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Genetic screening and instrumental biomarkers in ATTRv amyloidosis: a focus on molecular diagnosis and treatment response to RNA silencers through Neurophysiology, Bioelectrical Impedance Analysis and Handgrip strength

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TABLE OF CONTENTS

To Sicilian patients affected by rare disease and their families

Introduction

Hereditary transthyretin amyloidosis with polyneuropathy (ATTRv) is an adult-onset, rare and multisystemic disease, affecting the sensorimotor and autonomic functions along with other organs, especially the heart, the gastrointestinal tract, eyes, and kidney(1). ATTRv is caused by the accumulation of abnormal amyloid fibrils originating from mutations in the *TTR* gene; TTR mutations display incomplete penetrance and present an autosomal dominant pattern of inheritance(2,3). Clinical phenotype is heterogeneous and often unpredictable therefore the diagnosis is very difficult and, in most cases, delayed. In most cases, ATTRv is a devastating disease with a lethal outcome in a period of 2–15 years after the clinical onset in the absence of treatment, but recent new drugs for ATTRv are very promising and they might change the survival in patients with polyneuropathy. Consequently, misdiagnosis of ATTRv carries high costs for the community in terms of mortality and inappropriate treatments(4), although several treatment options available are particularly effective in treating early disease stages(5). In the recent past, the diagnosis of ATTRv required genetic testing performed in the presence of a strong clinical suspicion and a positive biopsy(4,6). However, the role of biopsy has become questioned due to the broad availability of genetic testing(7,8). Indeed, more recent diagnostic

algorithms suggest anticipating and often replacing the biopsy in the diagnostic workup(1). Consequently, based on published literature and expert opinions, symptom clusters and specific "red flags" have been recently proposed to facilitate an earlier diagnosis(9,10). However, there is still a need for new strategies to find undiagnosed individuals and implement existing evidence-based guidelines to improve ATTRv care. The last evidence suggests that ATTRv should be suspected if progressive peripheral sensory-motor neuropathy is observed in combination with one or more of the following conditions: autonomic dysfunction (erectile dysfunction, orthostatic hypotension, syncope), cardiomyopathy, gastrointestinal symptoms, unexplained weight loss, bilateral carpal tunnel syndrome, lumbar canal stenosis, renal impairment, ocular involvement (vitreous opacities) and/or family history of polyneuropathy, cardiomyopathy or ATTRv (9,11). Finally, because ATTRv is an autosomal dominant genetic condition, screening at-risk relatives of individuals with ATTRv (*cascade* testing) is highly effective in identifying additional individuals who require treatment(10).

The second issue explored in this project is the need for biomarkers of disease activity and response to treatment in ATTRv. Patisiran, a small interfering RNA acting as TTR silencer approved in Italy in 2020, has been shown to stabilize the course of polyneuropathy in ATTRv, but its administration is bound to the presence of

neuropathy as a manifestation(12). Consequently, a meticulous clinical assessment is essential. Of interest, muscle strength can be measured through handgrip tools, which evaluate the force that a person is able to produce grasping an object. Handgrip tools are easy and cost-effective providing a quantitative measure of distal strength. Apart from a progressive sensory-motor neuropathy and cardiomyopathy, autonomic dysfunction, gastrointestinal problems, and unexplained weight loss are relevant symptoms of ATTRv, but they are self-reported symptoms difficult to demonstrate and consequently often underestimated. Some questionnaires are commonly used in clinical practice, but an instrumental evaluation of dysautonomia through neurophysiological instruments is not systematically performed. Bioelectrical Impedance Analysis (BIA) is a very sensitive tool to examine the composition of the body tissues in polyneuropathies and conditions with dysautonomia. Hence, BIA might accurately estimate the body composition in terms of muscle and fat masses as well as water contents in ATTRv patients. To our knowledge, there are no studies assessing body composition through bioimpedance analysis in ATTRv patients. Taken together, HGS and BIA might have a potential in the assessment of the severity of the disease and the beneficial effects of treatments. Of note, the use of instrumental biomarkers for the evaluation of tissue damage in ATTRv might lead to a higher sensitive and specific approach; moreover, these biomarkers might

contribute to detect the exact clinical onset of the disease in carriers of TTR mutation giving them the opportunity to be early treated when the polyneuropathy starts. In this project, we aim to develop strategies for genetic screening in ATTRv and explore instrumental biomarkers for disease activity and monitoring response to treatment with RNA silencers.

Chapter I. Transthyretin: the role in physiology and disease

Transthyretin (TTR) is a 55 kDa homotetrameric protein composed of 127-residue β-sheet-rich subunits that is produced in the liver and it is found mainly in the serum, cerebrospinal fluid, and acqueus fluid(13). Almost all TTR is synthesized and secreted by the liver, while only a small contribution on TTR production depends on the choroid plexus and the retinal pigment epithelium. The main function of TTR is the transport of thyroxine (T4) and retinol (vitamin A)-binding protein complexes in the serum and cerebrospinal fluid.

Amyloidosis is a gain-of-toxic function protein-misfolding disease in which amyloidogenic TTR assembles into amyloid fibrils in extracellular spaces, leading to organ dysfunction(14).

The presence of amyloid deposits in tissues is usually confirmed when a typical green- yellow-orange birefringence is found in cross-polarized light in Congo red-stained sections (Figure IA)(15). However, it is important always to stress the origin of the β-fibrils in order to avoid misunderstanding. Given the broad use of the word "amyloid" in human pathology, several classes of amyloid fibrils may be distinguished(15). For the medical in vivo situation, and to be included in the amyloid nomenclature list, "amyloid" still means mainly extracellular tissue deposits of protein fibrils, recognized by specific properties, such as

green-yellow birefringence after staining with Congo red(15). It should also be underlined that amyloid fibril in vivo contain associated compounds, particularly serum amyloid P-component (SAP) and proteoglycans, mainly heparan sulfate proteoglycan in addition to the main protein (i.e., TTR). With this definition there are presently 36 human amyloid proteins of which 14 appear only associated with systemic amyloidosis and 19 as localized forms. Three proteins can occur both as localized and systemic amyloidosis.

Amyloid transthyretin amyloidosis (ATTR)

Amyloid transthyretin amyloidosis (ATTR) is a progressive and systemic disease. The nomenclature committee of the Internal Society of Amyloidosis has defined two major forms of ATTR amyloidosis: wild-type amyloidosis designed "ATTRwt" amyloidosis and hereditary ATTR amyloidosis named "ATTRv" (v for variant)(15). The first form is also known as "senile" systemic amyloidosis, while the second is known as "familial amyloid polyneuropathy" (FAP). A third form of ATTR amyloidosis has been reported in patients who received livers from ATTRv patients, which is also known as "acquired" ATTR amyloidosis after domino liver transplantation.

ATTRwt amyloidosis

ATTRwt amyloidosis has classically been regarded as a

cardiomyopathy found in the elderly, whereas carpal tunnel syndrome has also been recognized as a major manifestation of the disease(6,16). Also, other studies have also suggested an association between spinal canal stenosis and wild-type TTR deposition in ligaments(17).

ATTRv amyloidosis

ATTRv amyloidosis is characterized by polyneuropathy, cardiomyopathy, oculoleptomeningeal involvement, but the clinical phenotype is heterogeneous and depends on the specific mutation(5). In addition to variant TTR, the deposition of wild-type TTR plays a significant role even in ATTRv amyloidosis, particularly in patients with late-onset Val30Met ATTRv(18).

ATTR amyloidosis after domino liver transplantation

Liver of patients with ATTRv is usually not compromised, hence, it might be transplanted into a patient with severe liver disease because of the shortage of donor livers. As a result, there is possibility of amyloid deposition in recipients of livers from ATTRv patients(14). This condition is also known as ATTR amyloidosis after domino liver transplantation. The main duration between domino liver transplantation and the first detection of ATTR symptoms is about 8 years. However, recipients complain only sensory but not autonomic symptoms similarly to late-onset forms of ATTRv (14).

In recent years the number of newly diagnosed patients has significantly

increased leading to an expansion of the clinical spectrum of ATTR amyloidosis(5). This is a consequence of increased awareness and progress in diagnostic techniques for this disease. Furthermore, the new insight in the pathophysiology of ATTR has carried out the discovery of disease-modifying therapies such as TTR stabilizers and gene-silencing agents.

Pathophysiology: misfolding of TTR, aggregation of amyloid fibrils, and tissue damage

TTR is stable in its homotetrameric form, but the dissociation of tetramers into monomers causes misfolding of TTR, resulting in aggregation of amyloid fibrils(19). Amyloidogenic mutations in the *TTR* gene result in the production of variant TTR that is more instable compared to wild-type, even if dissociation and subsequent aggregation might occur also in wild-type TTR. Indeed, deposits of wild-type TTR are found in the heart of almost half of patients with Val30Met ATTRv(20–22). Also, in ATTRv patients treated with liver transplantation cardiac amyloidosis may develop as a result of wild-type TTR deposition, particularly in elderly males(23,24). Indeed, apart from the full-length TTR, C-terminal fragments starting at about amino acid position 50 are also found in fibrils from patients with ATTRwt and late-onset ATTRv(22). These fragments suggest that cleavage of TTR might destabilize native tetrameric structures, accelerating misfolding.

The chronological sequence of how amyloid fibrils are formed is still poorly understood(14). Some studies employing electron microscopy suggest that deposition of nonfibrillar TTR might occur before amyloid fibril formation(25). Studies in cardiac deposits suggested that TTR aggregation into fibrillar structures tends to occur in association with basement membrane because of the expression of some components (i.e., laminin, fibronectin)(20). Evidence from nerve biopsy in ATTRv proved a thickening or reduplication of basement membrane surrounding endoneurial vessels, which was considered a unique feature of diabetic neuropathy in relation to the accumulation of advanced glycation end products. Of interest, the impact of amyloid deposition in neighboring tissues differs depending on the exact morphology of amyloid fibrils(20,22). In early-onset forms amyloid deposits present long and thick fibers with a good affinity to Congo red accompanied by strong birefringence under polarized light (Figure IA). Conversely, amyloid fibrils are shorter and finer in late-onset ATTRv with weak affinity to Congo red, resulting in weak birefringence under polarized light (Figure IA). Hence, it seems that large fibrils might cause distortion of neighboring tissues, particularly those associated with small-diameter nerve fibers. For example, in the heart large fibrils might invade and cause degeneration and atrophy of myocardial cells inducing cardiac conduction abnormalities. By contrast, small fibrils seem to have a minor influence in neighboring tissues. In the heart short fibrils might have a less conspicuous effect until massive amyloid

11

deposition cause diastolic dysfunction.

Finally, apart from direct damage induced by amyloid fibrils to surrounding tissues, recent evidence has pointed out the role of nonfibrillar TTR(25). On this perspective, TTR oligomers may participate in the process of neurodegeneration and damage to tissues explaining why such abnormalities (i.e., disruption of blood-nerve barrier, swelling of the nerve trunk, etc.) might occur before the formation of amyloid fibrils.
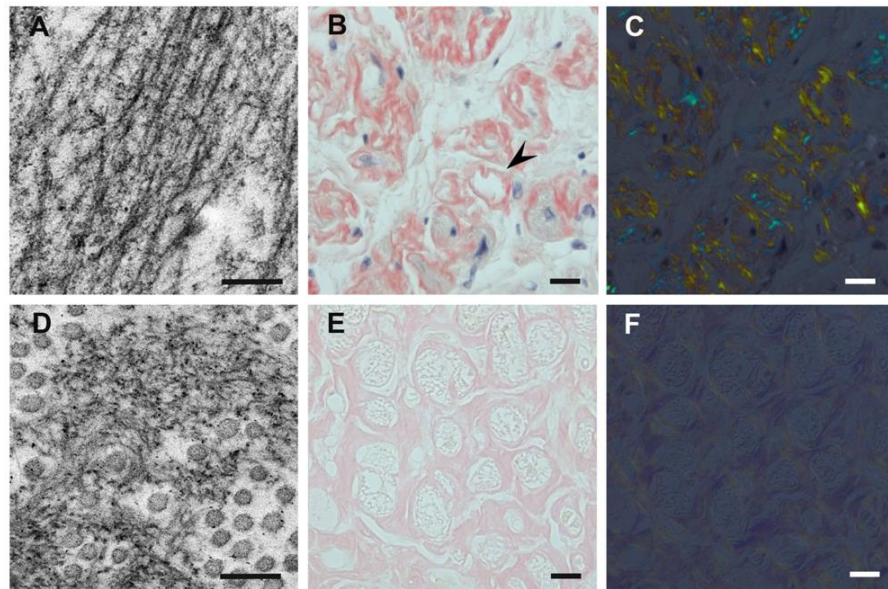

Figure IA. Differential characteristics of amyloid deposits between patients with conventional early-onset Val30Met ATTR amyloidosis from endemic foci (a–c) and patients with late-onset Val30Met ATTR amyloidosis from nonendemic areas (d–f). Biopsy specimens of the sural nerve (a, d) and autopsy specimens of the heart (b, c, e, f). Uranyl acetate and lead citrate staining specimens (a, d). Alkaline Congo red staining specimens (b, c, e, f). In early-onset patients from endemic foci, amyloid fibrils tend to be long and thick on electron microscopy (a). On light microscopy, amyloid deposits tend to be highly congophilic
(b) and exhibit a strong apple-green birefringence (c) in early-onset patients from endemic foci. Atrophy and degeneration of myocardial cells result in the formation of amyloid rings (arrowhead). In late-onset

patients from nonendemic areas, amyloid fibrils are generally short and thin on electron microscopy (d). Circular structures with a diameter of 50–70nm are collagen fibers. On light microscopy, amyloid deposits are generally weakly congophilic (e) and exhibit a faint apple-green birefringence (f) in late-onset patients from nonendemic areas. Scale bars 0.2μm (a, d) and 10μm (b, d, e, f). From Koike et al, 2020(14)

Chapter II. Hereditary transthyretin amyloidosis (ATTRv)

Hereditary transthyretin amyloidosis with polyneuropathy (ATTRv) is caused by point changes in the *TTR* gene, located on chromosome 18 (18q12.1)(26). Single amino-acid mutations in the *TTR* gene determine a deposition of mutant TTR amyloid which is toxic for several tissue and organs. This rare condition was reported for the first time by Andrade in 1952 in Portugal in a large group of patients with ATTRv with Val30Met mutation (p.Val50Met, according to the Human Genome Variation Society recommendation) (5,27). Then other larger foci have been discovered in Japan by Araki and in Sweden by Andersson. Hence, until 30 years ago the disease was considered extremely rare and confined to some peculiar endemic foci(28). However, the most recent past had taught that ATTR is diffuse worldwide. For many years ATTR was an intractable disease with only symptomatic therapies available. In the 21st century, effective disease modifying therapies have been developed such as stabilizers of tetrameric form of TTR and gene-silencers(29,30). As a result, life span of ATTR patients was significantly prolonged in the last years(31).

ATTRv is an adult-onset multisystemic disease, affecting the sensory-motor and autonomic functions along with other organs, especially

heart, gastrointestinal tract, eyes, and kidney(32). ATTRv has an autosomal dominant pattern of inheritance and worldwide its global prevalence is estimated at up to 38000 persons(2,3). It is encoded by the *TTR gene* located in the 18th chromosome. The TTR protein forms tetramers constituted by monomers rich in beta sheet structure(33). The presence of missense mutations conducts to a less stable tetramer by altering the amino acid sequence, thus favoring its dissociation. The misfolded monomers aggregates generating amyloid fibrils, which precipitate into tissues(25,33). In other words, genetic mutations determine a conformational change of the protein leading to the formation of amyloid fibrils.

Clinical presentations of ATTRv

The most frequent TTR mutation is Val30Met with an early onset (<50 years) in endemic areas, whereas a late onset (>50 years) phenotype is prevalent in non-endemic areas(34–36). In peripheral nerves, amyloid fibrils cause a rapidly progressive peripheral sensory-motor polyneuropathy with significant disability, while in the heart they generate a cardiomyopathy which may influence the progression of the disease and survival of ATTRv patients(37).

Typical presentation of ATTRv is a progressive length-dependent sensory-motor polyneuropathy, which usually begins with loss of thermal and pain sensation in the feet and slowly ascends up the limbs(6). Another type of clinical appearance starts with focal deficits

resulting from local deposits of amyloid(38,39). Both are associated with variable autonomic disturbances and extra-neurological manifestations, especially a cardiomyopathy. Some patients with an early-onset presentation deteriorate quickly because of autonomic dysfunction and rapid progression of the sensory-motor deficit. Conversely, in many patients with a late onset (6th to 8th decade), the polyneuropathy progresses slowly, often with a cardiac involvement but with less autonomic dysfunction. Furthermore, as TTR can be synthetized by the choroid plexus and retinal pigment epithelium, the occurrence of oculoleptomeningeal amyloidosis has been also reported(40).

Diagnosis is based on family history, neurophysiological evidence of a prevalent axonal polyneuropathy, identification of amyloid deposits in the tissues, and detection of TTR mutation. The diagnosis can be challenging in sporadic cases and when clinical manifestations are not typical(6,41). Diagnostic pitfalls include inadequate attention of neurologists to autonomic symptoms, decreased nerve conduction velocity and increased protein content in the cerebrospinal fluid leading to a wrong diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP), no detection of amyloid at biopsy, coincident diabetes mellitus or monoclonal gammopathy(41).

Heterogenicity of ATTRv

Although TTR-FAP is a seemingly monogenic disease, literature

highlights the considerable phenotypic heterogeneity in patients with either the hereditary or sporadic form, in endemic and non-endemic areas. (40)

Early-onset ATTRv amyloidosis (endemic)

The typical form in endemic foci of Portugal and Japan presents an age at onset from the late 20s to early 40s, an almost 1:1 male-to-female ratio and predominant impairment of superficial compared to deep sensation with marked autonomic dysfunction and conduction cardiac disturbances. The main survival in patients from endemic foci is 12.6 years(27).

Late-onset ATTRv amyloidosis (nonendemic)

Despite having the same mutation (Val30Met), patients affected from Sweden, another endemic focus, display a later age at onset and the same happens in non-endemic countries with onset over 50 years. Also, these patients have a distinct phenotype with male preponderance, loss of all sensory modalities, mild autonomic dysfunction, and tardive heart failure from massive cardiac amyloid deposition. The main survival in patients from nonendemic foci is 7.3 years (27).

Genotype-phenotype correlations in ATTRv

Over 130 allelic variants have been described, most of which are amyloidogenic(26). Clinical manifestations depend mainly on the

existing variant. The age of onset ranges from the second to the ninth decade of life with a wide clinical spectrum. The great heterogeneity in penetrance data depend on phenotype, genotype, and environmental factors(42). Also, the penetrance of the disease in carriers of allelic variants in different areas is high variable, increasing the risk of suffering the disease with age. In Portugal the penetrance at 50 years is 80% compared with 18% in French population. These percentages increase with age, so at 70 years penetrance is 91% in Portugal and 50% in France(3). Of note, anticipation phenomenon has been reported in ATTRv and there is a risk of early onset with the mutation inherited from the mother(43,44). Val30Met (p.Val50Met) is reported in early-onset ATTRv and most cases of late-onset ATTRv from Europe(18,45). On the contrary, very few reports examined other mutations in detail, but often they described small numbers of cases, or miscellanea of mutations all together.

Val30Met

Val30Met (p.Val50Met) is the most studied mutation with similarities as well differences in age at onset, system/organ involvement and complications (18,45). Of interest, although Val30Met is the second most common amyloidogenic variant (5% of all affecting function variants), it represents more than 50% of the cases of FAP(26). This discrepancy might demonstrate the high aggressiveness of neurological manifestations as well as an increased awareness of this phenotype,

especially in endemic areas. Mean age of onset ranges from 32–35 years in endemic areas of Brazil, Portugal, and Japan to 56.7 years in Sweden(27). An even later age of onset (61 years) has been reported in sporadic cases in a series from a non-endemic area(46). In the same mutation, gender analysis reported a later age of onset in women in Portugal and Brazil, whereas no difference in Sweden, Cyprus, and Majorca. Possible factors contributing to the differences among populations, but also within populations, include associate polymorphisms, mitochondrial function, environmental or external causes as diet influencing amyloid deposition, and are now under investigation(14,19).

Recent advances in the understanding of ATTRv has come from the discovery of many patients with mutations other than Val30Met in the *TTR* gene, in which more than 130 mutations have been reported (27).

Val122Ile

Val122Ile (p.Val142Ile) has been associated to cardiomyopathy as the principal manifestation of ATTRv in the absence of neuropathy(40). Hence, since its discovery, Val122Ile is now considered a common cause of heart disease in African Americans originating from West African Countries. However, Val122Ile has been described also in patients no African descendent and according to data from genomic database 1:12666 Europeans carries this variant(26). Hence, Val122Ile

is currently recognized as the most common cause of ATTRv with cardiomyopathy worldwide. Studies from genomic database report that this variant represent 88% of amyloidogenic variant affecting functions(42).

Phe64Leu

Phe64Leu (p.Phe84Leu) variant was described in literature has producing a mixed phenotype. It was described in 1:9900 from genomic database, being mainly present in people of African descendent with a prevalence of 1:924(26).

Ile68Leu

Amyloidogenic variant Ile68Leu (p.Ile88Leu) has a prevalence of 1:23098 according to genomic database and was associated to people of European ancestry(26,47). The clinical manifestations are almost exclusively cardiac (Figure IIA) (48).

Such variants are associated to specific clusters: this is the case of *Thr60Ala* (p.Thr80Ala) reported in Northern Ireland and *Ala97Ser* (p.Ala117Ser) being the most frequently reported from Chinese populations(26).
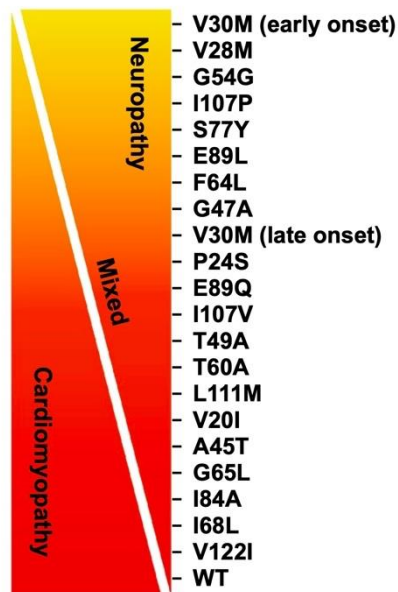
Figure IIA. Genotype–phenotype correlations in ATTR amyloidosis. ATTR amyloid transthyretin, WT wild type. From Adams et al. 2021. (16)

In conclusion, phenotypic variability of ATTRv is still poorly understood. Some amyloidogenic mutations present incomplete penetrance and variable expressivity within the same family, but it is also true that the same mutation might cause distinct phenotypes in different countries. Differences in natural history among mutations and within mutations may have a number of important consequences in planning measures to overcome diagnostic difficulty and therapeutic management. With the promise of new disease-modifying gene/RNA therapies on the horizon, it has become increasingly important to have a good knowledge of the natural history of the disease, according to

different mutations.

Chapter III. Epidemiology of ATTRv

ATTRv has an estimated prevalence of 1/100000 in Europe and United States. In endemic regions of Portugal, Japan, Brazil, and Sweden the prevalence ranges from 1/1000 to 1/10000 people (Figure IIIA) (3,40). In Majorca 5/100000 and in Cyprus 3.72/100000 people(49,50). ATTRv with cardiac phenotype has a higher frequency than polyneuropathy, probably due to the high diffusion of Val122Ile in the African population(26). However, the actual prevalence of ATTRv could be much higher than what has been reported to date and some authors have concluded that it could be as high as 40000 cases(3).
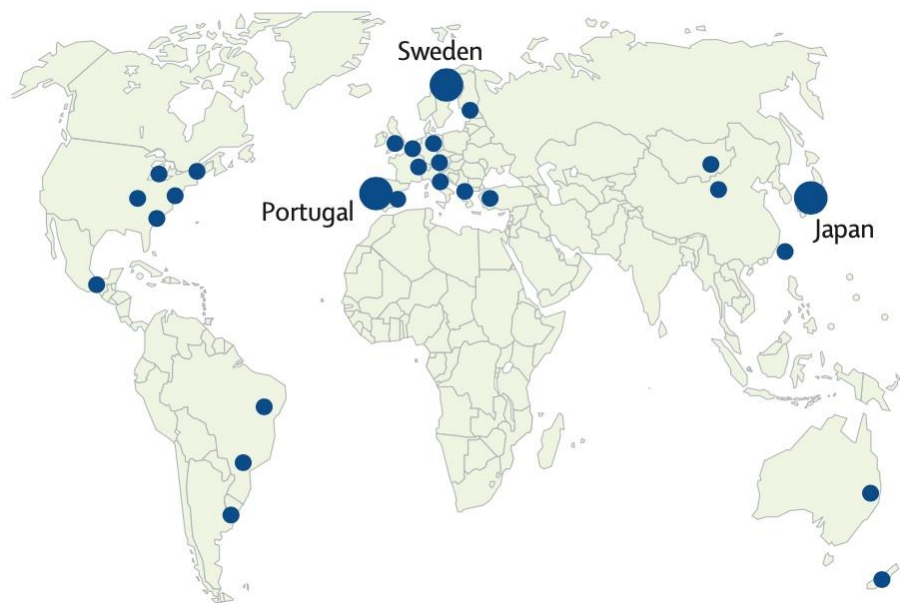

Figure IIIA. Geographic distribution of transthyretin familial amyloid polyneuropathy. From Planté-Bordeneuve et al, 2011 (51)

Epidemiological data from Italy

The Italian Registry for ATTRv amyloidosis involved all expert centers in the country, most likely including the majority of patients diagnosed in Italy with ATTRv until January 2019(52). Four hundred forty-seven living subjects were included in the Registry. Among them, 260 were patients, while 187 were asymptomatic carriers. Male/female ratio was 0.7/1 in asymptomatic carriers and 2.3/1 in patients. In that present survey, the national prevalence of ATTRv amyloidosis was 4.3/million, with quite variable regional differences (Figure IIIB). The prevalence was similar to that found in France (3–4/million) in Bulgaria (6.2/million) and Netherlands (2.6/million)(52). However, some considerations suggest that the Italian prevalence might be even higher: (i) the high number of unrelated patients (163 probands out of 260 patients) makes it likely that paucisymptomatic relatives of these may escape medical attention; (ii) participation to the Registry is on a volunteer basis, therefore some patients might not have been included; (iii) some Italian regions have no referral centers. Apart from some small regions, which however are close to referral centers in nearby regions, this is true for Sardinia, with 1.6 million of inhabitants and no ATTRv patient so far diagnosed.

Val30Met was the most frequent mutation in Italy, being carried by almost one-fourth of patients (52). Val30Met ATTRv is mainly characterized by a sensory polyneuropathy with a late onset (mean:

63.7years; range: 50–81) in most patients. During its course, the disease shows a mixed phenotype including heart and autonomic involvement. Males were more frequently affected, three times more than females, very similar to the ratio of the entire cohort (2.3/1).

In this series, Ile68Leu patients show the shortest disease duration (52). These data are probably due to the natural history of this variant that is characterized by fast course and high mortality (41% at 3 years and 63% at 5 years). The percentage of patients carrying the two cardiological mutations (Ile68Leu and Val122Ile) was higher than that recorded in the past in Italy and in other Western Europe countries. An increased awareness of the disease among cardiologists in the last few years might in part explain this finding. Moreover, the two mutations are common in two regions of central Italy, where two cardiologic referral centres are located.

Val122Ile amyloidosis seems to show two different phenotypes: the largest number of patients, originating from Tuscany, have the classic cardiological phenotype that resembles Ile68Leu amyloidosis (52). Differently, an already described patient from Sicily(53) and another from Apulia had symptoms of peripheral neuropathy without cardiac involvement.

Glu89Gln amyloidosis is one of the most aggressive variants with onset around the age of fifty (52). The disease starts usually with neuropathic symptoms, but heart dysfunction with heart failure and sudden death are major clinical issues during the disease course and in the late stage [8].

Patients have the shortest delay between symptoms onset and diagnosis, probably for the well-known geographical distribution of this mutation, but also because CTS, cardiac dysfunction and peripheral neuropathy often advance in parallel, making the diagnosis more straightforward following diagnostic algorithms proposed in the recent years.

In the Italian Registry, Phe64Leu ATTRv was the second most frequent variant(52). It is the most common in Southern Italy, especially in Apulia and Calabria. Considering the high number of people emigrated from Southern Italy since the late nineteenth century, it is reasonable that this mutation is scattered worldwide. Indeed, the mutation was firstly described in an American patient of Italian ancestry. Patients usually have a late onset. This variant, and the other mainly neuropathic variant Tyr78Phe(54), are characterized by a high number of sporadic patients and a long diagnostic delay. Interestingly, these two mutations are similar also for the high male/female ratio. In both cases, the clinical feature includes distal paraesthesias/CTS at onset and could be easily underestimated or misdiagnosed with other peripheral neuropathies.

Thr49Ala amyloidosis is probably the most peculiar in the Italian Registry(52). These patients present with early disease onset in 80% of cases, there is no difference in male/female prevalence and autonomic disturbances are remarkable. Indeed, orthostatic hypotension may be the inaugural symptom that remains isolated for many years. In line with the young age at onset in these patients, all have a positive family history, the oldest onset being at age of 55 years and the oldest

asymptomatic carrier being 47-year-old. Moreover, all adult family members have been genotyped and therefore, the penetrance of Thr49Ala ATTRv was 100% at the age of 56.
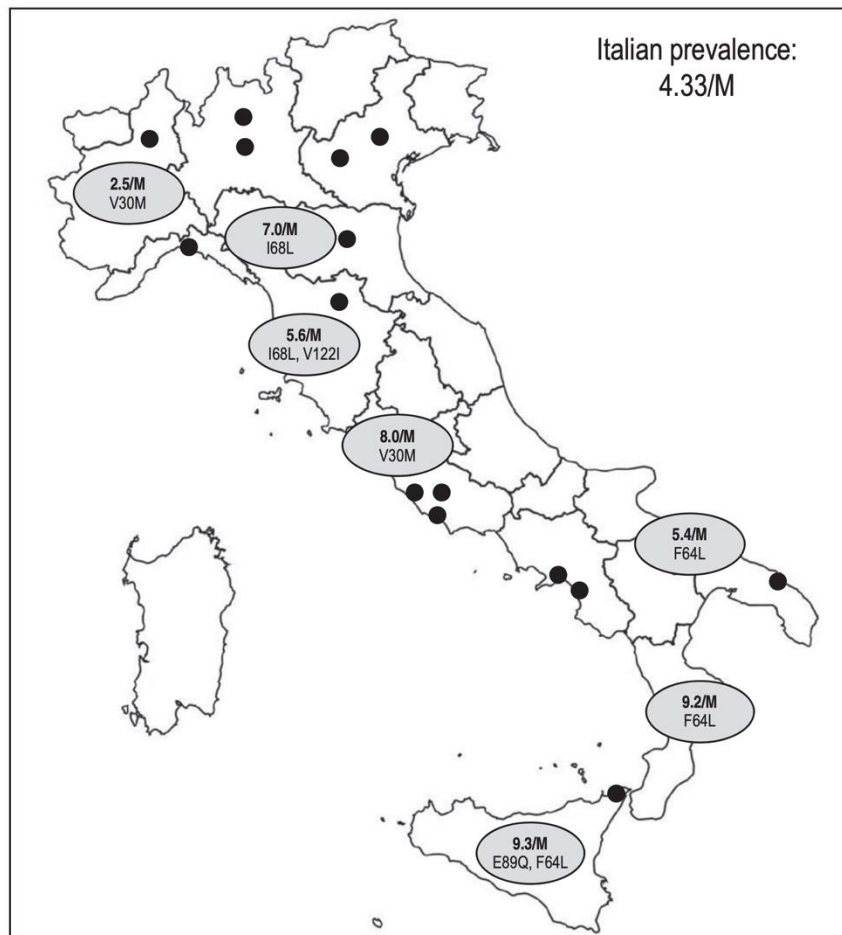
Figure IIIB. Prevalence (no. of patients/million) and more common mutations in the Italian regions with at least 10 affected patients each. Black circles indicate referral centers. From Russo et al, 2020 (52)

Epidemiological data from Sicily

A study from 2015 on ATTRv reported a total prevalence rate of 8.8/1000000 (Figure IIIC) (55). It is lower than the prevalence of 151, 104 and 3.72/100000 in endemic areas as North Portugal, North

Sweden, and Cyprus, respectively(27), but higher than that of 0.87–1.1/1000000 in an even endemic area as Japan and that of approx. 3-4/1000000 in France(28). We can assume that the prevalence is underestimated because of possible late onset, isolated cases, and diagnostic pitfalls.
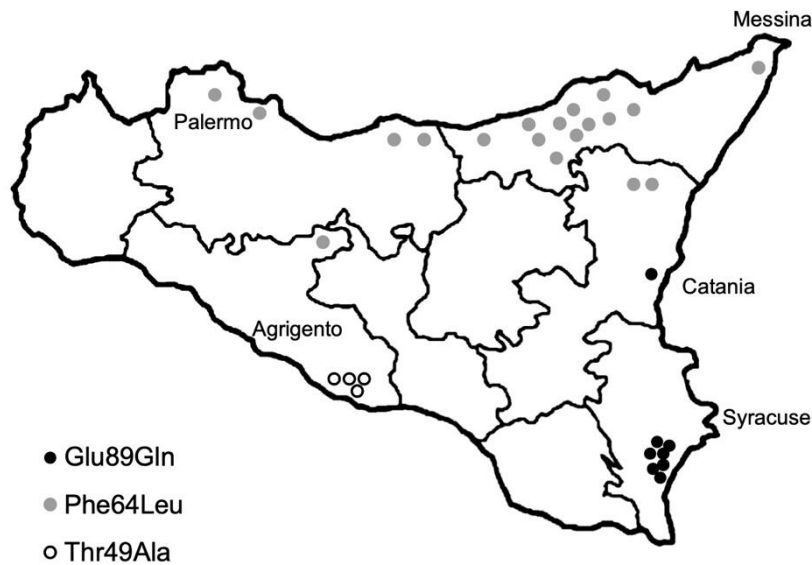
Figure IIIC. Geographical distribution of TTR-FAP patients' families in Sicily according to different mutations. From Mazzeo et al, 2015 (55)

This survey from Mazzeo et al, reported phenotype-to-genotype correlations in 76 patients belonging to 31 Sicilian families, carrying three different TTR mutations (Glu89Gln, Phe64Leu, Thr49Ala) geographically distributed in three major areas of the island. They could be inherited from three common ancestors, and haplotype analyses should be performed to confirm this hypothesis. It is summarized that there are three phenotypes of FAP in Sicily, each corresponding to a different TTR variant, which are homogeneous within and

28

heterogeneous between each other.

Glu89Gln

Glu89Gln mutation, characterized by onset in the 5th – 6th decade, prevalent distal paraesthesias/CTS as presenting symptoms, early heart dysfunction but with fatigue, palpitation, dyspnea appearing later, heart failure and sudden death as major cause of mortality followed by dysautonomia and cachexia.

Phe64Leu

Phe64Leu mutation, marked by familiarity reported in one-half of cases, late onset from 5th to 8th decade, prevailing distal paraesthesias/CTS at onset, organ involvement with severe peripheral neuropathy, moderate dysautonomia and mild cardiomyopathy, death from 7th to 9th decade for wasting syndrome followed by dysautonomia.

Thr49Ala

Thr49Ala mutation, distinguished by an earlier onset in the 5th decade, autonomic disturbances as inaugural symptoms which may remain isolated for many years, moderate polyneuropathy, dysautonomia and cachexia as major causes of mortality followed by cardiomyopathy.

In contrast, comparison among the three mutations revealed no sex

predominance, no sex difference in age of onset, same interval between onset and diagnosis from 1 to 11 years, same life expectancy from 3 to 20 years. However, duration of the disease appeared shorter in Glu89Gln patients (7.6 years), most likely due to the cardiomyopathy which represents the first cause of death (Figure IIID).
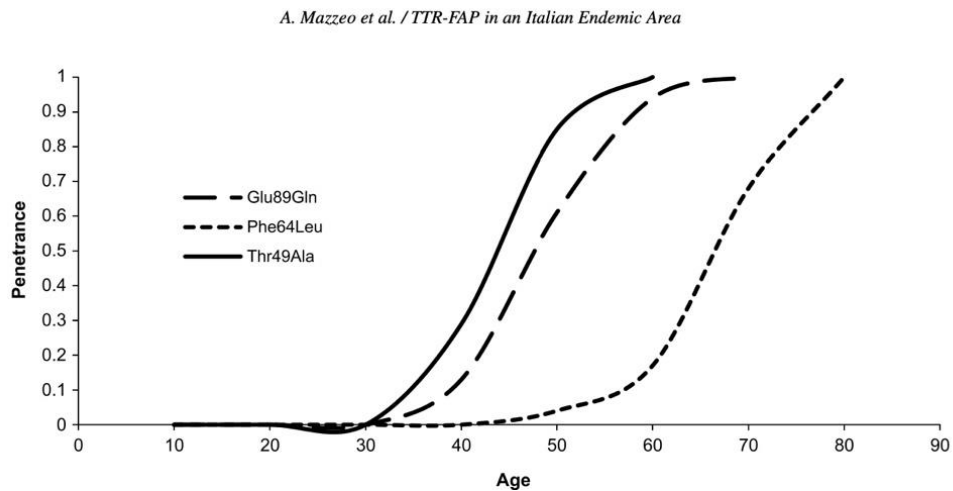
Figure IIID. Estimated penetrance curve according to mutation. From Mazzeo et al, 2015 (55).

Although the disease profile associated with mutations with an exclusively/predominantly cardiac involvement has been little defined, patients with non-endemic Val30Met or with non-Val30Met mutations display a more frequent and severe heart phenotype(18). The only large prospectively followed cohort of patients with one of the so-called "cardiac" mutations, Thr60Ala, showed in sixty patients that: i) clinical presentation was cardiac in 42% but 96% of the patients had echocardiographic evidence of amyloidosis at diagnosis; ii) the median

age of onset of symptoms was 63 years; iii) prognosis was poor, reflecting frequency and severity of cardiac involvement. Unfortunately, the absence of neurological symptoms and indolent course of cardiomyopathy may cause the patient to seek medical attention far along(41). Life expectancy in the Sicilian cohort was of 7.6 – 11.3 years in accordance with the known mean duration of the disease of 10 years(27).

Chapter IV. Diagnosis of ATTRv

The diagnosis of ATTRv represent a significant challenge for the clinician(16,41). Indeed, it is difficult to hypothesize such diagnosis at first presentation given its rarity within general population(41). Hence, the diagnostic process is driven by two components. The first is clinical suspicion, which permits a tentative diagnosis of ATTRv through patient's history and physical examination; the second is diagnostic confirmation using accurate diagnostic tools, including histopathology and genetic analysis. From a neurological perspective the diagnostic process starts with the exclusion of common causes of acquired progressive chronic sensory or sensory-motor polyneuropathy, which are very common in older people, through a complete workup including fast glucose, hemoglobin glycosylated, vitamins (B12, B6, E), serology for hepatitis B, C, and HIV-1, TSH, antiganglioside and anti-MAG antibodies, urine porphyrins, paraneoplastic antibodies, serum immunofixation electrophoresis, ANA, ANCA, SSA-Ro, SSB-La antibodies, cryoglobulins. Once excluded more common acquired disease, ATTRv patients should be considered.(16)

In the recent past, the diagnosis of ATTRv required genetic

testing performed in the presence of a positive biopsy for the presence of amyloid deposits(4,6). However, the role of biopsy has become no longer irreplaceable due to the broad availability of genetic testing(7,8). Indeed, more recent diagnostic algorithms suggest anticipating and often replacing the biopsy in the diagnostic workup(1).

Histopathology

Biopsy from several affected tissue can provide direct evidence of amyloid deposition through histopathological analysis. The first step is Congo red stain to visualize extracellular amyloid deposits and confirm the characteristic apple-green birefringence after cross-polarized light examination (Figure IVA, panels a and b). The second step is immunohistochemistry which can confirm that amyloid is formed by TTR protein (Figure IVA, panels c and d).
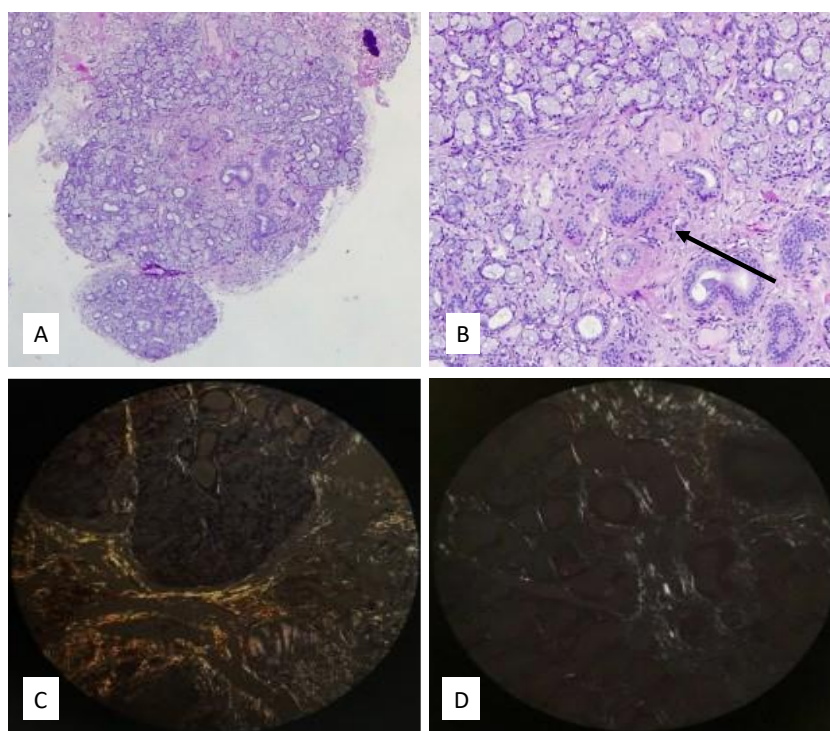
Figure IVA. Histologic view of the biopsy specimen in an ATTRv patient from our cohort. A) a fragment of minor salivary gland mainly composed of mucous secreting glands. At low magnification, amorphous eosinophilic material is centrally observed (H&E stain, original magnification 40x); B) The material mainly appears in the perivascular space and interspersed between the salivary ducts (arrows) (H&E stain, original magnification 100x); C) Congo Red stain confirmed the amyloid composition of the amorphous material with the characteristic "apple-green" birefringence under the polarized light. (Congo Red stain, original magnification 200x); D) Focal "apple-green" birefringence was also observed in the periglandular spaces. (Congo Red stain, original magnification 400x).

The most frequent site of biopsy are abdominal fat and rectum, followed by sural nerve (Figure IVB), labial salivary gland biopsy, and heart. It should be noted that while salivary gland biopsy and nerve biopsy are comparable, heart biopsy might be preferred in the presence of cardiac involvement(8). However, interpretation of biopsy specimens has certain challenges because

the sensitivity of non-cardiac tissue biopsy is low, especially for abdominal fat(8). Moreover, false negative results are quite frequent as biopsy sensitivity depends on the experience of the pathologist in reading the slides, even if immunohistochemistry significantly reduce the risk of misdiagnosis(16).
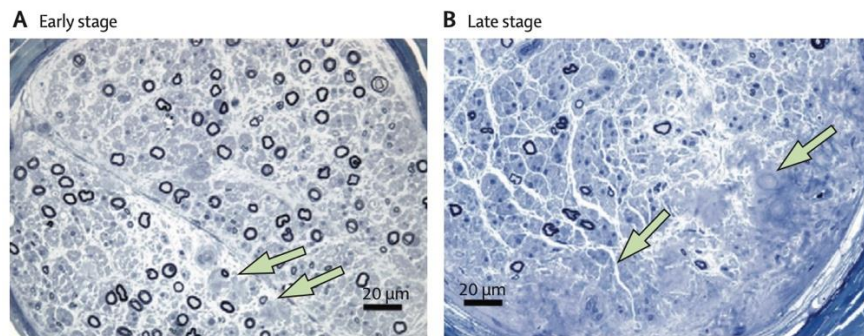
Figure IVB: Pathology at early and late stages of ATTRv. (A) 1 μm thick plastic section of a sural nerve biopsy specimen from a 42-year-old woman carrying the Val30Met mutation who had early symptoms of polyneuropathy. Note the presence of small amyloid deposits (arrows) and the preservation of larger myelinated fibres. (B) Sural nerve biopsy specimen from a 39-year-old man with the Val30Met mutation at a late stage of the disease, showing nearly complete disappearance of myelinated fibres and large endoneurial amyloid deposits. At this stage, endoneurial blood vessels are often invaded and destroyed by amyloid (arrows). Thionin blue staining was used on both samples. From Planté-Bordeneuve et al 2011 (51).

Genetic analysis

Genetic testing allows detection of specific amyloidogenic TTR mutations. The TTR gene is located on chromosome 18 (18q12.1), is divided into four exons and, despite being small, over 130 allelic variants have been reported(26). A targeted approach can be used in the case of family history, but TTR gene sequencing is necessary for the detection of already known and new amyloidogenic mutations.

Finally, in the presence of a diagnosis of ATTRv amyloidosis, meticulous neurological and cardiological assessment are needed to document organ involvement and start the appropriate treatment(16). If symptoms of neuropathy are present a careful clinical examination with electromyography, nerve conduction studies and autonomic testing (i.e., sympathetic skin response) enables diagnosis of polyneuropathy involving small or large fibers. If the patients display a cardiological onset, cardiac conduction disorders or infiltrative cardiomyopathy can be demonstrated through transthoracic echocardiography, scintigraphy with bone tracers and, in selected cases, cardiac MRI.

Pre-symptomatic genetic testing for ATTRv

If the result of genetic testing is positive in the proband, family members may undergo genetic counselling to be tested themselves; also, pre-symptomatic carriers should be monitored for the onset of the symptomatic disease to achieve early treatment. However, due to the incomplete penetrance of the disease, this arises some ethic issues(44). Recently, in order to determine when pre-symptomatic carriers of a TTR mutation should start a regular monitoring, it has been proposed to estimate the predicted age of onset of symptomatic disease (PADO)(56). PADO depends on the specific TTR gene mutation, the typical age of onset for that mutation and the age of onset in other members of the proband's family. International expert

consensus recommends that regular clinical monitoring should start

10 years before the PADO(56).

Chapter V. Available treatments in ATTRv: disease-modifying therapies

Liver transplantation was the first treatment of ATTRv, developed in Sweden in 1990(57). This approach has halted the progression of the disease, especially if performed in the early stages of the disease; however, as time goes on, neuropathy and cardiomyopathy continue to progress in some patients notwithstanding liver transplantation; also, ocular, and central nervous system manifestations cannot be prevented by liver transplantation because of the continue production of altered TTR from the eye and the choroid plexus.

This first approach aimed the inhibition of amyloidogenic TTR synthesis; similarly, gene-silencers, such as siRNA and antisense therapies have been developed and approved in 2018 for ATTRv(29,58). More recently, several promising therapeutic strategies are in the early phases of investigation, such as CRISPR-Cas9 system(59).

The second approach is the stabilization of the native tetramer structure of TTR; on this regard, diflunisal and tafamidis were developed demonstrating a good efficacy and amelioration of survival in patients affected by cardiomyopathy(60,61).

Finally, the third approach consists in removal of amyloid fibrils or

misfolded TTR through specific antibodies. This approach is recently under evaluation.

Liver transplantation

Liver transplantation aims a reduction in wild- type/variant TTR. As the main source of circulating TTR is the liver, liver transplantation has been performed since 1990 in patients with ATTRv amyloidosis. Long-term efficacy from the viewpoint of survival has been proven, particularly in early-onset Val30Met patients from endemic foci. A retrospective analysis of the data obtained from the Familial Amyloidotic Polyneuropathy World Transplant Registry suggested an early age of onset, short disease duration, and Val30Met mutation to be better predictors for survival(18). However, the progression of cardiomyopathy and neuropathy resulting from wild-type TTR deposition may occur even after liver transplantation, particularly in late-onset male patients, resulting in poor prognosis after liver transplantation in these patients(62).

TTR Stabilizers

As the dissociation of TTR tetramers into monomers is the crucial step for the subsequent process of protein misfolding and amyloid fibril formation (25,27), an approach to stabilize the native quaternary structure of TTR tetramers using small molecules that bind to thyroxin-binding sites has been proposed as a potential approach for the treatment

of not only ATTRv amyloidosis but also ATTRwt amyloidosis (61,63,64). In the early 2010s, randomized controlled trials suggested the efficacy of two orally administered TTR stabilizers (i.e., tafamidis and diflunisal) for ameliorating the progression of neuropathy in patients with ATTRv amyloidosis(61). As these TTR-stabilizing drugs can be administered orally, patients with late-onset ATTRv amyloidosis who were not eligible for liver transplantation also became targets for disease-modifying treatment. Another recent randomized controlled trial suggested the efficacy of tafamidis even for cardiomyopathy resulting from both ATTRv and ATTRwt amyloidosis(60).

Tafamidis is an analogue of thyroxine designed to stabilize TTR tetramers(61). A phase III clinical trial involving 128 patients with early-stage Val30Met ATTR amyloidosis who were randomly assigned in a 1:1 ratio to receive tafamidis (tafamidis meglumine) 20 mg once daily or placebo for 18 months suggested that tafamidis delayed the progression of neuropathy, although the primary endpoint could not be achieved(61). An open-label extension study for up to 6 years also demonstrated the slowing of neuropathy progression without any unexpected adverse events(65). In particular, patients who continued to receive tafamidis had less progression of neuropathy than those who switched to tafamidis following 18 months of placebo, warranting the need for early intervention(65). In addition to its efficacy on neuropathy, the efficacy of tafamidis on cardiomyopathy due to not

only ATTRv amyloidosis, but also ATTRwt amyloidosis was suggested by a recent phase III clinical trial involving 441 patients with ATTR amyloidosis(61). This study included 335 patients with ATTRwt amyloidosis and 106 patients with ATTRv amyloidosis who were randomly assigned in a 2:1:2 ratio to receive 80 mg of tafamidis, 20 mg of tafamidis, or placebo for 30 months and demonstrated reduced mortality and cardiovascular-related hospitalizations.

Diflunisal is a nonsteroidal anti-inflammatory drug that also acts to stabilize TTR (66). A study involving 130 patients with ATTRv amyloidosis who were randomly assigned in a 1:1 ratio to receive 250 mg of diflunisal twice daily or placebo for 2 years suggested that diflunisal can slow the progression of neuropathy, although 67 patients (27 diflunisal patients and 40 placebo patients) discontinued the treatment before completing the 2-year protocol(66). The demographics of patients in this study were different from those in the phase III trial of tafamidis for neuropathy in patients with ATTRv amyloidosis(61) because it included patients with relatively late disease onset, various disease severities, and non-Val30Met mutation.

Gene-Silencing Drugs
Theoretically, preventing the production of TTR efficiently ameliorates systemic organ dysfunction in ATTR amyloidosis because it not only prevents amyloid fibril formation, but also suppresses an increase in

toxic amyloid precursors, such as TTR oligomers(25). As described earlier, TTR seems to exert harmful effects even when fibrillar structures recognized as amyloid fibrils are not formed. Given that circulating variant TTR may induce microangiopathy, which plays a role as an initial lesion of organ damage(67), a strategy that eliminates circulating TTR is more reasonable than liver transplantation and TTR stabilizers(27). This strategy became a reality with the development of gene-silencing therapeutics, including small interfering RNA (siRNA) and antisense oligonucleotide (ASO)(27). In 2018, two randomized controlled trials of such gene-silencing agents (patisiran and inotersen) demonstrated an efficacy on neuropathy in patients with ATTRv amyloidosis(29,58).

Patisiran is an RNA interference therapeutic comprising siRNA formulated in a lipid nanoparticle: when the complex enters into hepatocytes, it selectively targets TTR mRNA, reducing both ATTRv and ATTRwt production(27). In a phase III trial, 225 patients with ATTRv amyloidosis with polyneuropathy were randomly assigned in a 2:1 ratio to receive patisiran intravenously (0.3mg/kg of body weight) or placebo once every 3 weeks(58). The results of this study were excellent because all endpoints, including the scores related to somatic and autonomic neuropathies, quality of life score, and exploratory cardiac measures, were better in patients who received patisiran than in those who received placebo. In particular, modified Neuropathy

Impairment Score+7 (mNIS +7, primary endpoint: 56% vs 4%), Norfolk QoL scores (51.4% vs 10.4%), gait speed (53% vs 13%) and Composite Autonomic Symptom Scale-31 (COMPASS-31) measure of autonomic symptoms. Besides, the use of patisiran was associated to an 81% reduction in serum TTR level. Finally, patisiran significantly improved quality of life, nutritional status, and activities of daily living. An analysis of several cardiac parameters in a pre-specified cardiac subpopulation of APOLLO study showed a beneficial effect on cardiomyopathy, suggesting that patisiran could halt or reverse the progression of cardiac symptoms of ATTRv amyloidosis patients(12). A recently published interim 12-month analysis of the global OLE study, including patients from APOLLO study and phase 2 OLE study, showed that patisiran is able to maintain a long-term efficacy with an acceptable safety profile in patients with ATTRv amyloidosis and polyneuropathy(68). These data also support the importance of an early treatment to halt or reverse the progression of polyneuropathy, dysautonomia, disability, malnutrition and QoL impairment.

Inotersen is a second-generation ASO designed to reduce the production of TTR(29). Parenterally administered ASO, in general, is rapidly transferred into various organs, with the highest concentration in the liver and kidneys(69). A phase III trial involving 172 patients with ATTRv amyloidosis who were randomly assigned in a 2:1 ratio to receive weekly subcutaneous injections of inotersen (300 mg) or

placebo for 15 months demonstrated significantly better primary endpoints represented by neuropathy impairment and quality of life scores(29). Because glomerulonephritis and thrombocytopenia were reported as severe adverse events, close monitoring of renal function and platelet count is required in patients receiving inotersen.

Vutrisiran, an RNA interference therapeutic that reduces TTR production, significantly improved multiple disease-relevant outcomes for ATTRv amyloidosis versus external placebo, with an acceptable safety profile in "HELIOS-A", a phase 3, global, open-label study(30). In this study vutrisiran was compared to an external placebo group (APOLLO study). Patients were randomized 3:1 to subcutaneous vutrisiran 25 mg every 3 months or intravenous patisiran 0.3 mg/kg every 3 weeks for 18 months. HELIOS-A enrolled 164 patients (vutrisiran, n = 122; patisiran reference group, n = 42); external placebo, n = 77. Vutrisiran met the primary endpoint of change from baseline in modified Neuropathy Impairment Score +7 (mNIS+7) at 9 months (p = 3.54×10^{-12}), and all secondary efficacy endpoints; significant improvements versus external placebo were observed in Norfolk Quality of Life-Diabetic Neuropathy, 10-meter walk test (both at 9 and 18 months), mNIS+7, modified body-mass index, and Rasch-built Overall Disability Scale (all at 18 months). TTR reduction with vutrisiran was non-inferior to within-study patisiran(30).

Management of ATTRv

ATTReuNET recommends a multidisciplinary approach to the management of ATTRv, including not only the diagnostic physician, but also a neurologist, a cardiologist and possibly an ophthalmologist in the initial assessment and subsequent reviews(70). The principal strategy is anti-amyloid therapy (Figure VA), but also symptomatic treatment and management of complication and genetic counselling are necessary.
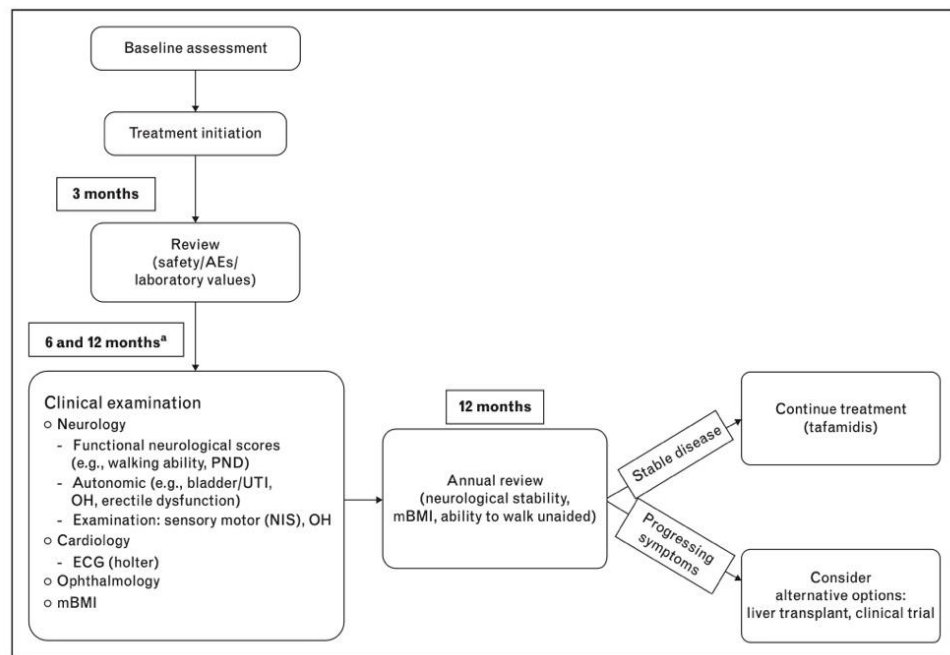
Figure VA. Algorithm for patient follow-up during treatment for TTR-FAP. AE, adverse event; ECG, electrocardiogram; mBMI, modified body mass index; NIS, Neurological Impairment Scale; OH, orthostatic hypotension; PND, modified polyneuropathy disability score; TTR-FAP, transthyretin familial amyloid polyneuropathy; UTI, urinary tract infection. a Quarterly basis for those with more advanced (stage II, III) disease unless responding well to treatment. From Adams et al, 2016 (70).

Patients should be assessed by neurologist and cardiologist biannually with monitoring maintained throughout their lives. Regular follow-up with a multidisciplinary team can help to diminish anxiety and acceptance of diagnosis. Patients with advanced disease (FAP 2-3) should be monitored shortly on a quarter basis, whereas patients well-responding to treatment can be followed up less regularly (70).

Chapter VI. The need for a screening for ATTRv

Clinical phenotype of ATTRv is heterogeneous and often unpredictable therefore the diagnosis is very difficult and, in most cases, delayed(40,50). In most cases, patients present with neurological onset with progressive sensory polyneuropathy, autonomic dysfunction, pain in hands and feet, and gait disorders associated with other systemic signs(40). However, none among these symptoms is enough specific for ATTRv, but there is a clinical overlap with common disorders causing polyneuropathy. As a consequence, misdiagnosis of ATTRv carries high costs for the community in terms of mortality and inappropriate treatments(4), although several treatment options are available being particularly effective in early disease stages(5). Delay in the time to diagnosis is a major obstacle to the optimal management of ATTRv. Indeed, with the exception of those clearly diagnosed in the presence of a family history, ATTRv patients wait several years between the emergence of the first symptom and an accurate diagnosis. This issue is relevant if we think that the disease progresses rapidly and irreversibility if unchecked and untreated. To counteract this situation, we should consider two important aspects: misdiagnosis and underdiagnosis of ATTRv.

Misdiagnosis of ATTRv

Due to the rarity of the disease, ATTRv patients are often mistaken for more widely seen disorders (Table VIA) (70). Common misdiagnoses include idiopathic axonal polyneuropathy, chronic inflammatory demyelinating polyneuropathy, lumbar spinal stenosis, diabetes, and chronic alcoholism. Less frequently, Charcot-Marie-Tooth neuropathy and motor neuron disease can mimic ATTRv(70).

Common Misdiagnoses	*ATTR Symptoms Contradicting Given Diagnosis*
Cardiac	
Hypertrophic cardiomyopathy	Discordant voltage to mass ratio
Hypertensive heart disease	Discordant voltage to mass ratio; intolerance to beta blockers; waning need for antihypertensives
Undifferentiated heart failure with preserved ejection fraction	Nondilated hypertrophic LV
Uncomplicated degenerative aortic stenosis	Reduced longitudinal strain Frequent low-flow, low-gradient paradoxical pattern Thickened atrioventricular valves
Neurologic	
Chronic inflammatory demyelinating polyneuropathy	Pain in the limbs, dysautonomia (erectile dysfunction, OH), symmetric polyneuropathy in upper limbs
Monoclonal gammopathy–associated neuropathy	Autonomic dysfunction (erectile dysfunction, OH)
Idiopathic axonal polyneuropathy	Dysautonomia (erectile dysfunction, OH), walking difficulties
CTS	Worsening of upper limb symptoms despite CTS surgery
Lumbar spinal stenosis	Failure to relieve symptoms in spite of spine surgery
Diabetic neuropathy	Walking difficulties
Amyotrophic lateral sclerosis	No upper motor neuron syndrome Reduction of amplitude of SNAP
Motor neuropathy	Reduction of amplitude of SNAP

Gastrointestinal

Inflammatory bowel syndrome	Absence of inflammation
Irritable bowel syndrome	Absence of or only minor abdominal pain; weight loss
Idiopathic diarrhea	Weight loss
Idiopathic bile acid malabsorption	
Pseudo-obstruction	Absence of or only minor abdominal pain or radiologic findings of intestinal obstruction

Table VIA Common Misdiagnoses of Disturbances Caused by ATTR Amyloidosis. ATTR Transthyretin amyloidosis, CTS Carpal tunnel syndrome, GI Gastrointestinal, LV Left ventricle, OH Orthostatic hypotension, SNAP Sensory nerve action potential. From Gertz et al 2020 (41)

Underdiagnosis of ATTRv

Apart from difficulties of knowing the true prevalence and penetrance of the disease, ATTRv is underdiagnosed due to the high clinical variability and the lack of specific symptoms and biomarkers. It should be considered that many centers do not have the possibility to perform genetic tests and in some settings the disease is still poorly understood(71). In a survey on ATTRv, the diagnosis was made within 6 months in only 35% of cases, with patients seeing more than five physicians before receiving a correct diagnosis(41). Moreover, underdiagnosis is proven by studies on large genomic databases that demonstrated high circulation of amyloidogenic variants in general population (from 1:1269 for variants affecting function with European ancestry to 1:230 considering individuals with African ancestry). Also, there are studies indicating that 32% of patients are undiagnosed(40)

and 5% of patients with hypertrophic cardiomyopathy have ATTRv(72). Finally, in studies on ATTRv, populations are not equally represented with European population being the majority(26). Hence, appropriate conclusions cannot be made for Asian or Latino groups.

A practical approach: clustering ATTRv symptoms in "red flags"

The main clinical features of ATTRv amyloidosis are carpal tunnel syndrome, peripheral sensory-motor neuropathy, cardiovascular manifestations (conduction blocks, cardiomyopathy, arrhythmia), autonomic manifestations (orthostatic hypotension, recurrent urinary tract infections, sexual dysfunction, sweating abnormalities) and gastrointestinal manifestations (nausea, vomiting, diarrhea, severe constipation, alternating episodes of diarrhea and constipation, unintentional weight loss), central nervous system manifestations (such as headache, progressive dementia, ataxia, seizures), ocular involvement (vitreous opacification, glaucoma) and nephropathy (proteinuria, renal failure) may rarely occur. Consequently, based on published literature and expert opinions, symptom clusters and specific "red flags" have been recently proposed to facilitate an earlier diagnosis of ATTRv(9,10). Potential "red-flag" symptom clusters that may warn of a diagnosis of transthyretin familial amyloid polyneuropathy are a progressive symmetric sensory-motor neuropathy plus ≥1 of the following: family history, early autonomic dysfunction, unexplained weight loss, gastrointestinal complaints, cardiac hypertrophy,

arrhythmias, ventricular blocks, cardiomyopathy, bilateral carpal tunnel syndrome, renal abnormalities, and vitreous opacities(10). However, there are no studies clearly demonstrating the actual value for each symptom and it is no clear which are the more effective driving to the correct diagnosis. Hence, there is still a need for new strategies to find undiagnosed individuals and implement existing evidence-based guidelines to improve ATTRv care. The last reviews and expert opinion suggest that ATTRv should be suspected if progressive peripheral sensory-motor neuropathy is observed in combination with one or more of the following conditions: autonomic dysfunction (erectile dysfunction, orthostatic hypotension, syncope), cardiopathy, gastrointestinal symptoms, unexplained weight loss, bilateral carpal tunnel syndrome, lumbar canal stenosis, renal impairment, ocular involvement (vitreous opacities) and/or family history of polyneuropathy, cardiopathy or ATTRv (9,11). Finally, being ATTRv an autosomal dominant genetic condition, screening at-risk relatives of individuals with ATTRv (*cascade* testing) is highly effective in identifying additional individuals who require treatment(10).

Family history

A family history of neuropathy is present in most ATTRv patients. Sometimes, these patients present parents with early loss in ambulation or even confined to bed. A family history of cardiopathy is also frequent, especially in males, with death for cardiac causes. However,

many ATTRv patients present in absence of familiarity, simulating a sporadic disease. In such cases, it is very difficult to achieve an early diagnosis and high suspicion should be kept in the presence of other red flags.

Sensory-motor polyneuropathy

Polyneuropathy is the principal hallmark of ATTRv, being considered the key feature and most disabling symptom of the disease. Neuropathy starts with axonal damage of sensory fibers as it is demonstrated by histopathological studies. A further proof for early sensory involvement is demonstrated by neurophysiological data from sudoscan, sympathetic skin responses, and laser evoked potentials. Sensory neuropathy causes pain in the early phases, which are followed by sensory disfunction with tactile and thermic hypoesthesia and loss of sensation and vibration sense. As a result, ambulation in ATTRv patients become uncertain and ataxic with frequent falls. Sensory neuropathy can be symmetric and length-dependent even if an early involvement of the upper limb is the rule. As the disease goes on motor damage worsens symptoms with often asymmetric damage in motor fibers of peroneal, tibial, median, ulnar, and radial nerves. A bilateral foot drop due to bilateral peroneal palsy is a typical presentation of motor axonal neuropathy. Some patients have preserved ambulation with severe and often asymmetric involvement in the upper limbs which might simulate amyotrophic lateral sclerosis (ALS). Of interest, motor neuropathy can affect also

52

cranial nerves, included hypoglossus nerve causing severe dysarthria and dysphagia, again simulating ALS. If we consider that the clinical onset is in the seventh decade, in which sensory neuropathy from all causes is very common, we can have a perception of the trouble with differential diagnosis with ALS, a disease in which a coexistence of sensory neuropathy due to comorbidities is not uncommon.

Cardiac involvement

Cardiomyopathy is one of the typical expected features of ATTRv(41). Reduced voltage in routine electrocardiograms as well as increased parietal walls in the left ventricle are the usual clinical presentation(27,33). Early diastolic dysfunction is the first sign of cardiac amyloidosis, but, as misfolded monomers aggregate in amyloid fibrils infiltrating the parietal walls, systolic dysfunction starts with progressive cardiomyopathy and high frequency arrhythmias. As a result, cardiac insufficiency and atrial fibrillation are the rule in advanced cardiac ATTRv, being the principal cause of death of these patients.

Echocardiographic findings of ATTRv can often lead to a misdiagnosis of hypertrophic cardiomyopathy, especially in older adults(41). Also, hypertensive cardiac remodeling and undifferentiated heart failure with preserved ejection fraction are further cardiologic misdiagnoses(41).

Cardiac deposits can be detected through cardiac MRI, especially with T1-mapping sequences, and scintigraphy with bone tracers (Figure

VIA). However, tracer uptake can be sparse or absent in patients with cardiopathy; indeed, it has been reported that Phe64Leu mutated patients might not display any uptake with bone tracers on scintigraphy despite having significant cardiopathy(73).
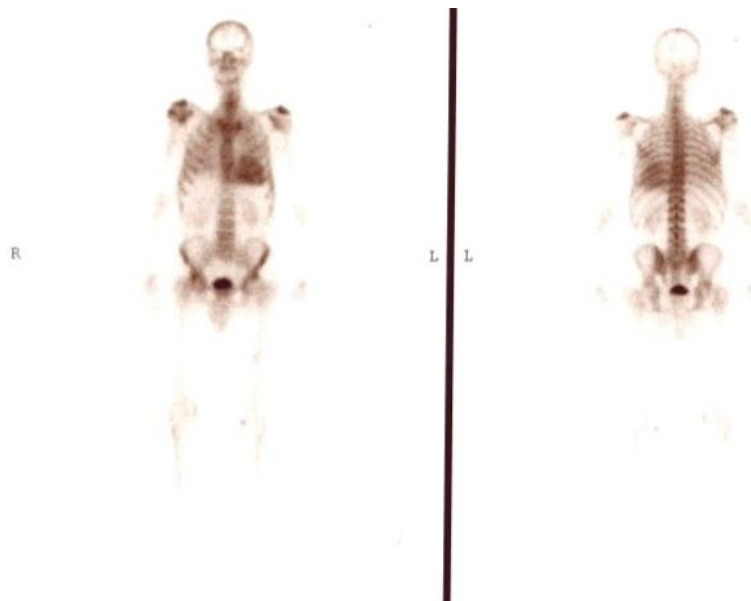
Figure VIA. A high (score 3, myocardial uptake > rib uptake) uptake in the heart is seen on 99mTc-HDMP bone tracer scintigraphy from a patient from our clinic affected by ATTRv with Val122Ile mutation.

Use of serum cardiac biomarkers such as troponin levels, N-terminal pro-brain natriuretic peptide plasma levels are also useful(72).

Cardiac involvement can occur in isolation, as reported in the pure cardiac phenotypes described many years ago, but the coexistence of a mixed phenotype is a more typical clinical phenotype. Specific mutations have been linked to cardiac phenotypes, in particular Ile68Leu and Val122Ile (p.Val142Ile)(47). Hence, cardiac presentation could be the first sign of ATTRv, or it might follow polyneuropathy(74). However, cardiomyopathy is a major determinant

of survival in ATTRv, significantly influencing the prognosis. Diagnosis of ATTRv cardiomyopathy can be obtained through a meticulous cardiac assessment with electro- and echocardiography with cardiology clinic every 6 months in patients with polyneuropathy. Management of cardiac insufficiency and fluid imbalance as well as oedema should be treated with symptomatic therapies (diuretics, antihypertensive agents). More than an effort should be done to achieve early recognition and treatment of this feared complication of ATTRv.

Carpal tunnel syndrome

Carpal tunnel syndrome (CTS) is a well-known symptom of ATTRv and appears very early in the disease course causing hand clumsiness and sensory deficits associated with weakness and hypotrophy in the tenar muscles(75). Also, CTS can be the first sign of the disease and is often present in middle aged carriers of *TTR* variants(76). Moreover, hand weakness progresses along with the aggregation of amyloid fibrils in the peripheral nerves and in the carpal ligament; as a consequence, ATTRv patients often complain of severe neuropathy in the median nerve with significant limitations and implications in the quality of life, due to difficulties in the execution of simple movements, such as turning a key or signing a document(75).

In a previous study from Sicily(55), CTS alone was the first symptom/sign in one third of the cohort, occurring bilaterally only in one patient. CTS remained the only manifestation for a period of 1–12

years. The occurrence of a short interval supports, as postulated by some authors, that the electrophysiological abnormality at the distal portion of the median nerve may be the consequence of polyneuropathy rather than an entrapment injury. On the other hand, the occurrence of a long interval between CTS signs and appearance of other complaints could suggest a coincidental presence of an idiopathic CTS, because of the high CTS prevalence of 7.8% in working populations(77).

Autonomic dysfunction

As autonomic sensory fibers are early involved in ATTRv, autonomic dysfunction is a frequent feature at disease onset and almost constantly reported during the course of ATTRv(78). As a consequence, gastric retention, chronic or alternating diarrhea and constipation, erectile dysfunction, orthostatic hypotension, and syncope are typical features.

Gastrointestinal symptoms

Chronic diarrhea or diarrhea alternating with constipation, unintentional weight loss often associated with early satiety and absence of abdominal pain are typical features of ATTRv(79). A gastrointestinal onset is not uncommon(45); hence a suspect should be kept even in absence of a manifest neuropathy. Chronic gastrointestinal disturbances might indirectly cause unexplained or unintentional weight loss, which is a typical feature of ATTRv(80). Finally, gastrointestinal tract containing mucosa and submucosa should be considered as a good site for biopsy,

where amyloid deposits may be detected(81,82).

Lumbar canal stenosis

Amyloid deposits can accumulate in the ligament flavus of the lumbar spine in advanced ATTRv disease(17). In these cases, patients present a classical lumbar canal stenosis with neurogenic claudication and severe difficulties in ambulation. Lumbar back pain is common, and it is also associated with amyloid deposits in the carpal tunnel with coexistence of CTS. Lumbar canal release is the gold standard approach when amyloid deposits cause significant motor involvement. In such Countries, histopathological analysis of ligamentum flavus is routinely performed, thus allowing a diagnosis in the majority of ATTR cases(17). The lack of neuropathy in these cases may be misleading for the diagnosis and EMG studies are needed to achieve a clinical and neurophysiological suspect.

Ocular involvement

Vitreal opacities are frequently encountered in ATTRv patients causing reduced or blurred vision and early cataract(41). However, this symptom is not easily assessed in the lack of an expert ophthalmologist and might be misdiagnosed due to the high incidence of cataract and diabetes at the typical age of onset in non-endemic ATTRv. Moreover, the eye is one of the sites of production of TTR, hence it is not a surprise that ocular involvement might occur in patients treated with gene

silencers or in liver transplant receivers.

Renal impairment

Nephropathy is commonly recognized as a main feature of systemic ATTRv(41). Indeed, up to 30% of patients present some degree of renal disfunction and amyloid deposits are frequently found in kidney specimens. Attention should be paid in the assessment of renal function of ATTRv patients to obtain a good care and compliance with therapies.

Ethnicity

The country of origin and ethnicity should always be considered when evaluating a patient suspected for ATTRv(26,50). Indeed, it is a well-known fact that ATTRv amyloidosis is endemic in Sweden, Portugal, and Japan, but it is also quite diffused in non-endemic countries, such as Italy. Indeed, each specific region of the world present peculiar characteristics and unique genotypes. For example, a specific mutation can be expected in endemic countries such as Portugal (Val30Met)(40). Conversely, it is also true that some mutations are almost always present in specific areas. For example, Val122Ile (p.Val142Ile) is a mutation diffused among Afro-Americans occurring in about 3% of people with that ethnicity(26). This variant has been frequently associated with prominent cardiomyopathy phenotype with polyneuropathy in a minority of cases. Furthermore, a founder effect can be recognized for specific mutations(26).

Recent algorithms

ATTRv with polyneuropathy should be suspected in any patient who has length-dependent small-fiber polyneuropathy with autonomic dysfunction and a family history of ATTR amyloidosis, unexplained weight loss, heart rhythm disorders, vitreous opacities, or renal abnormalities. In nonendemic countries, the disease may present as idiopathic rapidly progressive sensory motor axonal neuropathy or atypical CIDP with any of the above symptoms or with bilateral carpal tunnel syndrome, gait disorders, or cardiac hypertrophy (Figure VIB) (16).
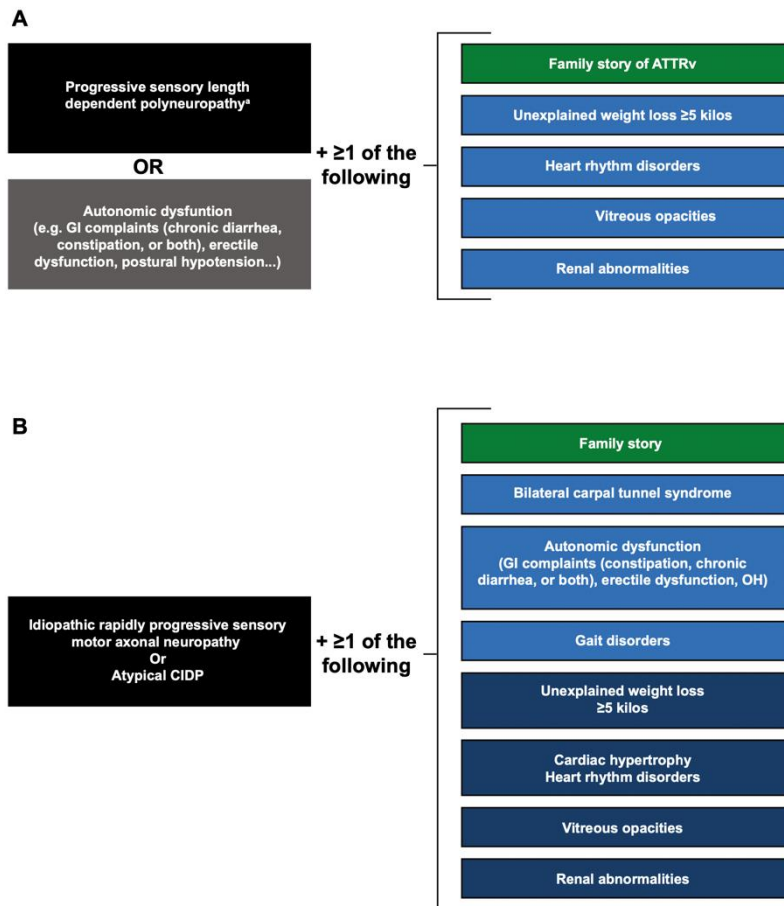
Figure VIB. Suspicion index for diagnosis of ATTRv amyloidosis with PN. A) In endemic areas. B) In nonendemic areas. ATTRv, hereditary transthyretin amyloid amyloidosis, CIDP, chronic inflammatory demyelinating polyneuropathy, GI, gastrointestinal, OH orthostatic hypotension. [a] No diabetes, no alcohol abuse, vitamin B12 deficiency. From Adams et al 2021(16).

Chapter VII. The need for instrumental biomarkers of disease activity in ATTRv polyneuropathy

Nowadays, several available treatment options are effective in early disease stages of ATTRv(31). Patisiran, a small interfering RNA acting as TTR silencer approved in Italy in 2020, has been shown to stabilize the course of polyneuropathy in ATTRv, but its administration is bound to the presence of neuropathy as a manifestation(47,83). Consequently, a meticulous assessment of neuropathy is essential. Moreover, self-reported symptoms difficult to demonstrate and consequently often underestimated. Some questionnaires are commonly used in clinical practice, but an instrumental evaluation of dysautonomia through neurophysiological instruments is not systematically performed. Some researchers are involved in the evaluation of serum biomarkers of neuropathy such as light chain neurofilaments(84).

Assessment of motor strength and large fibers

According to the most common scale to assess the overall burden of polyneuropathy(85), ATTRv evolves into three successive stages: in the first, patients have a sensory polyneuropathy leading to difficulty

in walking without assistance (FAP 1); in the second phase, there is a significant limitation in ambulation (FAP 2); finally, patients become wheelchair-bound or bedridden (FAP 3). Also, in most studies, neuropathy is assessed through nerve conduction studies, Norfolk QOL-DN questionnaires, Neuropathy Impairment Score (NIS), NIS-lower limbs, NIS+7 scale and Six-minute walking test (6MWT)(85,86). In late-onset ATTRv, the large fiber involvement is generally more evident and small-fiber symptoms can be absent or subclinical. Nerve conduction studies disclose a predominantly sensory or sensory-motor axonal distal symmetric polyneuropathy that is stabilized through RNA silencers.

Of interest, muscle strength can be measured through handgrip tools(87). Handgrip tools are easy and cost-effective providing a quantitative measure of distal strength. Handgrip strength (HGS) test evaluates the force that a person can produce grasping an object, a physical characteristic strictly connected to carrying out the activities of daily living (ADL)(88). Indeed, a decrease in handgrip strength, common in some conditions such as in carpal tunnel patients, can negatively affect the quality of life (QoL)(89). Moreover, it is widely recognized that handgrip strength represents a valid marker for physical health, and it is considered a predictor for risk of falls in elderly(90). Since HGS is performed just with a dynamometer, it is an easy and cost-effective tool that allow to measure the maximum isometric handgrip strength of hand and

forearm muscles(90). HGS provides a quantitative measure of distal strength, and several studies confirm that HGS can be used as a diagnostic and prognostic tool in several chronic diseases, such as sarcopenia(91), and acquired neuropathy(92), such as carpal tunnel syndrome (CTS)(93) and hereditary neuropathy, like Charcot-Marie-Tooth(94).

Assessment of dysautonomia

Apart from a progressive sensory-motor neuropathy and cardiomyopathy, accompanying symptoms are reported in ATTRv, such as autonomic dysfunction, as well as gastrointestinal problems and unexplained weight loss (9). These self-reported symptoms are difficult to demonstrate and consequently often underestimated(79). Some questionnaires, such as COMPASS-31 scale and CADT, are commonly used in clinical practice, but an instrumental evaluation of dysautonomia through neurophysiological instruments is not systematically performed in most centres(85).

Bioelectrical Impedance Analysis (BIA) is a very sensitive tool to examine the composition of the body tissues in polyneuropathies and conditions with dysautonomia. Hence, BIA might accurately estimate the body composition in terms of muscle and fat masses as well as water contents in ATTRv patients, representing an indirect measure of dysautonomia and gastrointestinal function. Although BIA has been used to evaluate sarcopenia in diabetic neuropathy and

AL amyloidosis(95,96), to our knowledge, there are no studies yet assessing body composition through bioimpedance analysis in ATTRv patients.

Assessment of small-fiber neuropathy

The diagnosis and follow-up of peripheral neuropathies involving small-diameter nerve fibers require specific examinations beyond conventional nerve conduction studies which only concern large-diameter nerve fibers. Among these tests, some are dedicated to the investigation of cutaneous innervation by the autonomic nervous system, mainly by unmyelinated sympathetic C fibers. Indeed, there is a possibility that many pre-symptomatic carriers might present mild signs of small-fiber neuropathy, which might escape to conventional evaluation with nerve conduction studies and clinical scales(97). However, a definite diagnosis of pure small fiber neuropathy relies on specific diagnostic testing, such as quantitative sensory testing, skin biopsy, laser-evoked potentials (LEPs), and electrochemical skin conductance (ESC), which require considerable resources that may not be widely available(98,99). This kind of evaluation is still evading in ATTRv. Skin biopsy allows the estimation of intraepidermal nerve fiber density (IENFD), which might be a reliable biomarker of small-fiber neuropathy in ATTRv(97). Also, LEPs are the most widely agreed neurophysiological tool for investigating small fiber damage(100).

Finally, measurement of ESC by Sudoscan® is increasingly becoming a widely used technique, because it allows a quick and simple assessment of the sudomotor function of the limb extremities(101,102). This technique is based on the principles of reverse iontophoresis and chronoamperometry and since its introduction in 2010, has been the source of many recent studies. In the clinical field, most of these publications concern the evaluation of diabetic polyneuropathy, for which the value of Sudoscan® no longer needs to be demonstrated. However, there is also evidence for a role for Sudoscan® in the testing of the autonomic nervous system in various peripheral neuropathies of other origins such as ATTRv. However, there are a few studies exploring this technique in ATTRv patients(103,104).

Heart rate variability (HRV) is a useful tool to evaluate the impairment of cardiac autonomic control(105). The HRV represents the change in the time interval between successive heartbeats(105,106). HRV provides an index of the parasympathetic nervous system activity(105,107), whereas possible inferences on sympathetic components have been revised and rejected(105,108). The relationship between HRV and parasympathetic activity has been extensively described(109). The analysis of HRV has been extensively employed to study changes of the sympathovagal balance that occurs upon physiological responses of healthy subjects as well as in patients affected by cardiac or neurological diseases. It

is now well- established that the HRV is reduced in individuals affected by diabetes(110–112). Previous studies have demonstrated that dysautonomia can affect the parameters of Heart Rate Variability (HRV)(110). However, little is known about the specific autonomic changes associated with ATTRv.

The disadvantage of such techniques is low availability and lack of normative ranges as well as documentation of accuracy. Taken together, instrumental biomarkers for ATTRv polyneuropathy are on demand. Non-invasive instrumental tools might have a potential in the assessment of the severity of the disease and the beneficial effects of treatments. However, clinical trials and real-world studies have demonstrated the effect of patisiran on PND score, NIS scale, and questionnaires(113), but there are no instrumental data on muscle strength, dysautonomia and heart rhythm. Of note, the use of instrumental biomarkers for the evaluation of tissue damage in ATTRv might lead to a higher sensitive and specific approach; moreover, these biomarkers might contribute to detect the exact clinical onset of the disease in carriers of TTR mutation giving them the opportunity to be early treated when the polyneuropathy starts.

Chapter VIII. General organization of the project

The entire project consisted of three phases: a first phase with genetic screening of patients affected by polyneuropathy in Western Sicily and a second phase perspective evaluation of instrumental biomarkers of disease activity and response to treatment in patients affected by ATTRv before and during therapy with RNA silencers (patisiran).

Aims and objectives

- The first phase (PART 1) of the project had the objective to define the frequency of ATTRv in a real-world setting in patients affected by sensory-motor polyneuropathy and clinical features associated with the onset of the disease.

- The second phase (PART 2) was focused on the definition of instrumental biomarkers for disease activity and response to treatment in the population of patients included from the first phase. In this phase, instrumental data by means of Nerve conduction studies, Heart rate variability, Handgrip strength, and BIA have been collected in ATTRv patients and compared with age- and sex-matched controls.

- In a third phase (PART 3), instrumental data from patients started on patisiran have been collected and then compared at baseline and after 9 and 18 months of follow-up to evaluate their potential to measure the pathophysiological alterations of ATTRv amyloidosis and the effects of therapy with patisiran.

Ethical approval

The study was approved by the Ethical Committee of Palermo on 13 July 2020 (V. n.7/2020), and it was conducted in conformity with the Declaration of Helsinki principles.

PART 1: genetic screening for ATTRv

In this study, we performed a genetic screening on patients presenting to our Clinic for neuromuscular diseases with a sensory or sensory-motor polyneuropathy and one or more clinical feature suggesting ATTRv. We hypothesize that a systematic screening for ATTRv might contribute to significantly reduce the diagnostic delay of ATTRv in non-endemic areas, as well as ensuring the early treatment for this rare inherited disease.

This first *phase* aimed to evaluate the role of genetic testing in the diagnosis of ATTRv in a Centre specialized in neuromuscular diseases to establish the impact of ATTRv in a real-life context. Patients suspected to have ATTRv based on specific "red flags"(9,10) were enrolled in a two-year period. In a second phase, they went through genetic testing and, in selected patients, a biopsy to confirm TTR deposits. Clinical data have been collected from patients undergoing to transthyretin genotyping in the Neurological clinic specialized in the diagnosis and care of neuromuscular diseases of Palermo (Policlinico "Paolo Giaccone"). For each patient undergoing genetic testing the presence of specific "red flags" was investigated through a detailed questionnaire. In screened-positive patients, main misdiagnoses and

disease duration have been investigated and a "cascade screening" was proposed to first-degree family members according to the PADO(114). Then, clinical data were compared in patients with positive and negative genetics to identify the role of each red flag in a real-life context.

Patients' population

Inclusion criteria were: 1) informed consent for genetic testing; 2) age > 18 years; 3) presence of at least two red flags: a) bilateral carpal tunnel syndrome, b) sensory ataxia, c) lumbar canal stenosis, d) gastrointestinal symptoms, e) unexplained weight loss, f) autonomic dysfunction (erectile dysfunction, orthostatic hypotension, syncope), g) cardiomyopathy, h) renal impairment, i) ocular involvement (vitreous opacities) and/or l) family history of polyneuropathy, o) cardiopathy or n) ATTRv. Exclusion criteria were: 1) lack of informed consent; 2) non eligibility to genetic testing. For cascade screening we proposed genetic testing according to expert consensus statements and updated guidelines, considering the "Predicted Age of Disease Onset" (PADO) (i.e., we screen patients with Phe64Leu after 55 years according with expected clinical onset)(1,4,9).

Statistical analysis

The statistical analysis was applied to compare the TTR Mutation groups (Negative and Positive). In particular, the Mann–Whitney U test was used to test the only continuous variable (Age) and Interquartile

Range (IQR) was reported for overall population, negative and positive groups. The Chi-Square test was applied for the other categorical variables, and the proportions were reported also for overall population, negative and positive groups.

A comparison between screened-positive and negative patients was performed for each red flags and clinical features. For statistical analysis a p-value lower than 0.05 was considered significant.

Results

A total of 229 patients underwent genetic testing and a mutation in the TTR gene was found in 47 patients (22%). In particular, depending on the presence of clinical symptoms of ATTRv amyloidosis, genetic screening allowed a diagnosis of ATTRv in 14 families and early recognition of 21 symptomatic ATTRv patients and 26 asymptomatic carriers of TTR mutation. A diagram in Figure P1A reports screening procedures and enrolled patients.

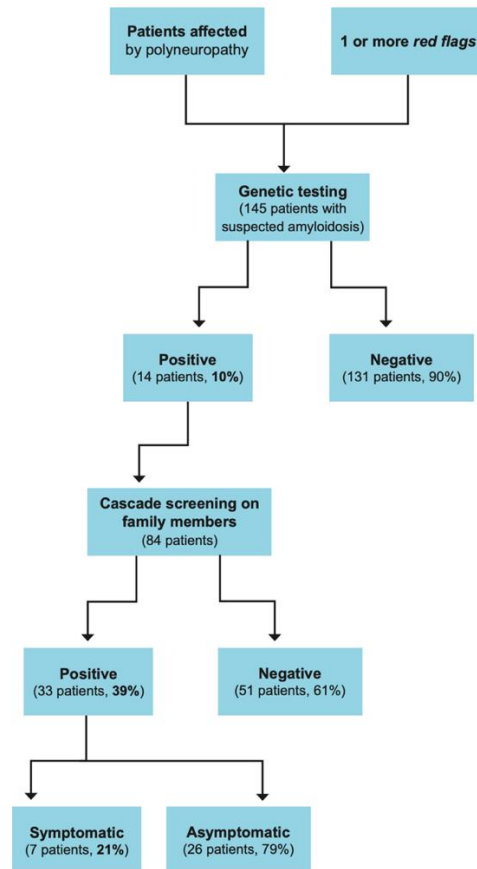**Figure P1A.** Screening procedures in patients affected by polyneuropathy.

Phase 1A: Screening patients affected by polyneuropathy

As a consequence of systematic screening, 145 patients affected by neuropathy underwent genetic testing resulting positive for mutations in the *TTR gene* in 14 cases (10%). Five mutations recurred among ATTRv patients with Phe64Leu being the most frequent genotype (Figure P1B).
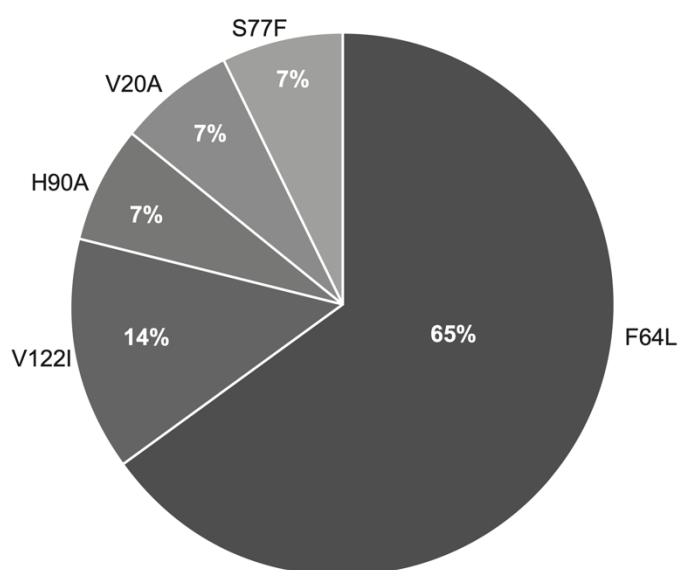
Figure P1B. Principal mutations encountered in the study sample.

Interestingly, His90Asn, Val20Ala and Ser97Phe have been reported for the first time in Sicily. Table P1A compares clinical findings in patients with positive and negative results in genetic testing.

Clinical features	ATTRv patients	Patients with Negative genetic testing	p-value
Gender (males)	8 (57%)	81 (62%)	0,47
Unexplained weight loss	9 (64%)	36 (27%)	0,007*
Bilateral carpal tunnel syndrome	10 (71%)	62 (47%)	0,07
Ataxia	8 (57%)	65 (50)	0,4
Lumbar canal stenosis	3 (21%)	31 (24%)	0,57
Gastrointestinal disturbances	9 (64%)	50 (38%)	0,05*
Autonomic dysfunction	9 (64%)	65 (50%)	0,22
Cardiopathy	5 (35%)	39 (30%)	0,42

Renal dysfunction	1 (7%)	17 (13%)	0,44
Ocular disorders	3 (21%)	35 (27%)	0,47
Family history of neuropathy	3 (21%)	16 (12%)	0,2
Family history of cardiopathy	8 (57%)	41 (31%)	0,05*

Table P1A. Clinical features in screened positive versus negative patients.

TTR mutated patients presented a higher frequency of unexplained weight loss (p=0.007), gastrointestinal symptoms (p=0.05) and family history of cardiopathy (p=0.05).

Bilateral CTS (10 patients, 71%), associated with unexplained weight loss, gastrointestinal disturbances, and autonomic dysfunction (9 patients, 64%) was the most recurring red flags among TTR-mutated patients. Of note, ataxia and family history of cardiomyopathy were present in 64% and 57% of positive cases, while family history of neuropathy was uncommon (21%). Renal and ocular dysfunction, as well as spinal lumbar stenosis were reported in a minority of cases. Finally, patients screening positive on genetic testing presented less frequently diabetes and autoimmune comorbidity compared to negative ones. Anorexia was the most common misdiagnosis (7%), followed by motoneuron disease (5%). Table P1B shows misdiagnoses in ATTRv patients detected through systematic screening.

Misdiagnoses	N=14 (%)
Anorexia nervosa	4 (29)
ALS	3 (21)
CIDP	2 (14)
Idiopathic polyneuropathy	2(14)
Diabetes	1 (7)
Cardiomyopathy	1 (7)
Lumbar canal stenosis	1(7)

Table P1B. Misdiagnoses in ATTRv patients. ALS, Amyotrophic Lateral Sclerosis; CIDP, Chronic Inflammatory Demyelinating Polyradiculoneuropathy.

Phase 1B: cascade screening on first-degree relatives with ATTRv

After diagnosis of 14 ATTRv symptomatic patients, 14 families have been detected. Hence, after diagnosis of the probands, a genetic screening was proposed to their first-degree relatives. Genetic testing was proposed to 104 relatives, but only 84 subjects (80%) gave consent for genetic testing. As a result, 33 individuals carrying TTR mutation have been detected (39%). After neurological evaluation, 7 patients (21%) presented sensory-motor polyneuropathy and were started on treatment, while 26 pre-symptomatic carriers of TTR mutation began regular follow-up.

Discussion

In the first phase of the study, we systematically evaluated the impact of clinical red flags, described in pivotal papers and guidelines, in predicting ATTRv diagnosis in a real-life setting. Genetic screening was offered to patients presenting with a sensory or sensory-motor polyneuropathy and one or more clinical feature suggesting ATTRv. The main result that should be underlined is that ATTRv amyloidosis is not uncommon in Sicily: indeed, a detection rate of 10% should encourage to formulate the clinical suspect of such treatable disease, especially considering its high mortality rates and costs for the society. The present survey is one of the most numerous on non-Val30Met patients reporting phenotype-to-genotype correlations. Studies regarding epidemiological data from different countries are very important worldwide and should be encouraged. As expected, most patients carried a Phe64Leu mutation (64%) and two subjects a Val122Ile mutation (14%). However, comparing the mutations' distribution found in previous study(55), several differences emerge: in our study, Phe64Leu frequency is almost twice, while the most prevalent mutation reported in that study (Glu98Gln) is absent in our population(55). Furthermore, we describe four mutations that were previously unreported in Sicily. The population referring to our clinic

was mostly descendent from Western Sicily ancestry (Palermo, Agrigento, and Trapani), while in the previous population studied were from the North and Eastern Sicily (Messina, Siracusa and Catania)(35). These new findings add new insight on the geographical distribution of ATTRv amyloidosis in our region (Figure P1C).
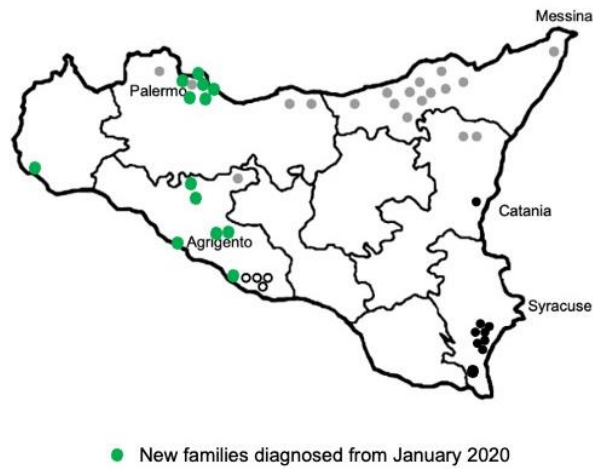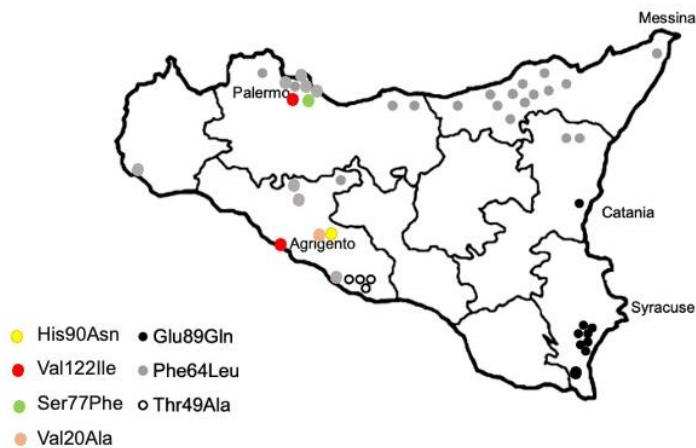

Figure P1C. In panel A new diagnoses are indicated in green; in B Geographical distribution of TTR mutations in Sicily is reported. Modified from Mazzeo et al, 2015(55).

From a diagnostic perspective, the clinical reasoning should be focused on misdiagnosis of ATTRv amyloidosis. Unfortunately, in such cases, the clinical presentation makes it difficult to distinguish ATTRv from other conditions, thus causing a significant misdiagnosis(34,71). Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), diabetes, sensory ataxia, and amyotrophic lateral sclerosis (ALS) are commonly considered misdiagnoses of ATTRv(34,71). Conversely, the most frequent misdiagnoses in our study were anorexia nervosa (4/14) and ALS (3/14); other misdiagnoses were CIDP (2/14), idiopathic neuropathy (2/14), diabetes, cardiomyopathy, and lumbar canal stenosis (1/14 each). It should be noted that CIDP was not confirmed as the most common misdiagnosis in our study, while anorexia and ALS explained 50% of cases. These results might be explained by the presence of axonal motor neuropathy and high frequency of significant weight loss in our population. In two cases, severe tongue atrophy (Figure P1D) and dysarthria have been reported in two unrelated women of 71 and 80 years carrying Phe64Leu and motor neuropathy. In such difficult cases, mild sensory deficits associated with depression had been overlooked due to the accompanying picture of relevant weight loss and motor impairment.

Figure P1D. Tongue atrophy in two patients carrying Phe64Leu mutation in the TTR gene misdiagnosed with ALS. In panel A an 80-years-old woman with a history of depression, anxiety, and weight loss of 10 kg in one year complaining of difficulty in ambulation and dysphagia; in B a 71-years-old woman with motor neuropathy and dysphagia.

Gastrointestinal involvement is frequent in ATTRv (80) and diarrhea, constipation or weight loss may be present since the onset of the disease, even anticipating neurological symptoms(45). Furthermore, gastrointestinal symptoms are insidious and can be misinterpreted as common conditions such as irritable bowel syndrome or functional dyspepsia, thus causing a relevant diagnostic delay (45). Of interest, while irritable bowel syndrome, unexplained malabsorption syndrome, protein-losing enteropathy secondary to ischemia and celiac disease have been hypothesized among the most common gastrointestinal misdiagnoses(41), anorexia nervosa was reported only anecdotally mimicking amyloidosis(115) (Table P1B). Weight loss and

gastrointestinal symptoms showed the same prevalence in TTR-mutated patients (64%), confirming their possible pathophysiological correlation(82,116). However, the prevalence of gastrointestinal symptoms did not reach a significant difference between the two groups, though a trend emerged; further analyses on larger populations might confirm the relevance of gastrointestinal involvement in ATTRv amyloidosis. Bilateral carpal tunnel syndrome (CTS), though being the most frequently recurring red flag, showed a high prevalence in both screening positive and negative patients, as it is also commonly associated to other causes of peripheral nerve disease (i.e., hereditary neuropathy, rheumatoid arthritis, diabetes mellitus, hypothyroidism)(117,118). However, our data confirm its strong association with ATTRv amyloidosis and, thus, its relevance as a red flag. Similarly, autonomic dysfunction and ataxia were often reported, but are too common in other forms of polyneuropathies to show a specific association with amyloidosis. Of interest, cardiomyopathy was frequently reported in family history of TTR-mutated patients (57%) but was uncommon in patients themselves (37%). This apparent inconsistency might be explained considering that cardiological phenotype can follow the onset of neuropathy in mixed phenotypes being hidden for several years(6). Indeed, Phe64Leu genotypes, which is characterized by predominant neurological involvement associated with mild cardiac involvement is advanced disease(55), was the most prevalent in our study sample; therefore, we hypothesize that this

phenotype might be more frequently found in older and unrecognized affected familiars rather than in younger patients with a more recent onset of symptoms. Furthermore, cardiac uptake on bone scintigraphy resulted only in one patient in this study. Musumeci et al have linked Phe64Leu mutation to a low bone scintigraphy sensitivity for the diagnosis of amyloid cardiomyopathy and our study seems to confirm this finding(73). Finally, sex distribution deserves some considerations: indeed, although ATTRv amyloidosis is considered a disease prevalent in males(13), M:F ratio was 6:8 in this sample of ATTRv patients. Phe64Leu mutation was reported as predominant in females (M:F ratio 1:22) in a population from Lazio(39); in Sicily, a M:F ratio of 16:12 was reported(55). It should be noted that the cited reports included asymptomatic carriers; similarly, our study included 26 asymptomatic carriers.

A last consideration from these data is the importance of cascade screening. Indeed, cascade screening of first-degree relatives allowed prompt recognition of further 7 ATTRv patients who presented mild sign of sensory neuropathy which were undetected before and overlooked by clinicians. These "lucky" patients had the opportunity to be early treated in the first stage of the disease (FAP 1) with good response to treatment with RNA silencer. Also, 26 asymptomatic carriers attend to regular follow-up. It is reasonable to state that the prognosis of both symptomatic and asymptomatic carriers of TTR mutation will improve thank to the early diagnosis due to the

availability of effective treatments.

PART 2: instrumental evaluation of ATTRv patients. Research for biomarkers of disease activity

In this second prospective phase, we described the instrumental assessment of a cohort of ATTRv patients. After the first phase of the project, patients affected by ATTRv diagnosed through genetic screening giving informed consent for study participation have been enrolled to the second phase.

The clinical and instrumental evaluation included: body weight and height; Coutinho stage; Neuropathy Impairment Score (NIS); Karnofsky performance status (KPS); Norfolk QOL Questionnaire; Six-minute walking test (6MWT); Nerve conduction studies (NCS); Handgrip measures; Bioimpedance analysis.

Coutinho stage

Familial amyloidotic polyneuropathy (FAP) staging is based on the walking ability of the patients: stage 0 is for asymptomatic patients; stage I identifies patients with symptoms but unimpaired ambulation; stage II patients require assistance for walking; stage III patients are wheelchair-bound or bedridden(85).

Neuropathy Impairment Score (NIS)

NIS evaluates strength, reflexes, and sensation(85). It is one of the most well-known clinical instruments to evaluate and quantify the burden of neuropathy. It ranges from 0 to 192 points, with higher scores indicating a worse impairment. We calculated the total score (NIS, muscle weakness, muscle stretch reflexes, and sensation), the motor component alone for the whole body (NIS-W, muscle weakness) and the composite sub-score, by anatomic region, for the upper limbs (right and left NIS-W_{UL}).

Karnofsky performance status (KPS)

The KPS score was used to quantify the subjects' ability to perform normal daily life activities and their need for assistance (ranging from 0 [dead] to 100 [normal; no complaints])(63).

Norfolk QOL Questionnaire

The Norfolk QOL(119) is a 35-item questionnaire designed to evaluate the impact on the patient's life of symptoms related to neuropathy, ranging from -2 (best QOL) to 138 (worst QOL). It has been used to assess quality of life in patients affected by ATTRv amyloidosis treated with tafamidis(61), inotersen(29), patisiran(120), and vutrisiran(30).

Six-minute walking test (6MWT)

6MWT is an easy clinical test that estimates the patient's performance

in daily activities; it is a measurement used to assess the functional capacity, i.e., the aerobic endurance(121). Although it has been extensively used for cardiopulmonary diseases, it has been used only anecdotally on familial amyloidotic neuropathy(62); however, Vita et al. proved its reliability in the evaluation of ATTRv patients with neuropathy but without cardiovascular involvement(122). 6MWT is conducted by measuring the distance covered in a period of 6 minutes by the patient, walking quickly on a flat, hard surface(123). Each participant was asked to walk as much as possible for 6 minutes in a flat corridor where two cones, placed 30 meters from each other, marked the turning points. At the verbal command of the researcher, each participant, placed upright next to one of the two cones, began to walk to the other cone, and then back, and so on. The distance walked in 6 minutes measured in meters was recorded, i.e., the 6-minute walk distance.

Nerve conduction studies (NCS)

NCS were performed in both median nerves for all subjects enrolled according to standard procedures (i.e., bipolar surface stimulating electrodes delivering rectangular pulses 0.1-0.5ms in duration and recording electrodes placed over the recording site with a ground electrode placed between recording and stimulation electrodes)(124). In particular, the study protocol was defined as follows:

-for upper limb SNAPs: stimulation at wrist and registration from II

digit (medial nerve) and V digit (ulnar nerve).

-for upper limb CMAPs: stimulation at the wrist and elbow and recording from abductor pollicis brevis (APB) for median nerve; stimulation at the wrist and elbow 4 cm distal from the medial epicondyle of the humerus and recording from abductor digiti minimi (ADM) muscle for ulnar nerve.

-for lower limb SNAPs: stimulation at posterior-lateral calf, recording from lateral malleolus for sural nerve.

-for lower limb CMAPs: stimulation at the medial malleolus and popliteal fossa, recording from abductor hallucis brevis (AHB) muscle for tibial nerve; stimulation at anterior ankle and popliteal fossa, recording from extensor digitorum brevis (EDB) muscle for peroneal nerve.

Distal motor latency (DML), compound motor action potential (CMAP) amplitude and motor conduction velocity (MCV) have been analyzed in motor conduction studies. Distal sensory latency (DSL), sensory nerve action potential (SNAP) amplitude and sensory conduction velocity (SCV) have been analyzed in sensory conduction studies.

Handgrip test (HGS)

Each participant carried out the handgrip test as recommended by the American Society of Hand Therapists(125), that is, in a sitting position with back leaning against the backrest of the chair and elbow joint positioned at a 90° angle. At the verbal command of the researcher, each

participant had to tighten the handle of a mechanical dynamometer (KernMap model 80K1 - Kern®, Kern & Sohn GmbH, Balingen, Germany) exerting the maximum isometric handgrip strength for 3 s (Figure P2A). Each participant performed 3 trials both with the dominant and the non-dominant hand with 3 min rest between trials. The best trial was considered for statistical analyses(126).

Figure P2A. A subject during the Handgrip test in the left hand.

Bioelectrical Impedance Analysis (BIA)

BIA is a non-invasive, radiation-free examination that evaluates body composition using a device that allows to estimate the quality of different tissues (e.g., fat and muscle) since it is able to measure the different electrical conductivity of the tissues (Figure P2B). In detail, the body is crossed by a low voltage alternating current generated by the device which, in turn, measures the impedance (resistance and reactance of the tissues) (127). BIA allows to estimate body cell mass

(BCM), extracellular water (ECW), fat mass (FM) and fat-free mass (FFM). Prior to the BIA evaluation, age and weight of each participant were entered into the software (Bodygram, Akern; Montacchiello, Pisa, Italy). Then, each participant was asked to lie down on a cot and the surface electrodes were applied on the right-hand and right-foot and the device (BIA 101, Akern; Montacchiello, Pisa, Italy) measured the body composition.
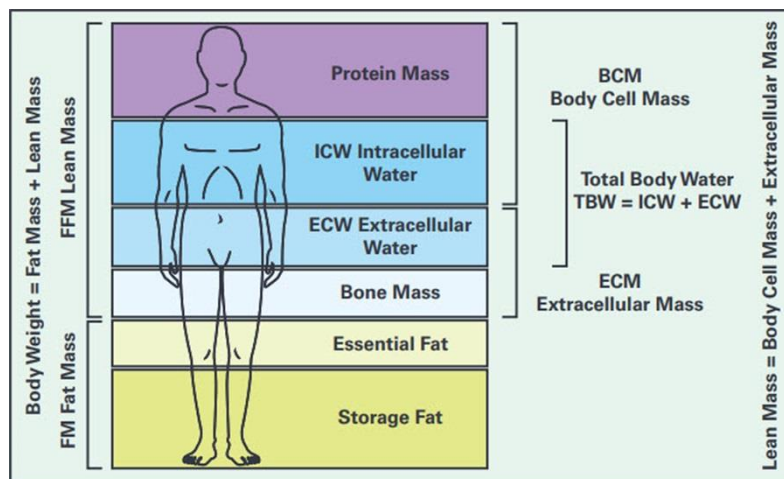
Figure P2B. Body composition assessed by Bioelectrical Impedance Analysis (BIA).

Heart rate variability (HRV)

Bipolar electrocardiogram (EKG) recordings from lead I of a 12- lead EKG were carried out utilizing the EKG channel of the EBN- Neuro EEGNet System (EBN Neuro–Florence Italy). EKG data were sampled at a frequency of 256 Hz and exported from the EBN system (EEGNET, Florence, Italy) in the European Data Format (EDF). All data were subsequently processed using dedicated software for HRV analysis

(Kubios, HRV software version 2.1, University of Eastern Finland, Kuopio, Finland). The software identified QRS complexes and R peaks using a multiscale wave- let-based peak detection algorithm. Before proceeding with the HRV analysis, all the RRI samples were visually inspected to remove any artifacts, extrasystoles, and erroneously detected R waves or insertions of missed R beats. A short-term recording analysis (49) (time-series length = 5 min) was performed to assess heart rate variations in time and frequency domains as well as non-linear analysis.

The time-domain methods are derived from the beat-to-beat R.R. interval values in the time domain. HRV parameters that measure the variability within the R.R. time intervals in the time-domain assessed in terms of (1) mean R.R. (the mean heart rate in a precise R.R. sequence); (2) SDNN (standard deviation of all R.R. intervals); (3) RMSSD (root mean square of the difference of adjacent R.R. intervals); (4) pNN50 (the percentage of successive R.R. intervals differing more than 50 ms); (5) HRV triangular index (integral of the density of the R.R. interval histogram divided by its height), and (6) TINN (baseline width of the R.R. interval histogram). According to the current literature, short-term analysis of SDNN and RMSSD is the most reliable HRV time-domain parameters. SDNN assesses sympathetic and parasympathetically mediated HRV variations. Frequency-domain measurements estimate the distribution of absolute or relative power into four frequency bands. The power spectral density (PSD) of the R.R. series was calculated

using parametric methods (based on self-regressive models, AR). PSD was analyzed by calculating the frequency of waves for the different frequency bands. According to the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (1996), H.R. oscillations should be analyzed taking in consideration selected frequencies: Very Low Frequency (VLF, 0–0.04Hz), Low Frequency (LF, 0.04–0.15 Hz), and High Frequency (HF, 0.15–0.4 Hz). The most common frequency domain parameters include the powers of the bands VLF, LF, HF expressed in absolute (VLF [ms2], LF [ms2], and HF [ms2]) and relative values (VLF%, LF%, and HF%), the normalized power of the LF and HF bands (LF n.u. = LF [ms2]/(total power [ms2] − VLF [ms2]; HF n.u. = HF [ms2]/(total power [ms2] − VLF [ms2]), and the LF/HF ratio.

Spectral analysis allows the discrete analysis of different autonomic components. HF band reports the parasympathetic components, whereas the interpretation of the LF band is controversial. VLF, LF, and HF bands can be expressed in absolute (ms2) or relative (expressed in % or n.u.) units, but absolute values are preferred(105).

Statistical Analysis

The Shapiro-Wilks test was used to detect normality among the distribution of variables. If data presented a normal distribution, a paired t-test was used to detect differences between groups. If variables were not normally distributed, a Wilcoxon signed rank test was adopted

instead. Subgroup analysis concerning gender was carried out on significant parameters. Absolute differences between post and pre-values have been calculated. Spearman's correlation was finally carried out to identify associations among performance and clinical variables. Significance was set at 0.05 for all analysed variables.

Part of this project has been published on January 2022(87).

Results

Patient Demographics and Clinical Features

Twenty patients affected by TTR amyloidosis (66.1±8.37 years, 8 females) and 30 controls (61.1±11.56 years, 16 females) participated in the study. Genetic testing confirmed a mutation in heterozygosis in the *TTR* gene in all patients enrolled. F64L (p.F84L) mutation was encountered in 15 patients, followed by E89Q (p.E109Q) in 2, V122I (p.V142I) in 2 and H90A (p.H110A) in 1 patient. The most frequent symptoms were carpal tunnel syndrome (50%), cardiomyopathy (44%), weight loss (38%) and autonomic dysfunction (28%). Controls have been selected among patients' relatives who screened negative to genetic testing for *TTR* mutations. All patients underwent a complete neurological assessment, neurophysiological evaluation, and handgrip analysis. No significant differences were retrieved for anthropometric characteristics between the two groups (Table P2A).

Table P2A. Descriptive characteristics of participants.

	Age (years)	Height (cm)	Weight (kg)
TTR	66.1±8.37	166.2±10.90	70.5±18.56
Controls	61.1±11.56	166.1±9.02	77.2±12.07

*Data are presented as means±st.dv. *significant.*

Clinical evaluation

Fifteen patients presented mild symptoms (FAP I) and 5 patients had moderate-severe symptoms (FAP II). ATTRv patients presented a mean NIS of 37.15 ± 30.71, NIS-W 18.54 ± 22.4. In the right upper limb NIS-W_{UL} scored 3.83 ± 5.49, while in the left was 3.61 ± 4.9.

The data indicate that at increased pathology severity as indicated by FAP, increased presence of bilateral carpal tunnel syndrome and increased overall symptoms of neuropathy (Table P2B). A concomitant decrease of the CMAP amplitude of the median nerve was also observed (Figure P2C). FAP degree alone may explain 45% of the decrease of the median nerve amplitude. Also, when severity of disease is associated to bioimpedance analysis (BIA), we observe increased presence of weight loss with increased severity, which is associated to a reduction only in lean muscle mass. Increased FAP is also strongly associated to a reduction of the phase angle.
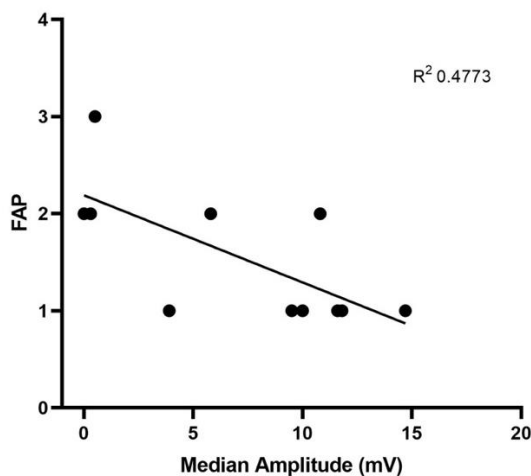
Figure P2C. Correlation between FAP grade and CMAP amplitude in the median nerve.

Handgrip measures

When evaluating grip strength for both hands, significant differences were retrieved between patients and controls in the right (handgrip right, HGS_R, TTR 21.1 ± 13.0 kg vs Control 29.4 ± 11.3 kg, p=0.017) and left (handgrip left, HGS_L, TTR 22.2 ± 10.7 kg vs Control 31.0 ± 11.3 kg, p=0.007, Figure P2D).

Figure P2D. Handgrip measures in ATTRv patients and controls for the right and left hands.

Neurophysiological data

In the right median nerve, a significant difference was reported between ATTRv patients and controls for DML and MCV (Table P2B). In the left median nerve, the difference was more pronounced and

demonstrated for all parameters (DML, CMAP amplitude and MCV). A significant correlation was found between the right median nerve DML and CMAP amplitude (-0.34, p=0.049), as expected due to the presence of CTS.
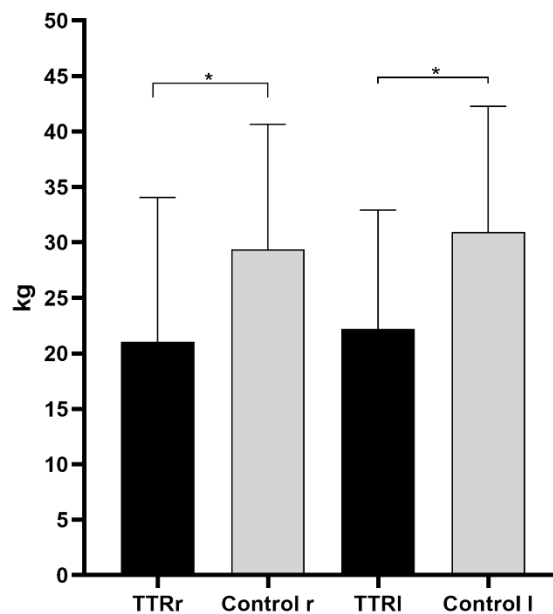
	ATTRv patients	Controls	P value
Right median nerve			
DML (ms)	4.4±1.6	3.0±0.4	<0.0001*
CMAP Amplitude (mV)	7.4±5.0	8.9±2.2	0.66
MCV (m/s)	48.4±5.0	56.6±5.1	<0.0001*
Left median nerve			
DML (ms)	4.4±1.6	3.2±0.7	0.012*
CMAP Amplitude (mV)	5.6±2.9	8.9±1.0	0.001*
MCV (m/s)	48.2±4.4	53.4±2.5	<0.0001*

Table P2B. Neurophysiological variables relative to the EMG of the median nerve in ATTRv patients and controls. *Data are presented as means±st.dv; NA, not applicable; FAP, familiar amyloid polyneuropathy; TTR, transitiretine; DML, distal motor latency; CMAP, compound motor action potential; MCV, Motor Conduction Velocity. *Mann Whitney U test, p<0.05.*

Correlations between clinical, neurophysiological and handgrip measures. Clinical and neurophysiological variables for the ATTRv group (Described in Table P2B) have been further analyzed and related to the HGS.

Correlations between clinical and HGS measures. Total NIS score was negatively correlated with both HGS_R (r=-0.45, p=0.033) and HGS_L (r=-0.50, p=0.015). Also, the motor sub-scores NIS-W were negatively

correlated with HGS$_L$ (r=-0.44, p=0.035); a similar correlation was present for the right side, but it was not statistically significant (r=-0.39, p=0.06). NIS-W$_{UL}$ scores were negatively correlated with homolateral HGS measured for both the right (r=-0.43, p=0.039) and left upper limbs (r=-0.46, p=0.027).

Correlations between clinical and neurophysiological measures. Total NIS score was negatively correlated with CMAP amplitude of both right (r=-0.58, p=0.014) and left (r=-0.61, p=0.01) median nerves. The motor sub-scores NIS-W was negatively correlated with the CMAP amplitude of the left median nerve (r=-0.44, p=0.035). NIS-W$_{UL}$ scores were not correlated with any neurophysiological data.

Correlations between neurophysiological and HGS measures. The CMAP amplitude of the right median nerve showed a positive correlation with HGS$_R$ (r=0.54, p=0.026). Similarly, we found a positive correlation between CMAP amplitude of the left median nerve and HGS$_L$ (r=0.56, p=0.02). No significant correlations were established between DML and VCM of both median nerves and HGS$_R$ or HGS$_L$.

Bioelectrical Impedance Analysis (BIA)
Body composition was not significantly different between ATTRv patients and controls. In particular, the patients exhibited a slightly reduced weight, BCM, FM, and FFM compared to controls, even if no

significant difference was demonstrated (Table P2C). Conversely, a not significant reduction in the PA was observed in ATTRv patients.

	ATTRv patients	*Controls*	*P value*
FFM (kg)	*49.8±10.5*	*52.6±10.0*	*0.46*
BCM (kg)	*24.0±8.8*	*26.8±7.0*	*0.116*
FM (kg)	*20.8±11.5*	*23.9±8.1*	*0.29*
TBW (l)	*37.7±8.6*	*38.7±7.8*	*0.59*
ECW (l)	*19.4±5.7*	*18.8±4.1*	*0.66*
PA	*5.2±2.4*	*5.5±1.4*	*0.16*

Table P2C. The table shows data on body cell mass (BCM), extracellular water (ECW), fat mass (FM), fat-free mass (FFM) and phase angle (PA) in the two groups.

Heart rate variability (HRV)

The two groups showed no differences in any parameters: MeanRR (p=0.16), SDNN (p=0.16), RMSSD (p=0.17), TINN (p=0.087), pNN50 (p=0.12), VLF [ms2] (p=0.052), VLF% (p=0.23), LF [ms2] (p=0.34), LF% (p=0.43), HF [ms2] (p=0.43), HF% (p=0.47), LF/HF ratio (p=0.53). Hence, only VLF [ms2] showed a different distribution in ATTRv patients and controls (33.2±39.6 vs 1448.8±5738.6 ms2).

Discussion

Nowadays, ATTRv is a tractable disorder, in which a prompt diagnosis is needed, and biomarkers are on demand(128). Many molecules have been studied for cardiac phenotypes, such as serum retinol-binding protein 4 or B-type natriuretic peptide and transtiretin(129). However, there are only a few studies on biomarkers for nerve damage in ATTRv. Of interest, serum neurofilament light chain has been recently studied in ATTRv(130), but there are only preliminary results available(128), showing a correlation with the severity of polyneuropathy, thus proposing NfL as a biomarker for nerve damage showing a significant decrease after therapy with Patisiran(130).

Handgrip test is a rapid, simple, and non-invasive tool that can be used for strength evaluation in patients affected by neuromuscular diseases. Furthermore, it is an attractive tool since it can be easily performed and does not require expensive equipment (Figure P2A). Several studies have showed how handgrip test is altered in polyneuropathies, such as diabetic one, in which patient show a significant reduction of strength(131). Moreover, some authors tried to use it as an outcome measure, showing promising results in CMT(132). However, to date, few authors used handgrip test in the evaluation of familial ATTRv, showing significant strength reduction(133).

Here, we aim to confirm an alteration in handgrip strength in patients affected by ATTRv and to correlate HGS data with clinical and neurophysiological findings. In particular, since the high prevalence of carpal tunnel syndrome in ATTRv(75), we argued to correlate the reduced strength due to CTS with pathological findings in the median nerve motor conduction studies.

The present data directly compared NCS from the median nerve and HGS in patients affected by ATTRv and healthy controls(87). HGS demonstrated a significant reduction of strength in both right and left hands in patients compared to controls (Figure P2B); also, the entity of strength reduction was similar in the right and left side. Moreover, NCS showed a prolonged DML in both right and left median nerves in patients, but no in controls (Table P2B), while CMAP amplitude was different only in left median nerves of ATTRv patients (p=0.012). This result might be explained by the high prevalence of CTS in the dominant hand (more often right) in general population, because also controls presented a similar mean right CMAP amplitude of the median nerve. Moreover, when present, CTS in ATTRv is usually bilateral, underlining the importance of detecting left (non-dominant) or bilateral CTS in ATTRv, as a specific finding of amyloidosis, less often encountered in routine NCS(75,124). Indeed, while bilateral CTS is an early sign of ATTRv, CTS in the dominant hand is the rule in general population, especially in the elderly(134).

Our data showed that the combined use of neurophysiology and HGS

might represent a simple and unexpensive way to assess the motor compromission in ATTRv. As expected, DML of the right median nerve was negatively correlated to the CMAP amplitude, but not with HGS in ATTRv patients. Conversely, CMAP amplitude of both right and left median nerve was positively correlated with left HGS. Indeed, the CMAP amplitude is an expression of the number motor fibers and consequently the motor units who can generate the maximum strength(135). On the contrary, DML depends on direct compression and local demyelination in the median nerve at wrist in CTS, which is not related with ATTRv itself and it is not related with motor strength. Hence, we can assume that reduced strength measured at HGS reflects CMAP amplitude in the median nerve.

Of interest, relevant correlations have been reported between CMAP amplitude of the median nerve and both NIS and HGS measures. This finding suggests that a reduction in CMAP amplitude of the median nerve might cause a consequent increase in NIS scores and a reduction of strength measured by HGS. However, when assessing the upper limbs alone (without considering the score from the lower limbs), the correlations between clinical and neurophysiological data disappear; on the contrary, the relationship between NIS-WUL and HGS for both the right and left side persist. These data support the use of HGS measures, suggesting that they might be more sensitive than neurophysiology alone in the assessment of the strength in the upper limbs. Indeed, despite being a very sensitive and reliable tool in the clinical onset,

conventional neurophysiology might be less reliable in the follow-up, when motor nerves become not elicitable or too altered to allow an immediate correlation with residual strength(136,137). On this perspective, HGS may offer the possibility to quantify the residual strength in the hand and appreciate even little changes that might escape to conventional neurophysiology.

Regarding BIA, not significant difference was found between patients and controls. This result might be explained by the low study sample, as a reduction in some parameters seem to be coherently present in ATTRv patients.

HRV data seem to suggest minimal differences, but these results are not significant too, apart from VLF which was reduced in ATTRv patients compared to controls. However, as recommended by the European Society of Cardiology (ESC) guidelines(105), VLF assessment in a short- term EKG analysis is of dubious value, and its interpretation should be avoided. Hence, more studies are needed to explore the role of HRV as a marker of disease.

PART 3: instrumental evaluation of ATTRv patients treated with RNA silencer. Research for biomarkers of response to treatment

For the third part of the project all patients who participated to phase 2 have been proposed to start the phase 3. Exclusion criteria were the absence of symptoms of ATTRv polyneuropathy or the treatment with therapies other than patisiran. Patients enrolled have been evaluated at the time of starting therapy with patisiran and after 9- and 18-months of follow-up.

Study procedures

Patients enrolled received the first dose of patisiran on T0. The treatment with patisiran was scheduled as for therapeutic protocol (0.3 mg per kilogram of body weight once every 3 weeks). Clinical and instrumental evaluation were performed at T0 (enrollement), T1 (after 9 months) and T2 (after 18 months) visits.

Patient Demographics and Clinical Features

Sixteen patients affected by ATTRv amyloidosis satisfied inclusion criteria and participated in the study, but one dropped out due to a femur fracture after T0 and another one month before the end of the study due to an ischemic stroke with fatal outcome. Hence, fifteen (66.4±7.8

years, 6 males) completed the follow-up at 9 months and 14 patients the follow-up at 18 months. The most frequent symptoms were carpal tunnel syndrome (80%), gastrointestinal disturbances (60%), ataxia (50%) weight loss and autonomic dysfunction (45%).

Clinical and instrumental evaluation

Clinical and instrumental assessment was the same of the phase 2. The clinical and instrumental evaluation included: body weight and height; Coutinho stage; Neuropathy Impairment Score (NIS); Karnofsky performance status (KPS); Norfolk QOL Questionnaire; Six-minute walking test (6MWT); Nerve conduction studies (NCS); Handgrip measures; Bioimpedance analysis.

Statistical Analysis

The Shapiro-Wilks test was used to detect normality among the distribution of variables. If data presented a normal distribution, a paired t-test was used to detect differences between baseline and post-9-month and 18-months of treatment. If variables were not normally distributed, a Wilcoxon signed rank test was adopted instead. Subgroup analysis concerning gender was carried out on significant parameters. Absolute differences between post and pre-values have been calculated. Spearman's correlation was finally carried out to identify associations among performance and clinical variables. Significance was set at 0.05 for all analysed variables.

Part of this project has been published on December 2022(138).

Results

T1: Evaluation at 9 months of follow-up

Genetic testing confirmed a mutation in heterozygosis in the *TTR* gene in all patients enrolled. F64L (p.F84L) mutation was encountered in 11 patients, followed by E89Q (p.E109Q), V122I (p.V142I), H90A (p.H110A) and S77F (p.S97F) in 1 patient, respectively. Table P3A shows clinical data of 15 ATTRv patients included in the study at baseline and after 9-months follow-up. Twelve patients presented mild symptoms (FAP stage 1) and 3 patients had moderate-severe symptoms (FAP stage 2).

Clinical scales

Among clinical variable and scales included, FAP stage, KPS, NIS, NIS-W, Norfolk, and COMPASS-31 scale resulted unchanged following 9 months of treatment with patisiran. Nerve conduction studies demonstrated axonal neuropathy in all subjects in absence of significant differences between baseline and follow-up evaluations. However, a significant increase after 9 months of treatment was observed for the 6MWT (229.6±72.6 vs 260.9±69.8 m, p = 0.033).

	Baseline	**Post 9 month**	**p**
Anthropometric and demographics			
Age (years)	66.4±7.8	66.9±7.7	ns
Height (cm)	163.0±10.9	163.0±10.9	ns
Weight (kg)	70.3±19.8	73.1±21.1	0.044
Clinical evaluation			
FAP stage	1.13±0.5	1.13±0.5	ns
Karnofsky performance status	72.7±13.8	75.3±14.1	ns
NIS	30.9±29.2	31.4±25.6	ns
NIS-W	14.6±17.8	14.7±14.5	ns
Norfolk	51.0±31.6	47.8±29.5	ns
COMPASS-31	18.9±9.1	19.8±9.2	ns
6MWT (m)	229.6±72.6	260.9±69.8	0.033
Body Composition			
FFM (kg)	48.8±11.9	52.0±12.1	0.005
BCM (kg)	23.1±7.5	26.4±7.8	0.014
FM (kg)	21.5±9.8	20.7±8.5	0.012
TBW (l)	36.5±9.2	38.4±9.1	ns
ECW (l)	19.0±4.9	18.7±4.5	ns
Strength			
HG R	20.6±13.8	21.4±13.9	ns
HG L	22.5±11.8	22.1±10.9	ns

Table P3A. Body composition, strength and 6-minute walking test outcomes. Data are presented as means±st.dv; ns not significant. Ns= not significant. FAP: Familial amyloidotic polyneuropathy; NIS: neuropathy impairment score; NIS-W: neuropathy impairment score muscle weakness; 6MWT: 6-minute walking test; BCM: Body Cell Mass; ECW: Extra Cellular Water; FFM: Free Fat Mass; FM: Fat Mass; HG: Hand Grip; L: Left; R: Right; TBW: Total Body Water.

Bioelectrical Impedance Analysis (BIA)

Body composition significantly changed following the 9-months pharmacological treatment. Figure P3A shows data FM, FFM and BCM at baseline and after 9 months of treatment with patisiran. In particular, the patients exhibited an increase in FFM (pre 48.8±11.9kg vs. post 52.0±12.1 kg, p = 0.005), which also translated to an increase in BCM

(pre 23.1±7.5 vs. post 26.4±7.8 kg, p = 0.014). Conversely, a significant decrease in FM (pre 21.5±9.8 vs. post 20.7±8.5 kg, p = 0.012) was observed. Overall, this led to an increase in body weight from 70.3±19.8 to 73.1±21.1 kg (p = 0.044). No significant differences neither in TBW and ECW were observed.
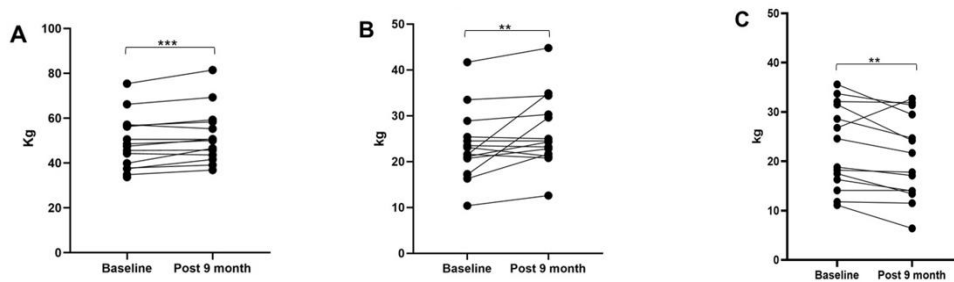

Figure P3A. Body composition at baseline and after 9 months of treatment with patisiran. Panel A represents individual patiens data for FFM: Free Fat Mass; Panel B represents individual patients' data for BCM: Body Cell Mass; Panel C represents individual patients' data for FM: Fat Mass. **p<0.05; ***p<0.01.

Handgrip strength (HGS)

Regarding performance measures, despite increased muscle mass was observed, no significant differences have been evaluated for measures of the upper limbs, neither for the right nor the left limb. Details of performance measures are shown in Table P3A.

Correlational analysis

Differences between male and female participants were also calculated for all above-mentioned variables. No significant gender difference was observed for any analysed variable. Correlational analysis detected that

significant and meaningful associations were present among the 6MWT and BCM (r=0.63, p=0.012), the 6MWT and the Norfolk scale (r=-0.76, p=0.001) and the 6MWT and both HG (r=0.61, p=0.016, right and r=0.60, p=0.016 left). In addition, the Norfolk scale was also negatively correlated with BCM (r=-0.61, p=0.016), and both HG (r=-0.69, p=0.005, right and r=-0.68, p=0.004 left). respectively. While BCM was also positively associated to the HG measures (r=0.79, p=0.001, right and r=0.85, p=0.001 left).

T2: Evaluation at 18 months of follow-up

Table P3B shows clinical data of 14 ATTRv patients who concluded the study with 18-months of follow-up. Unfortunately, one patient died after an ischemic stroke unrelated to ATTRv. Genetic testing confirmed a mutation in heterozygosis in the *TTR* gene in all patients enrolled. F64L (p.F84L) mutation was encountered in 11 patients, followed by E89Q (p.E109Q), V122I (p.V142I), and H90A (p.H110A) in 1 patient, respectively.

Clinical scales

Figure P3B shows the severity of disease assessed by NIS, Norfolk, and COMPASS-31 in the study population according to genotype. NIS was higher in Phe64Leu mutation, as expected for the typical neuropathic phenotype, while Norfolk scores underlined high impact in quality of life from His90Asn mutation. COMPAS-31 displayed a more severe

impairment following Glu89Gln.
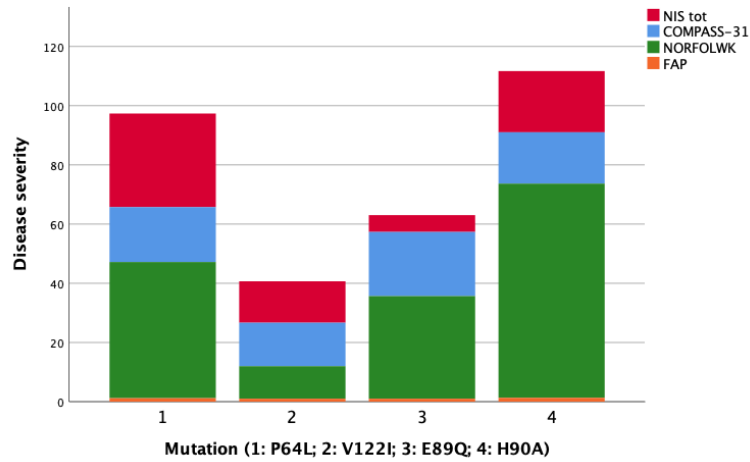
Figure P3B. Severity of ATTRv assessed by NIS, Norfolk, and COMPASS-31 in several mutations in the *TTR* gene.

Also, in accord with data from APOLLO study, a not statistically significant reduction in mean scores from clinical scale was observed (NIS, Norfolk and COMPASS-31, Figure P3C) at 9 and 18 months of follow-up.
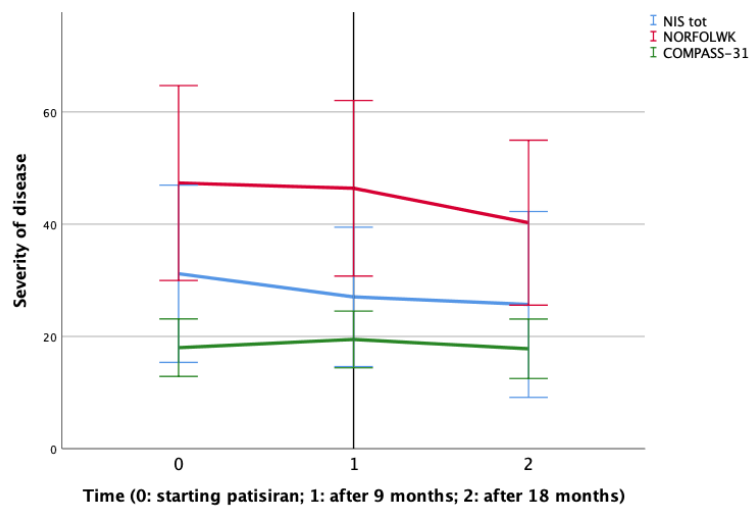

Figure P3C. Severity of ATTRv assessed by NIS, Norfolk and COMPASS-31 at baseline (T0) and after 9 (T1) and 18 (T2) months of follow-up.

A reduction of NIS score was more pronounced at T1 (about 3 points less), with a stabilization at T2 (further 1 point less), while Norfolk seemed to have a reduced improvement at T1 with a benefit at T2 evaluation (6 points less at T2). Finally, COMPASS-31 seems not change at T1 and T2. Moreover, reduction of scores from clinical scales was accompanied by an increase in mean 6MWT (Figure P3D). Of interest, an increase of about 24 mt was observed at T1 and further 9 mt at T2 evaluation. Nerve conduction studies demonstrated axonal neuropathy in all subjects in absence of significant differences between baseline and follow-up evaluations.

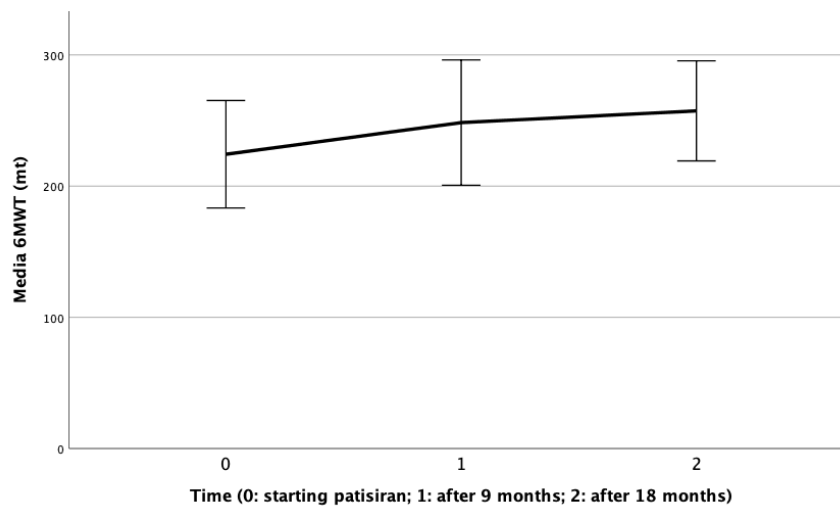**Figure P3D.** Severity of ATTRv assessed by 6MWT at baseline (T0) and after 9 (T1) and 18 (T2) months of follow-up.

Bioelectrical Impedance Analysis (BIA)

Considering analysis on 14 patients who completed the study, no significant differences have been found in BIA parameters. However, similarly to analyses performed at 9 months, Body composition changed following the 9-months pharmacological treatment, but not at

18 months. In particular, the patients exhibited a slight increase in FFM associated with an increase in BCM at 9 months. Conversely, a minimal decrease in FM was observed. Overall, this led to an increase in body weight from 69.5±19.6 to 71.1±18.0 kg. No significant nor absolute differences were observed these parameters at 18 months (Figure P3E). Finally, phase angle (PA) was altered in ATTRv patients. Moreover, a not significant increase of the phase angle (PA) was observed at 9 months with stabilization at 18 months.
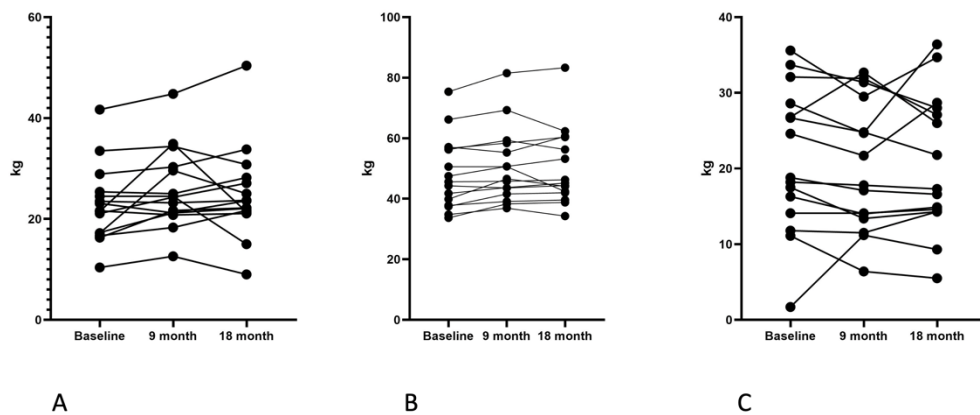
Figure P3E. Body composition at baseline and after 9 and 18 months of treatment with patisiran. Panel A represents individual patients' data for BCM: Body Cell Mass; Panel B represents individual patients' data for FFM: Free Fat Mass; Panel C represents individual patients' data for FM: Fat Mass.

Heart rate variability (HRV)

SDNN (22.2±22.8ms) and RMSSD (25.7±32.1ms) values indicated cardiac involvement (considering normal SDNN>50ms; RMSSD>30ms) in ATTRv. Moreover, LF/HF > 2 at baseline suggested an orthosympathic predominance, which tended to be reduced after

treatment at T1 and T2. However, no differences have been reported in any parameters among T0, T1 and T2 evaluations (MeanRR, SDNN, RMSSD, TINN, pNN50, VLF, VLF%, LF, LF%, HF, HF%, LF/HF ratio).

Correlational analysis

Correlational analysis detected that significant and meaningful associations were present between the 6MWT and FAP (r=-0.55, p<0.001), NIS (r=-0.71, p<0.001), Norfolk (r=-0.65, p<0.001), NAK (r=-0.53, p<0.001), Karnofsky (r=0.72, p<0.001), HGS_r (r=0.64, p<0.001), HGS_l (r=0.57, p<0.001), BCM (r=0.55, p<0.001), FFM (r=0.39, p=0.007), PA (r=0.56, p<0.001). In addition, PA was also negatively correlated with FAP (r=-0.41, p=0.05), NIS (r=-0.52, p<0.001), Norfolk (r=-0.31, p=0.04) and positively correlated with 6MWT. Of interest, a correlation was found between SDNN and clinical scales: NIS (-0.44, p=0.008) and Karnofsky (0.44, p=0.007). Bilateral HGS negatively correlated with NIS, FAP, 6MWT and Norfolk (for all p<0.01).

	Baseline	**9 months**	**18 months**	**P**
<u>Anthropometric and demographics</u>				
Age (years)	65.9±7.7	66.4±7.5	68.1±6.9	ns
Height (cm)	162.3±11.4	162.3±11.4	162.3±11.4	ns
Weight (kg)	69.5±19.6	71.1±18.0	71.3±19.9	ns
<u>Clinical evaluation</u>				
FAP stage	1.1±0.5	1.1±0.5	1.1±0.5	ns
Karnofsky performance status	72.0±13.7	74.7±14.1	74.0±15.5	ns
NIS	31.2±28.5	27.1±22.4	25.7±29.9	ns
NIS-W	14.9±17.2	11.8±10.4	12.9±16.3	ns
Norfolk	47.3±31.4	46.4±28.2	40.3±26.5	ns
COMPASS-31	18.0±9.3	19.5±9.1	17.8±9.6	ns
6MWT (m)	224.3±73.9	248.4±86.2	257.3±68.9	ns
<u>Body Composition</u>				
FFM (kg)	48.3±12.0	50.7±12.4	50.2±12.6	ns
BCM (kg)	22.8±7.7	25.8±7.9	25.0±9.2	ns
FM (kg)	21.2±9.6	20.1±8.6	20.6±9.2	ns
TBW (l)	36.0±9.3	37.3±9.3	37.6±9.3	ns
ECW (l)	18.8±4.8	18.1±4.6	18.7±4.8	ns
PA (°)	4.9±1.1	5.5±1.3	5.3±1.4	ns
<u>Strength</u>				
HG R	21.8±13.4	22.2±13.6	19.6±12.2	ns
HG L	23.3±11.4	23.2±10.1	20.1±9.2	ns

Table P3B. Body composition, strength and 6-minute walking test outcomes. Data are presented as means±st.dv; ns not significant. Ns= not significant. FAP: Familial amyloidotic polyneuropathy; NIS: neuropathy impairment score; NIS-W: neuropathy impairment score muscle weakness; 6MWT: 6-minute walking test; BCM: Body Cell Mass; ECW: Extra Cellular Water; FFM: Free Fat Mass; FM: Fat Mass; HG: Hand Grip; L: Left; R: Right; TBW: Total Body Water.

Discussion

Data from 18 and 9 months of follow-up after treatment with the RNA-silencer patisiran confirm stabilization of polyneuropathy in ATTRv. First of all, patisiran was safe, and no patients reported side effects. In particular, a positive trend of reduction of NIS, Norfolk scores while improving 6MWT and Karnofsky performance status. A not-significant reduction of Norfolk QOL mean scores (-3.5) was reported in our population at 9 months with a more pronounced effect at 18 months, in line with data from the APOLLO trial population (-6.7 ±1.8). Also, our data confirm real-life experience reporting worsening of Norfolk QOL scores in the first 6 months of treatment with improvement after 12 months(113). Our observations show that a trend of improvement can be seen after 9 months of treatment, confirming that there might be a latency period before observing benefits with Norfolk QOL.

BIA demonstrated detrimental effect of amyloid accumulation in body tissues, as shown by very low phase angle (PA). PA is a well-known predictor of morbidity and mortality in various diseases reflecting inflammatory and degenerative damage. However, we observed increasing PA after 9 months of treatment with patisiran with stabilization at 18 months. Moreover, the principal finding of the third

phase of the study is a significant increment in body weight accompanied by relevant changes in body composition in ATTRv patients 9 months after the start of treatment with the RNA silencer patisiran. More in detail, the more pronounced increment was on fat-free mass (FFM) and body cell mass (BCM), that are both expression of muscle mass; also, the increase of muscle mass was accompanied by a reduction of fat mass (FM) without any modification in the water content. Moreover, as most patients presented unexplained weight loss before treatment, the weight gain was characterized by an increment of muscle mass instead of fat. This finding might support a role for patisiran in reorganization of motor units in hypotrophic muscles since a reduction of amyloid deposition might interrupt axonal damage, favouring reinnervation and neurotrophic processes. Moreover, the benefits on the patient health cannot be explained by the stabilization of neuropathy alone: an improvement in gastrointestinal manifestations might also have guaranteed a better absorption of micronutrients, thus solving a deficiency status. These findings are in line with the preservation of residual motor strength demonstrated by clinical scales (stable FAP stage and NIS) and HGS and the beneficial effect on 6MWT. Finally, it should be noted that beneficial effects detected through BIA were obtained at 9 months with no further effects at 18 months (stabilization of body weight and BIA parameters).

A second useful insight regards the use of HGS tool in ATTRv patients.

HGS is a simple and cheap tool able to evaluate distal strength in several conditions, including hereditary polyneuropathies(94). Moreover, Anbarasan et al, demonstrated how it represents a functional outcome measure that significantly improved after mini carpal tunnel release(93). A previous study from our group has proved that HGS is reduced in ATTRv patients, probably because of bilateral carpal tunnel syndrome and polyneuropathy(87). Furthermore, HGS has showed a negative correlation with NIS scores, while it positively correlated with the neurophysiological evaluation of the median compound motor action potential amplitude(87). In the present study, despite an increase of muscle mass and a positive correlation with BCM (r=0.79, p = 0.001, right and r=0.85, p = 0.001 left), HGS did not show any significant variation after 9 months of therapy. However, such finding is encouraging and may account for a preservation of patient's distal strength. Furthermore, HGS positively correlated with 6MWT (r=0.61, p = 0.016, right and r=0.60, p = 0.016 left), suggesting that HGS might be related to exercise capacity, as already shown in different conditions(139). Finally, Norfolk QOL scale and HGS presented a negative correlation (r=-0.69, p 0.005, right and r=-0.68, p 0.004 left), supporting a relationship between HGS and patient's quality of life.

Of interest, 6MWT showed a significant improvement of patients' autonomy: the mean walked distance increased by 31.3 m after treatment, even if there were no significant changes in upper limb strength (however, it should be noted that 6MWT showed a correlation

with both HGS). On this regard, it should be considered that HGS assess the strength in upper limbs, while 6MWT evaluates the global ambulation process; hence, an increase in walking distance might not be related to an improvement of strength alone, as balance and coordination also play a fundamental role. Moreover, the correlation between 6MWT and BCM suggests that the increase of functional capacity confirmed by the 6MWT could depend not only on the stabilization of the neuropathy, but also on the improvement in the absorption of nutrients and the subsequent increase in metabolic capacity of muscles. Of note, these findings suggest that BIA might estimate the overall functional capacity of the patient detecting improvements better than clinical scales.

Finally, HRV was not able to detect beneficial effect from treatment in ATTRv patients, although revealing some abnormalities due to dysautonomia.

Limitations

Our study presents several limitations that should be addressed. The study sample size is quite small. These data are referred to a screening period in a limited area of Western Sicily in a tertiary referral Neurological clinic; hence, a selection bias is expected. Also, all patients came to our attention for neuropathy and were evaluated from a neurologist as a first approach; therefore, cardiac history should be over or underestimated. A further limitation comes from the concept of "red flag", which, can be self-reported from the patient, described in a specialist's report, demonstrated in an instrumental examination with different grades of precision in the clinical assessment. Hence, the assessment of such red flags might be poor and incomplete (i.e., erectile dysfunction), due to underreporting or undervaluation. Regarding data on instrumental evaluations, selection bias and the low number of patients might have led to interpreting errors. Also, the evaluation of upper limbs on HGS might have carried an underestimation of benefits on lower limbs. Also, we did not use modified BMI (considering the serum albumin levels), which is a useful biomarker in malnutrition status. Future studies are needed to validate the use of these promising tools in clinical practice.

Conclusions

ATTRv amyloidosis is a treatable, inherited, progressive, and fatal disease with late onset in non-endemic countries that is increasingly diagnosed worldwide. Diagnosis of ATTRv amyloidosis is difficult with a relevant diagnostic delay, misdiagnosis, and high costs for the community due to mortality and disability. Also, this paper underlines the impact of patisiran in patients affected by ATTRv amyloidosis from non-endemic areas in Sicily.

This analysis of data from screening procedures in Western Sicily highlighted a high prevalence, absence of the common Val30Met mutation, and presence of several TTR variants with clinical characteristics homogeneous within and heterogeneous between each other. Many patients initially received a wrong diagnosis with consequent significant diagnostic delay. Hence we suggest early TTR gene testing to confirm ATTRv amyloidosis.

Neurologists should be aware of diagnostic pitfalls of ATTRv and gene sequencing should be done in all suspected cases. Indeed, a systematic screening for ATTRv in neurological setting might contribute to significantly reduce the diagnostic delay of ATTRv in non-endemic areas, as well as ensuring the early treatment for this rare inherited disease. Unexplained weight loss associated with carpal tunnel

syndrome and polyneuropathy represent the most common presentation of ATTRv in Western Sicily. Anorexia and ALS might represent overlooked misdiagnoses and more attention should be paid on their evaluation in the presence of polyneuropathy. Genetic testing should be also encouraged in relatives of diagnosed cases when they are able to understand its medical, social, and psychological consequences. Moreover, a multidisciplinary approach including gastroenterologist and cardiologist can improve the management of symptoms. Also, the increased awareness and attention to ATTRv amyloidosis might reduce the diagnostic delay, favoring an earlier start of the appropriate treatment.

Good knowledge of the natural history of the disease according to different TTR mutations allow clinicians to optimise multiprofessional care for patients and to offer carriers a personalized follow-up to reveal first signs of the disease.

The present data also show that patisiran is effective and safe in improving both neurological and cardiovascular symptoms of ATTRv amyloidosis, and to maintain a good QoL, independently form the stage of the disease and the involved mutation.

Punctual and detailed instrumental biomarkers are on demand for ATTRv to measure the severity of the disease burden and to monitor progression and/or response to treatment. Indeed, instrumental evaluation of ATTRv patients might detect mild changes which could be often underrecognized by clinical evaluation alone. Handgrip test

and Bioelectrical Impedance Analysis are rapid, simple, and non-invasive tools that can be easily performed and do not require expensive equipment. Moreover, BIA might represent useful tools to assess the effects of multiorgan damage in ATTRv and to monitor disease progression and response to treatments. More data are still needed for HGS. Our data denote that patisiran stabilizes polyneuropathy and preserves motor strength by increasing muscle mass after 9 months of treatment. We believe that HGS and BIA might find an application in clinical practice due to their low cost and high reliability. Further studies are needed to confirm our findings and clarify a possible role of such tools in the diagnostic process.

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