre-symptomatic carriers who were consecutively evaluated at our amyloidosis referral Centers (Sant'Andrea and Agostino Gemelli University Hospitals) between January 2020 and November 2022. Subjects were considered eligible for the study if they carried a known amyloidogenic variant in *TTR*, were older than 18 years, and did not show any clinical signs and symptoms of polyneuropathy and cardiomyopathy. We used the term "late-onset" to specify that, even if pre-symptomatic, all carriers belonged to families where all affected members had their disease onset after 50 years. In details, we included in our study those subjects with normal neurological examination, a Neuropathy Impairment Scale (NIS) of 0-2 [15], and normal sural sensory nerve action potential (SNAP) amplitude. All subjects were routinely evaluated in our Centers by expert cardiologists to disclose any possible heart involvement. Subjects with other known cause or risk factor for peripheral neuropathy, such as diabetes, alcohol abuse, vitamin B12 deficiency, paraproteinemia, or hypothyroidism, were excluded from our study [16]. Each subject underwent clinical assessment, NCS, questionnaires, QST, and skin biopsy. Clinical assessment and NCS were carried out at our amyloidosis referral Centers prior to enrolment. Questionnaires, QST, and skin biopsy were performed at the peripheral neuropathy and neuropathic pain unit of the Department of Human Neuroscience at Sapienza University of Rome.

All data were collected on a structured form using standardized protocols by staff members (clinical examination and questionnaires: AT, GA, MS, MG, AL, MC; NCS and QST: ML, LF, SM, FF, NE, RC; skin biopsy collection and analysis: EG, PF, GDP).

This protocol was approved by Sapienza University Ethical Committee (UNTTR-19-1, protocol number 275 SA_2019 19/12/2019). Each enrolled subject provided a written informed consent, and

all study data were obtained and elaborated in accordance with our institutional ethical committee regulations.

Clinical examination and questionnaires

We performed a detailed neurological examination, using bedside tools. Patients were examined for negative (tactile, vibration, pinprick, and thermal hypoesthesia) and positive symptoms and signs (spontaneous pain, allodynia, and pinprick hyperalgesia). Touch sensitivity was assessed with a piece of cotton wool, vibration with a tuning fork (128 Hz), and pinprick sensation with a wooden cocktail stick, as recommended [17].

Neuropathy Impairment Scale (NIS) [15] was calculated for each participant. Neuropathic pain, autonomic disturbances, and small fiber related symptoms were evaluated through the *Douleur Neuropathique-4* (DN4) [18] and Small Fiber Neuropathy-Symptoms Inventory Questionnaire (SFN-SIQ) [19]. PADO was estimated as previously reported [4] and the difference between the age of pre-symptomatic carriers and PADO (delta-PADO) was consequently calculated.

Nerve conduction study

NCS was performed by surface recording electrodes with standard placement. SNAP amplitude and conduction velocity of sural, ulnar, median, and superficial radial nerves, and compound motor action potentials amplitude and motor conduction velocity of peroneal, tibial, ulnar, and median nerves were recorded. Recording methods adhered to the recommendations of the International Federation of Clinical Neurophysiology [20]. Skin temperature was maintained between 34° and 36°C. Data were compared with age-adjusted normative ranges [21]. Sural SNAP amplitude was considered as NCS main outcome variable.

Quantitative sensory testing

QST was performed on the subjects' feet by trained examiners following the standardized protocol of the German Research Network on Neuropathic Pain [22]. In detail, the following QST parameters were evaluated: cold and warm detection thresholds (CDT and WDT); cold and heat pain thresholds (CPT and HPT); mechanical detection and pain thresholds (MDT and MPT); and vibration detection threshold (VDT).

In each subject, a short demonstration of the different QST procedures in the radial nerve territory of the hand, as a practice area, was performed before the test. Using log-transformed raw values for each measured variable and a large, widely accepted dataset of normative values [23], a z-score was calculated for each QST variable ($z\text{-score} = \text{value of the patient} - \text{mean value of control subjects} / \text{standard deviation of control subjects}$) [23]. Negative z-scores indicated a loss of perception, whereas positive z-scores indicated a gain of perception. Values exceeding 0 ± 1.96 standard deviations (SDs) were considered abnormal.

CDT and WDT were considered as main QST small fibre related outcome variables; MDT and VDT were considered as main large fibre related outcome variables.

Skin biopsy

Skin specimens were obtained using a sterile 3 mm disposable punch after local lidocaine anaesthesia from the distal leg, 10 cm above the lateral malleolus, and the proximal thigh, 20 cm under the antero-superior iliac spine.

Using an indirect immunofluorescence staining protocol for protein gene product 9.5 (PGP 9.5) and collagen type IV, intraepidermal nerve fibre density (IENFD) was assessed on three randomly chosen 50 μm section for each subject, as previously reported [24].

Sections were examined through a fluorescence microscope (Leica NB) with appropriate wavelength filters. IENFD was calculated according to the guidelines of the European Federation of Neurological Societies and Peripheral Nerve Society [25]. Epidermal linear length was measured through Image-J

to obtain a linear density (number of fibres/mm). Normative values from an internationally recognized dataset were used [26]. Leg/thigh ratio, i.e., the ratio between distal and proximal IENFD, was used in subjects with reduced small fibre density to discriminate whether patients had a length or non-length dependent small fibre loss [27], with values ≤ 0.48 consistent with distal axonopathy and values >0.48 consistent with peripheral nerve damage without a distal gradient. Distal and proximal IENFD were considered as main outcome skin biopsy variables.

STATISTICAL METHODS

A preliminary univariate analysis was performed to describe the main demographic, clinical, and diagnostic test variables in ATTRv pre-symptomatic carriers. Since the normal distribution was rejected for all the considered continuous variables, as verified with the Kolmogorov-Smirnov test, quantitative variables were expressed as their median with inter-quartile range (IQR) and were compared by the non-parametric Mann-Whitney U test. Categorical variables were expressed as percentage frequencies and were compared using the Fisher's exact test. Correlations between variables were verified using the Spearman's correlation test. Simple linear regression was used to assess linear relations between selected correlated variables. No adjustment for multiple comparisons was adopted since all comparisons were pre-planned and our a priori intent was to test each variable independently. A P-value lower than 0.05 was considered significant. Analyses and graphics were performed with GraphPad Prism 9.5.1 statistical software.

RESULTS

Clinical and demographic variables.

Data from 40 pre-symptomatic late-onset ATTRv carriers from 24 ATTRv families (M/F 18/22; V30M/non-V30M 26/14; median age 49, IQR 42.3-59.5) were analysed. No evaluated subjects presented alternative causes of polyneuropathy, and all accepted to participate to the study. Subjects' demographic, clinical, and diagnostic test variables are described in Table 1. All non-V30M subjects carried the p.Phe84Leu (F64L) variant, with the exception of two subjects carrying the p.Ala140Ser (A120S) and p.Arg54Thr (R34T) variants.

Median PADO was 63 years (IQR 59-68.7), with significant difference between V30M and non-V30M subjects (60 vs 65 years, $p=0.01$). Median delta-PADO was 13.5 years (IQR 8.3-20.7), with comparable values between V30M and Non-V30M carriers.

Diagnostic test variables.

Out of 40 carriers, 20 (50%) had reduced distal IENFD. Of the 20 carriers with reduced distal IENFD, 15 also underwent a proximal biopsy and 11 (73%) showed a leg/thigh ratio greater than 0.48, suggesting a non-length dependent pattern of small fibre loss (Figure 1).

According to the z-score analysis of QST variables, CDT was altered in 9 carriers (23%), WDT in 6 (15%), with z-values exceeding -1.96 SD, indicating a loss of perception. Overall, CDT and/or WDT were altered in 11 carriers (27.5%). CPT z-values exceeded $+1.96$ SD in 3 subjects, indicating a gain of function with cold hyperalgesia; HPT z-values exceeded $+1.96$ SD in 2 subjects with heat hyperalgesia and -1.96 SD in 2 subjects with loss of function; MPT z-values exceeded $+1.96$ SD in 8 subjects, indicating mechanical pain hyperalgesia; MDT was impaired with loss of function in 5 carriers (13%), VDT in 1 (3%), with z-values exceeding -1.96 SD.

Seven carriers (17.5%) had both distal skin biopsy and CDT and/or WDT abnormalities, with a median delta-PADO of 11 yrs (IQR 2-14), and none of them reported significant symptoms at DN4 (0, IQR 0-1) and SFN-SIQ (3, IQR 1-4). Delta-PADO did not differ between subjects with and without skin biopsy and CDT/WDT abnormalities.

Stratifying subjects for a delta-PADO higher or lower than 10 years, we found no differences of distal IENFD reduction or QST alterations frequencies in the two groups (Figure 2). We found 2/23 subjects with combined IENFD/QST alterations in the > 10 years delta-PADO group vs 5/17 subjects in the < 10 years delta-PADO group (Figure 2). This higher prevalence of combined QST/IENFD alterations in subjects with lower delta-PADO did not reach statistical significance.

Correlations between the main diagnostic test outcome variables related to large (i.e., sural SNAP, MDT, VDT) and small nerve fibres (distal and proximal IENFD, CDT, WDT, MPT, HPT, CPT) are reported in Supplementary 1.

Delta-PADO positively correlated with sural SNAP ($r=0.416$, $p=0.004$) and z-values of CDT ($r=0.314$, $p=0.028$), WDT ($r=-0.294$, $p=0.034$) and MDT ($r=-0.382$, $p=0.012$) (Supplementary 1). Simple linear regression models showed a linear relation between delta-PADO and sural SNAP (R^2 0.1100, $p=0.0366$), CDT (R^2 0.1441, $p=0.0205$), and MDT (R^2 0.1183, $p=0.0431$) (Figure 3). No significant correlation was found between delta-PADO and HPT, CPT, MPT, and VDT.

DISCUSSION

In this multicentric prospective study, we evaluated NCS, QST, and skin biopsy parameters in a cohort of late-onset ATTRv pre-symptomatic carriers with heterogeneous *TTR* amyloidogenic variants from a non-endemic area, and analysed their relationships with predicted age of disease onset (PADO) [4].

We found that small fibre impairment, as detected by skin biopsy and/or QST thermal thresholds (CDT/WDT), the reference standard diagnostic tests for small fibre neuropathy diagnosis [5], was a frequent finding in ATTRv pre-symptomatic carriers. In detail, half of the examined subjects (50%) had IENFD reduction at skin biopsy from a distal site. Our results are consistent with previous skin biopsy studies [7-9], which showed that pre-symptomatic carriers of *TTR* mutations had intermediate IENFD reductions compared to ATTRv-PN patients, who were more severely denervated. In contrast to our findings, a previous study reported skin biopsy alterations almost in the totality of pre-symptomatic carriers [14], possibly reflecting differences in study populations in terms of genetic heterogeneity and skin biopsy timing.

Our study reported a lower frequency of QST respect to skin biopsy abnormalities. Overall, 27.5% of mutation carriers had small fibre related QST thermal abnormalities, with CDT and/or WDT impairment. Few studies have investigated QST in pre-symptomatic carriers [12, 28, 29], reporting heterogeneous frequencies of alteration in small fibre related QST parameters, possibly reflecting peculiar genotypic and phenotypic background of the different study populations.

Our QST findings showed a concomitant functional involvement of small myelinated A δ fibers, as assessed by CDT, CPT, and MPT, and of non-myelinated C fibers, as assessed by WDT and HPT, thus suggesting that both small fibers subsets are involved at pre-symptomatic early stages in ATTRv-PN. Consistently with a previous study, showing that CPT was the most frequently abnormal sensory modality in pre-symptomatic carriers [12], we found a slightly higher prevalence of QST A δ fibers related abnormalities, as shown by the higher frequency of CDT and MPT alterations. Interestingly, a small number of patients also showed large-myelinated fibers related QST abnormalities, with MDT impairment, thus suggesting a subclinical early involvement of A β fibers, which goes unnoticed to standard NCS.

Intriguingly, 17.5% of pre-symptomatic carriers in our study showed both QST (CDT and/or WDT) and skin biopsy alterations, thus fulfilling criteria for small fibre neuropathy diagnosis according to a widely recognized reference standard [5]. Combined QST/IENFD alterations seem to have a higher prevalence in those subjects with lower (<10 years) delta-PADO (29% vs 9%) (Figure 2), but with no statistical significance, possibly due to the small sample number. Interestingly, all the lower delta-PADO pre-symptomatic carriers with any QST alterations also showed a reduced distal IENFD (Figure

2). According to recent consensus on ATTRv pre-symptomatic carriers' follow-up [4], disease onset may be confirmed by two altered diagnostic tests in absence of clinical symptoms or signs, opening the possibility of a very early initiation of disease modifying therapies in these subjects. However, criteria for small fibre neuropathy diagnosis should be applied in patients, based on a clinical suspicion driven by symptoms or signs compatible with small fibre impairment [5]. Since our carriers were completely asymptomatic, further longitudinal studies are needed to better assess the meaning of these subclinical alterations of QST and skin biopsy in pre-symptomatic mutation carriers, and to establish whether they represent the onset of a small fibre neuropathy with a significant impact on the disease course.

Coherently with our previous smaller report [11], most of the subjects (73%) showing distal IENFD reduction had a leg/thigh ratio greater than 0.48, suggesting a non-length dependent pattern of small fibre damage [27]. Similarly, neuropathological, and neuro-imaging studies found a proximal involvement in ATTRv-PN [30-33]. These findings contradict the strict length-dependent neuropathy pattern found in a large French population of mixed late and early-onset ATTRv subjects, possibly reflecting profound genotype/phenotype differences in the studied populations, [14].

In our cohort of pre-symptomatic carriers, delta-PADO, i.e., the difference between the age of pre-symptomatic carriers and PADO, significantly correlated with the outcome measures of functional tests assessing both large (sural SNAP and MDT) and small fibers (CDT and WDT), showing a linear relation with sural SNAP amplitude, MDT, and CDT. Namely, NCS and QST parameters, i.e., sural SNAP, MDT, and CDT significantly deteriorated as the PADO approached. Consistently with QST findings of our study, a longitudinal study in a small sample of five ATTRv pre-symptomatic carriers recently showed that QST parameters significantly vary along time [28]. Similarly, the significant linear correlation we found between delta-PADO and sural SNAP amplitude is coherent with previous longitudinal studies reporting NCS changes in pre-symptomatic carriers [34]. Conversely, although we reported a high prevalence of skin biopsy abnormalities in our study population, we did not find any significant correlation between delta-PADO and IENFD, the main skin biopsy variable related to small-fibre damage.

Our findings suggest that sensory threshold assessment by QST, in particular CDT and MDT evaluation, may be a valuable tool for monitoring nerve function in pre-symptomatic carriers, and that serial combined QST and NCS evaluation could be useful in ATTRv pre-symptomatic carriers' follow-up, also given their non-invasiveness, cost-effectiveness, and easy repeatability over time. The lack of a clear correlation between IENFD and delta-PADO suggests that skin biopsy may not be an optimal tool to follow up pre-symptomatic carriers. Though skin biopsy is widely recognized as the gold standard test for small fibers morphometric assessment and is a useful tool to detect small fibre damage in TTR mutations carriers, the high frequency of early skin biopsy abnormalities in ATTRv pre-symptomatic carriers could affect its efficacy for monitoring nerve function deterioration over time due to a ceiling effect. On the other hand, we cannot exclude that skin biopsy parameters other than IENFD, like morphological abnormalities or autonomic innervation variables, could better correlate with delta-PADO and be more suitable to follow up the neurodegenerative process. Having said that, our results suggest that QST/IENFD combined analysis is able to detect those pre-symptomatic ATTRv carriers with sub-clinical combined small nerve fibre depletion/dysfunction, with implications in the follow-up schedule of this ATTRv population subset and, potentially, earlier disease detection and disease-modifying treatments initiation.

Limitations:

The cross-sectional design could be seen as a potential limitation of our study. PADO is a fast and practical parameter for clinical use and is currently the only available clinical measure to predict disease onset, providing a guide for ATTRv pre-symptomatic carriers monitoring schedule [4]. PADO depends on the specific TTR mutation, its typical age of onset, and the age of onset in other family

members. However, genetic anticipation has been reported in some TTR mutations and significant variability in age of onset exists within families, thus showing how complex it is to precisely predict disease age onset [35].

Hence, longitudinal studies are warranted to assess the evolution of NCS, QST, and skin biopsy parameters along time in ATTRv carriers and patients, thus contributing to better investigate optimal disease biomarkers.

ACKNOWLEDGMENT: ALNYLAM Pharmaceuticals Inc. provided financial support for the present study as an Investigator-Initiated Research study to LL and GA. Moreover, this work has been supported by the Italian Ministry of Health Young Researcher Project Grant (GR-2021-12372306 to AR, GP, and LL) as a part of a wider project on biomarker research in ATTRv.

CONFLICTS OF INTEREST: GA, LL and SM received speaker honoraria from SOBI and ALNYLAM and travel grants from SOBI, ALNYLAM and Akcea therapeutics. MC received travel grants and speaker honoraria from ALNYLAM. ML speaker honoraria, travel grants and consulting fees from SOBI, ALNYLAM and PFIZER.

DATA AVAILABILITY STATEMENT: Original supporting data are electronically stored at our Institution (NESMOS, Sapienza University of Rome) and are available on request. Please write an e-mail to the corresponding author (LL) to have full access to the original data.

REFERENCES

1. Adams, D., et al., *Hereditary transthyretin amyloidosis: a model of medical progress for a fatal disease*. Nat Rev Neurol, 2019. **15**(7): p. 387-404.
2. Adams, D., et al., *Rapid progression of familial amyloidotic polyneuropathy: a multinational natural history study*. Neurology, 2015. **85**(8): p. 675-82.
3. Ioannou, A., M. Fontana, and J.D. Gillmore, *RNA Targeting and Gene Editing Strategies for Transthyretin Amyloidosis*. BioDrugs, 2023. **37**(2): p. 127-142.
4. Conceição, I., et al., *Early diagnosis of ATTR amyloidosis through targeted follow-up of identified carriers of TTR gene mutations*. Amyloid, 2019. **26**(1): p. 3-9.
5. Devigili, G., et al., *Diagnostic criteria for small fibre neuropathy in clinical practice and research*. Brain, 2019. **142**(12): p. 3728-3736.
6. Yang, N.C., et al., *Clinical presentations and skin denervation in amyloid neuropathy due to transthyretin Ala97Ser*. Neurology, 2010. **75**(6): p. 532-8.
7. Masuda, T., et al., *Early skin denervation in hereditary and iatrogenic transthyretin amyloid neuropathy*. Neurology, 2017. **88**(23): p. 2192-2197.
8. Ebenezer, G.J., et al., *Cutaneous nerve biomarkers in transthyretin familial amyloid polyneuropathy*. Ann Neurol, 2017. **82**(1): p. 44-56.
9. Chao, C.C., et al., *Skin nerve pathology: Biomarkers of premanifest and manifest amyloid neuropathy*. Ann Neurol, 2019. **85**(4): p. 560-573.
10. Freeman, R., et al., *Cutaneous amyloid is a biomarker in early ATTRv neuropathy and progresses across disease stages*. Ann Clin Transl Neurol, 2022. **9**(9): p. 1370-1383.
11. Leonardi, L., et al., *Skin biopsy and quantitative sensory assessment in an Italian cohort of ATTRv patients with polyneuropathy and asymptomatic carriers: possible evidence of early non-length dependent denervation*. Neurol Sci, 2022. **43**(2): p. 1359-1364.
12. Tozza, S., et al., *Quantitative Sensory Testing in Late-Onset ATTRv Presymptomatic Subjects: A Single Center Experience*. Biomedicines, 2022. **10**(11).
13. Conceicao, I., et al., *Quantitative sensory testing: a good tool to identify subclinical neuropathy in ATTRV30M amyloidosis patients?* Amyloid, 2022: p. 1-5.

- Accepted Article
14. Leonardi, L., et al., *Skin amyloid deposits and nerve fiber loss as markers of neuropathy onset and progression in hereditary transthyretin amyloidosis*. Eur J Neurol, 2022. **29**(5): p. 1477-1487.
 15. Quan, D., et al., *Impact of baseline polyneuropathy severity on patisiran treatment outcomes in the APOLLO trial*. Amyloid, 2023. **30**(1): p. 49-58.
 16. England, J.D., et al., *Practice parameter: the evaluation of distal symmetric polyneuropathy: the role of laboratory and genetic testing (an evidence-based review)*. Report of the American Academy of Neurology, the American Association of Neuromuscular and Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation. Pm r, 2009. **1**(1): p. 5-13.
 17. Cruccu, G., et al., *EFNS guidelines on neuropathic pain assessment: revised 2009*. Eur J Neurol, 2010. **17**(8): p. 1010-8.
 18. Spallone, V., et al., *Validation of DN4 as a screening tool for neuropathic pain in painful diabetic polyneuropathy*. Diabet Med, 2012. **29**(5): p. 578-85.
 19. Galosi, E., et al., *The diagnostic accuracy of the small fiber neuropathy symptoms inventory questionnaire (SFN-SIQ) for identifying pure small fiber neuropathy*. J Peripher Nerv Syst, 2022. **27**(4): p. 283-290.
 20. Stålberg, E., et al., *Standards for quantification of EMG and neurography*. Clin Neurophysiol, 2019. **130**(9): p. 1688-1729.
 21. Chen, S., et al., *Electrodiagnostic reference values for upper and lower limb nerve conduction studies in adult populations*. Muscle Nerve, 2016. **54**(3): p. 371-7.
 22. Rolke, R., et al., *Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): standardized protocol and reference values*. Pain, 2006. **123**(3): p. 231-43.
 23. Magerl, W., et al., *Reference data for quantitative sensory testing (QST): refined stratification for age and a novel method for statistical comparison of group data*. Pain, 2010. **151**(3): p. 598-605.
 24. Galosi, E., et al., *Functional and morphometric assessment of small-fibre damage in late-onset hereditary transthyretin amyloidosis with polyneuropathy: the controversial relation between small-fibre-related symptoms and diagnostic test findings*. Amyloid, 2022: p. 1-8.
 25. Lauria, G., et al., *European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society*. Eur J Neurol, 2010. **17**(7): p. 903-12, e44-9.
 26. Provitera, V., et al., *A multi-center, multinational age- and gender-adjusted normative dataset for immunofluorescent intraepidermal nerve fiber density at the distal leg*. Eur J Neurol, 2016. **23**(2): p. 333-8.
 27. Provitera, V., et al., *The role of skin biopsy in differentiating small-fiber neuropathy from ganglionopathy*. Eur J Neurol, 2018. **25**(6): p. 848-853.
 28. Bekircan-Kurt, C.E., et al., *The functional and structural evaluation of small fibers in asymptomatic carriers of TTR p.Val50Met (Val30Met) mutation*. Neuromuscul Disord, 2022. **32**(1): p. 50-56.
 29. Papagianni, A., et al., *Clinical and apparative investigation of large and small nerve fiber impairment in mixed cohort of ATTR-amyloidosis: impact on patient management and new insights in wild-type*. Amyloid, 2021: p. 1-9.
 30. Hanyu, N., et al., *Peripheral nerve pathological findings in familial amyloid polyneuropathy: a correlative study of proximal sciatic nerve and sural nerve lesions*. Ann Neurol, 1989. **25**(4): p. 340-50.
 31. Granata, G., et al., *Ultrasound evaluation in transthyretin-related amyloid neuropathy*. Muscle Nerve, 2014. **50**(3): p. 372-6.

32. Podnar, S., et al., *Peripheral nerve ultrasonography in patients with transthyretin amyloidosis*. Clin Neurophysiol, 2017. **128**(4): p. 505-511.
33. Leonardi, L., et al., *High-resolution ultrasound of peripheral nerves in late-onset hereditary transthyretin amyloidosis with polyneuropathy: similarities and differences with CIDP*. Neurol Sci, 2022. **43**(5): p. 3387-3394.
34. Castro, J., et al., *Changes in nerve conduction studies predate clinical symptoms onset in early onset Val30Met hereditary ATTR amyloidosis*. Eur J Neurol, 2022. **29**(3): p. 826-832.
35. Grandis, M., et al., *Recommendations for pre-symptomatic genetic testing for hereditary transthyretin amyloidosis in the era of effective therapy: a multicenter Italian consensus*. Orphanet J Rare Dis, 2020. **15**(1): p. 348.

Table 1. Demographic, clinical, and main diagnostic test variables in ATTRv pre-symptomatic carriers

ATTRv carriers n=40		
<i>Continuous variables</i>	<i>median (IQR)</i>	<i>medium z-score (SD)</i>
Age, years	49.0 (42.3-59.5)	
PADO, years	63.0 (59-68.7)	
Delta-PADO, years	13.5 (8.3-20.7)	
DN4	0.0 (0.0-1.0)	
SFN-SIQ	3.0 (1.0-4.0)	

Sural SNAP amplitude (μ V)	15.0 (11.0-19.1)	
Distal IENFD, n fibres/mm	10.7 (6.9-14.1)	
Proximal IENFD, n fibres/mm	14.1 (11.7-19.0)	
Leg/thigh ratio	0.7 (0.6-0.9)	
CDT (32-0 °C, z-score)	26.9 (23.6-28.8)	-1.090 (1.119)
WDT (32-50 °C, z-score)	38.1 (36.1-42.6)	-0.418 (1.223)
CPT (32-0 °C, z-score)	10.5 (0.6-19.0)	0.071 (1.113)
HPT (32-50 °C, z-score)	45.8 (43.2-48.9)	0.028 (1.373)
MPT (0-512 mN, z-score)	20.7 (10.2-49.4)	1.331 (1.169)
MDT (0-512 mN, z-score)	4.0 (1.2-9.2)	-0.509 (1.455)
VDT (0-8, z-score)	8.0 (6.3-8.0)	0.040 (1.236)
<i>Categorical variables</i>		<i>n (%)</i>
Female gender	18 (45%)	
TTR mutation, n of val-30-met	26 (65%)	
CTS	15 (38%)	
Distal IENFD reduction	18 (45%)	
CDT and/or WDT impairment	10 (25%)	
QST and IENFD abnormalities	8 (20%)	

Table 1. Demographic, clinical, and main diagnostic test variables in ATTRv pre-symptomatic carriers. Continuous variables are expressed as median with interquartile range (IQR); categorical variables are expressed as number of patients presenting the selected variable (n) and relative percentages (%). PADO: Predicted age of disease onset; Delta-PADO: difference between PADO and subjects' age; DN4: Douleur neuropathique en 4 questions questionnaire (0-10); SFN-SIQ: Small fibre neuropathy symptoms inventory questionnaire (0-39). Sural SNAP: sural sensory nerve action potential; Distal and proximal IENFD: intraepidermal nerve fibre density of PGP9.5 immunoreactive fibres from the distal and proximal site; leg/thigh ratio: ratio between distal and proximal IENFD, as calculated in subjects with distal IENFD reduction; CDT: cold detection threshold; WDT: warm detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MPT: mechanical pain threshold; MDT: mechanical detection threshold; VDT: vibration detection threshold; TTR: transthyretin; CTS: carpal tunnel syndrome.

Figure 1. Exemplificative skin biopsy images from a distal and a proximal site from two ATTRv pre-symptomatic carriers with normal and reduced intraepidermal nerve fibre density (IENFD). The subject with altered IENFD shows both distal and proximal IENFD reduction. PGP 9.5 immunoreactive fibers are marked in red, Collagen IV immunoreactive structures in green. Calibration bars: 200 μ .

Figure 2. Graphical representation of intraepidermal nerve fibre density (IENFD) and quantitative sensory testing (QST) alterations frequencies in ATTRv pre-symptomatic subjects stratified for distance from predicted age of disease onset (PADO).

Figure 3. Simple linear regression models assessing linear relations between Delta-PADO and Sural SNAP, CDT, and MDT. Delta-PADO: difference between PADO and subjects' age; Sural SNAP: sural sensory nerve action potential; CDT: cold detection threshold; MDT: mechanical detection threshold.

Supplementary 1. Correlation matrix showing Spearman's r coefficients for the main diagnostic test variables. PADO: Predicted age of disease onset; Delta-PADO: difference between PADO and subjects' age; Sural SNAP: sural sensory nerve action potential; Distal and proximal IENFD: intraepidermal nerve fibre density from the distal and proximal site; CDT: cold detection threshold; WDT: warm detection threshold; CPT: cold pain threshold; HPT: heat pain threshold; MPT: mechanical pain threshold; MDT: mechanical detection threshold; VDT: vibration detection threshold.

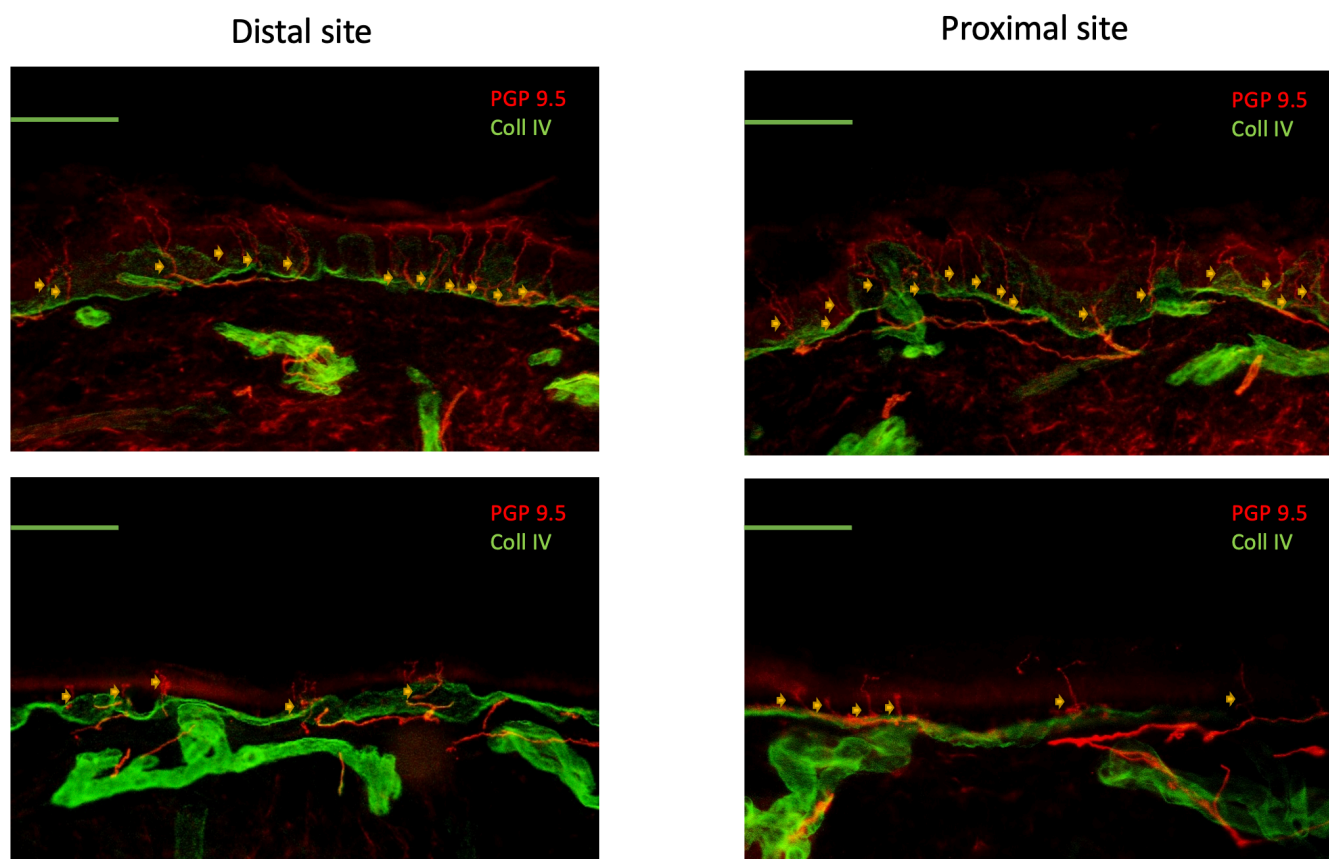


fig1_600dpi.tiff

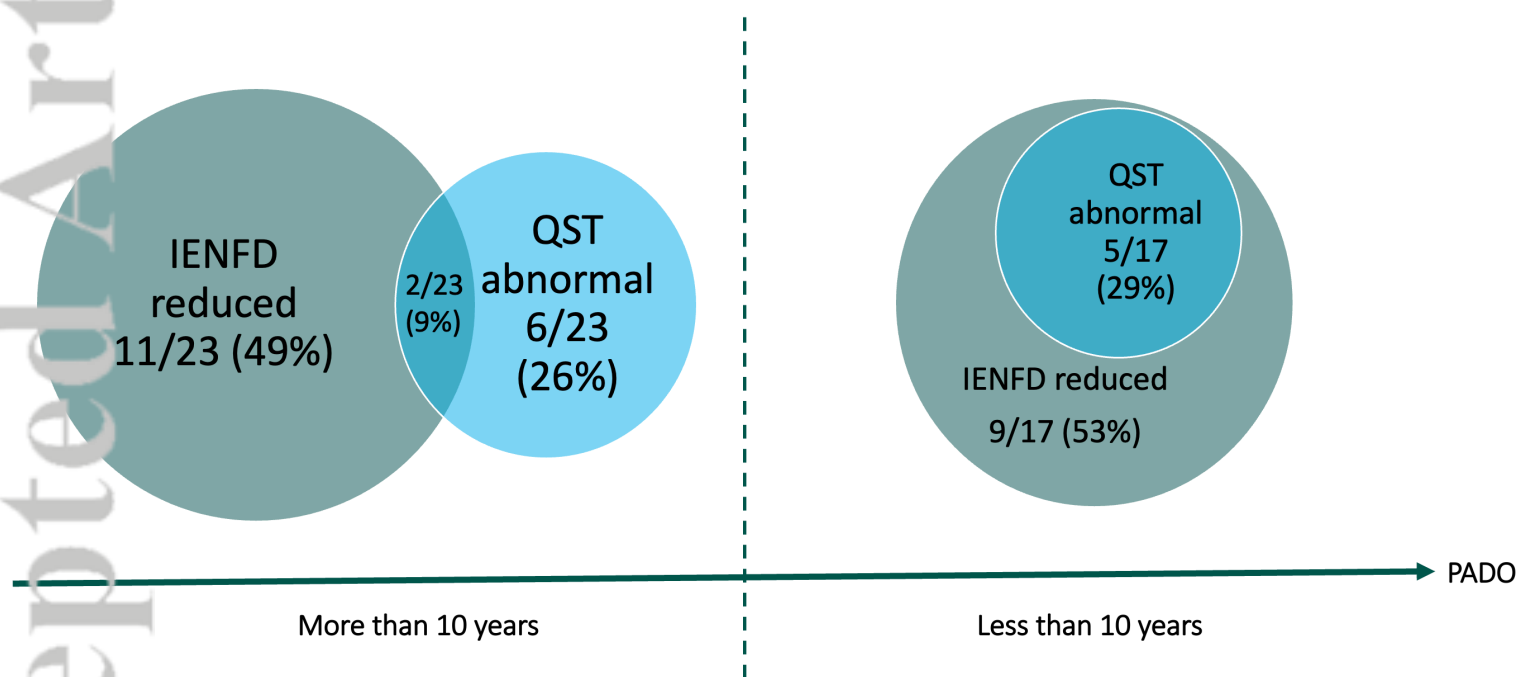
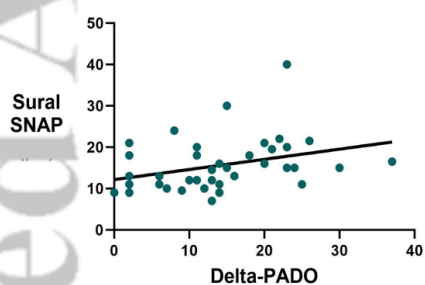
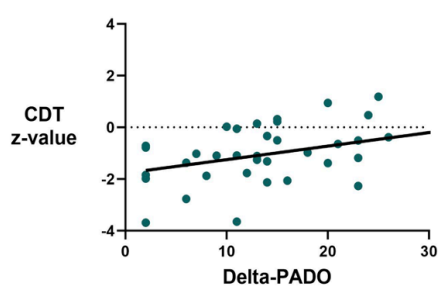


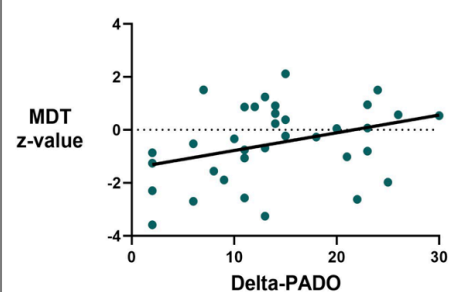
fig2_600dpi.tiff



R^2 0.1100, $p=0.0366$



R^2 0.1441, $p=0.0205$



R^2 0.1183, $p=0.0431$

Fig3_600DPIdef.tiff