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Developing of new machine-learning tools for early diagnosis of hereditary transthyretin amyloidosis polyneuropathy

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To my daughter, to my family.

Chapter 1 – General Introduction

Hereditary transthyretin amyloidosis (ATTRv) is a rare, progressive, and potentially life-threatening systemic disease caused by pathogenic variants in the **transthyretin (TTR)** gene. Mutations destabilize the native TTR tetramer, promoting its dissociation into monomers prone to misfolding. These misfolded proteins self-assemble into amyloid fibrils that deposit extracellularly in multiple tissues, progressively impairing organ function (1).

From a historical perspective, the first detailed description of hereditary amyloidosis was provided by Corino de Andrade in 1952, who observed families in northern Portugal with progressive sensorimotor neuropathy, autonomic dysfunction, and weight loss, later identified as carrying the Val30Met mutation (2). Since then, ATTRv has been recognized worldwide, with more than 150 amyloidogenic variants reported, the majority being missense substitutions (3).

1.1 Molecular Basis and Pathophysiology

TTR, also known as prealbumin, is a 55-kDa homotetrameric transport protein mainly synthesized in the liver, with minor contributions from the choroid plexus and retina. Its physiological function is the transport of thyroxine (T4) and retinol-binding protein–vitamin A complex (4).

Mutations in the TTR gene, located on chromosome 18q12.1, reduce the kinetic stability of the tetramer. The critical step in amyloidogenesis is the dissociation of the tetramer into monomers, which then misfold and aggregate into oligomers and insoluble fibrils (5). These fibrils are deposited in peripheral nerves, the myocardium, gastrointestinal tract, eyes, and kidneys. Importantly, both mutant and wild-type TTR may contribute to deposition, explaining disease progression even after liver transplantation (6).

Recent studies also suggest that toxic oligomers play a role in cell dysfunction before fibril deposition, contributing to neurotoxicity and cardiotoxicity (7). This dual mechanism, fibril deposition and oligomer toxicity, underlies the clinical heterogeneity observed in ATTRv.

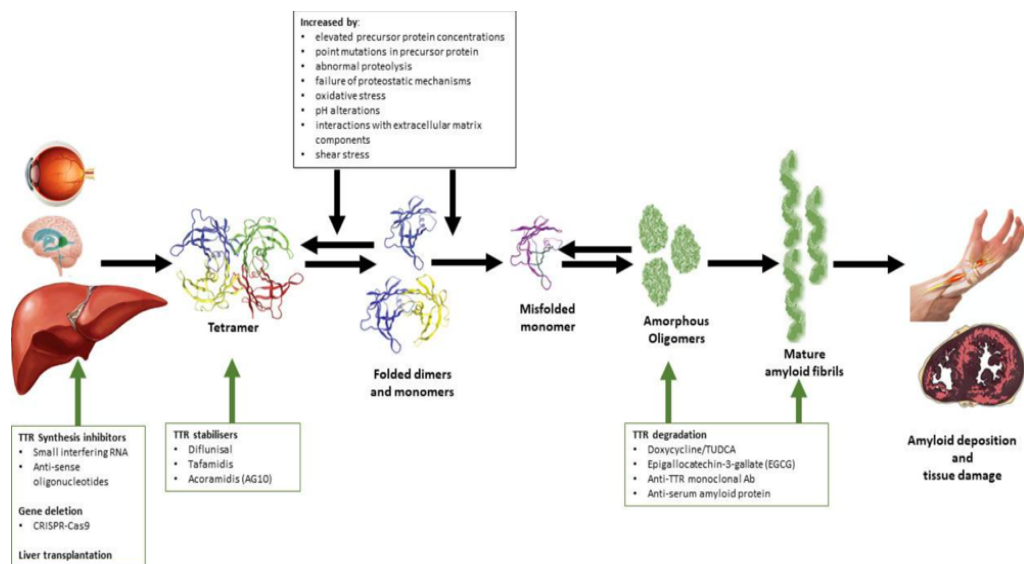


Figure 1. Mechanisms by which TTR mutants exert pathogenesis and therapeutic strategies for ATTRv (5)

1.2 Inheritance and Genetic Heterogeneity

ATTRv is inherited in an autosomal dominant manner, though penetrance is incomplete and highly variable across mutations and populations. Carriers of the same mutation may show strikingly different phenotypes, ranging from early-onset neuropathy to late-onset cardiomyopathy (8,9).

- ***Val30Met (p.Val50Met)*** is the most common mutation worldwide, with endemic clusters in Portugal, Sweden, and Japan. Early-onset forms manifest with neuropathy and autonomic dysfunction, whereas late-onset forms often show cardiomyopathy (10).
- ***Val122Ile (p.Val142Ile)*** is highly prevalent in African Americans (~3–4% carriers), associated with late-onset cardiomyopathy (11,12).
- ***Thr60Ala (p.Thr80Ala)*** is typically a late-/middle-onset mixed phenotype with prominent cardiomyopathy and length-dependent neuropathy; carpal tunnel is common. Reported in the Italian Registry as a minor variant (13).
- ***Glu89Gln (p.Glu109Gln)***, endemic in Bulgaria and Italy, has a mixed phenotype: neuropathy at onset (often 5th–6th decade) with early cardiac involvement; mortality is frequently cardiac (14).
- ***Phe64Leu (p.Phe84Leu)***, enriched in Southern Italy (incl. Sicily; also reported in Apulia/Calabria); late-onset severe axonal neuropathy with mild–moderate cardiac disease; nerve biopsy may lack detectable amyloid relatively often (15).

- ***Ile68Leu (p.Ile88Leu)*** has predominantly cardiac amyloidosis with frequent (often under-recognized) neurologic involvement; overall, a mixed phenotype with worse prognosis when both systems are affected (16).

Genetic heterogeneity contributes to diagnostic challenges and necessitates genotype-specific counseling and therapeutic planning.

Age of onset is another key discriminator: from the third decade in Portuguese Val30Met families to over 60 years in late-onset or cardiogenic variants. This variability often delays diagnosis, especially in non-endemic regions (17).

1.3 Clinical Heterogeneity

ATTRv presents with a wide spectrum of clinical phenotypes that can be broadly categorized into neuropathy-predominant, cardiomyopathy-predominant, and mixed forms.

The **neuropathy-predominant** form of ATTRv typically manifests initially with length-dependent sensory symptoms, including burning sensations in the feet, paresthesias, and progressive sensory loss. As the disease advances, motor weakness becomes apparent, which may ultimately lead to loss of ambulation. Autonomic dysfunction frequently accompanies these sensory and motor changes, with features such as orthostatic hypotension, gastrointestinal dysmotility, and erectile dysfunction significantly reducing quality of life. This pattern is classically seen in early-onset Val30Met (also

Val50Met) ATTRv seen in endemic regions such as Portugal and Japan (9,18).

Cardiac manifestations dominate the **cardiomyopathy-predominant** form. Restrictive cardiomyopathy, diastolic dysfunction, conduction blocks, and arrhythmias are frequent features. This phenotype is strongly associated with the Val122Ile and Ile68Leu mutation, which is relatively common in African American populations and typically manifests as late-onset disease (12,19).

In the **mixed phenotype**, patients display both neuropathic and cardiac involvement, often accompanied by other systemic manifestations. Ocular complications such as vitreous opacities and glaucoma, renal dysfunction including proteinuria and chronic kidney disease, and leptomeningeal involvement may all occur. Variants such as Glu89Gln and Thr60Ala are well-known examples associated with this heterogeneous clinical presentation (14,20).

1.4 Diagnostic Challenges

Despite increasing awareness of ATTRv, it remains underdiagnosed and often misdiagnosed. Several factors contribute to these diagnostic delays: the disease is rare; its clinical presentations overlap significantly with more common disorders; and penetrance can vary widely, even among carriers of the same mutation.

In the neuropathy-predominant forms, patients may initially present with features resembling chronic inflammatory demyelinating polyneuropathy (CIDP), diabetic neuropathy, or idiopathic axonal neuropathy. For example, an Italian study by Cortese et al. (2017) examined medical records of 150 patients with ATTRv and found that many were initially misdiagnosed with more common neuropathies, including CIDP (21).

On the cardiac side, ATTRv (and wild-type ATTR) cardiomyopathy often mimics hypertrophic cardiomyopathy (HCM) or hypertensive heart disease. In a study of patients with hypertrophic phenotypes, several were found to carry pathogenic TTR variants, raising the possibility that some cases ascribed to HCM are actually amyloid (22).

Therefore, **misdiagnosis** arises both in non-specific neuropathic symptoms and in cardiac hypertrophy phenotypes, delaying correct treatment. Awareness of these mimic conditions and use of red flag features (e.g. family history, multisystem signs, autonomic dysfunction, carpal tunnel syndrome) are essential for earlier recognition (1).

Symptoms of neuropathy (neurologic)				
Sensory		Motor	Autonomic	
Pain (stabbing, shocks, contact allodynia, burning)		Distal muscle weakness and atrophy (unless superimposed myopathy)	Erectile dysfunction	
Altered sensation (touch or temperature)		Tripping	Lightheadedness/orthostatic hypotension/syncope/presyncope	
Tingling, prickling sensations		Foot drop	Genitourinary problems (incontinence, incomplete emptying, increased urinary frequency)	
Imbalance	Walking difficulties	GI manifestations (diarrhea, constipation, early satiety, motility dysfunction) ^a		
	Difficulty opening jars	Loss of hair/sweating abnormalities		
	Loss of dexterity	Heat intolerance		
	Difficulty climbing stairs/getting up off a chair	Blurred vision, dry eyes		
		Dry mouth		
Signs/symptoms from other organs/body systems (non-neurologic)				
Cardiac		Musculoskeletal	Ophthalmologic	Renal
Features of hypertrophic cardiomyopathy		CTS, most often bilateral	Vitreous opacities	Renal failure
HFrEF		Dupuytren's contracture	Glaucoma	Proteinuria
Arrhythmia		Rotator cuff injury	Dry eyes	Hematuria
Peripheral edema		Lumbar stenosis	Abnormal conjunctival vessels	
Shortness of breath		Tendon rupture	Cataracts	

Table 1. Possible neurologic and non-neurologic manifestations of ATTRv amyloidosis (23).

Abbreviations: ATTRv, hereditary transthyretin (v for variant); CTS, carpal tunnel syndrome; GI, gastrointestinal; HFpEF, heart failure with preserved ejection fraction.

a: GI manifestations in ATTRv amyloidosis can also be of nonautonomic origin, with deposition of amyloid within the GI system resulting in symptoms such as abdominal pain, esophageal reflux, nausea, constipation, and early satiety.

Despite growing awareness of hereditary transthyretin amyloidosis ATTRv, it remains underdiagnosed and frequently misdiagnosed. Several factors contribute: its rarity, clinical overlap with more common diseases, and variable penetrance.

Studies have shown that **diagnostic delay** in ATTRv can be substantial. For example, in a Portuguese cohort of apparently sporadic patients, the median delay from symptom onset to diagnosis was approximately 3.5 years (1262

days) (24). This delay correlates with poorer outcomes and reduced efficacy of disease-modifying treatments, since therapies are more effective if started earlier in disease course.

To mitigate this, experts have proposed a set of **red flags** or clinical clues that should trigger suspicion of ATTRv and genetic testing. Typical red flags include bilateral carpal tunnel syndrome, unexplained weight loss, early autonomic dysfunction, ataxia, and a family history of neuropathy or cardiomyopathy (1). However, while these features are widely recognized, their predictive value (i.e., how strongly each contributes to reducing diagnostic delay) remains under study.

1.5 Therapeutic Advances

The therapeutic landscape of ATTRv has undergone a profound transformation over the past two decades. In the past, treatment options were restricted to supportive care, focusing mainly on symptom management, including pain control, autonomic dysfunction, and conventional therapy for heart failure (25).

The first disease-modifying intervention was orthotopic liver transplantation (OLT), aimed at suppressing the production of TTR. Although OLT significantly improved outcomes in selected early-onset Val30Met cases, its effectiveness proved limited in late-onset and non-Val30Met variants, largely

due to continued deposition of wild-type TTR and the risks associated with major surgery and lifelong immunosuppression (6,26).

A major step forward was the development of TTR tetramer stabilizers. Tafamidis demonstrated the ability to slow neurological decline in familial amyloid polyneuropathy and later improved survival and reduced hospitalizations in patients with ATTR cardiomyopathy (27,28).

Diflunisal, a repurposed NSAID, also slowed neuropathy progression, though its chronic use is constrained by renal and gastrointestinal toxicity (29).

More recently, gene silencing therapies have revolutionized the field. Patisiran, an small interfering RNA (siRNA) delivered intravenously, improved neuropathy scores, quality of life, and some cardiac measures (30).

Inotersen, an antisense oligonucleotide, showed efficacy in slowing disease progression, though with a need for careful safety monitoring due to risks of thrombocytopenia and renal events (31).

Eplontersen, a next-generation N-acetylgalactosamine (GalNAc) -conjugated antisense oligonucleotide, demonstrated comparable efficacy with monthly subcutaneous dosing and an improved safety profile in the NEURO-TTRansform trial (32).

Vutrisiran, a next-generation subcutaneous siRNA, offers similar benefits with a more convenient quarterly dosing schedule (33).

Looking to the future, in vivo gene editing with CRISPR-Cas9 (NTLA-2001) has shown unprecedented potential: a single infusion induced sustained reductions in circulating TTR levels in first-in-human studies, suggesting the possibility of a one-time curative approach (34).

In the Nexiguran Ziclumeran phase 1 trial (NEJM, 2025), a single infusion achieved >90% sustained TTR reduction with favorable safety, confirming the potential for a one-time curative therapy(35). Long-term studies are ongoing to evaluate editing durability, off-target risks, and patient selection criteria.

Taken together, these advances emphasize the critical importance of early diagnosis, since available treatments effectively stabilize the disease but cannot reverse organ damage once established.

1.6 Rationale for Machine Learning (ML)

Given the remarkable phenotypic heterogeneity of ATTRv, the frequent diagnostic delays, and the availability of therapeutic options that require early initiation for maximal benefit, there is an urgent need for innovative diagnostic tools. **Machine learning (ML)** provides a powerful data-driven framework that goes beyond traditional statistical approaches. Unlike linear models, ML can capture complex non-linear interactions among clinical features, assign relative weights to red flags, and ultimately help prioritize patients for early genetic testing, where suspicion of ATTRv is highest.

A recent Italian study evaluated the use of ML (XGBoost with SHAP explainability) to support early diagnosis of ATTRv. Among 184 patients with neuropathy, the model achieved fair performance (AUC-ROC $\approx$ 0.75, accuracy $\approx$ 71%). Key predictive features for a positive genetic test were unexplained weight loss, cardiomyopathy, gastrointestinal disturbances, and

ataxia, whereas diabetes, autoimmunity, ocular and renal involvement were associated with negative tests. Findings suggest that ML may help reduce diagnostic delays and guide genetic testing in non-endemic areas (36).

On the cardiological side, recent studies have shown that artificial intelligence (AI) can enhance early detection of transthyretin amyloid cardiomyopathy (ATTR-CM). An AI- Electrocardiography (ECG) and AI-echocardiography model identified disease signatures up to three years before clinical diagnosis, highlighting their value in preclinical screening. Another multimodality AI approach in patients with severe aortic stenosis demonstrated that CT-derived strain analysis, integrated with other imaging and clinical features, effectively discriminated coexisting ATTR-CM. Finally, deep learning applied to nuclear scintigraphy achieved very high accuracy (AUC >0.9) in both detection and automated grading of ATTR-CM, supporting its potential role in standardized and scalable diagnostic workflows (37–39).

Taken together, these findings suggest that the integration of ML and AI into diagnostic workflows could represent the next frontier in ATTRv. By enhancing the recognition of disease-specific patterns, such tools may shorten diagnostic delays, reduce rates of misdiagnosis, and facilitate timely access to disease-modifying therapies, ultimately improving outcomes for affected patients.

Chapter 2 – Epidemiology and Clinical Spectrum of ATTRv

2.1 Global epidemiology: from “endemic” islands to a worldwide disease

ATTRv was historically considered a geographically restricted disorder after the seminal description by Corino de Andrade of large kindreds from northern Portugal with early-onset neuropathy and dysautonomia (subsequently linked to the Val30Met variant) (2). Soon after, additional foci were recognized in northern Sweden and Japan, establishing a triad of “endemic” regions where carrier frequency and disease penetrance are higher than elsewhere (10,40). Over time, improved molecular diagnostics, active family screening, and awareness of late-onset and cardiogenic phenotypes have revealed a truly global distribution of ATTRv with both large families and apparently sporadic cases on every continent (41).

Epidemiological studies indicate that ATTRv is generally considered a rare disorder in non-endemic areas, with an estimated prevalence of about 1 case per 100,000 inhabitants in Europe and the United States. In contrast, certain regions display much higher prevalence due to founder effects and clustering within extended families. In classical endemic areas, such as northern Portugal, parts of Japan, Brazil, and Sweden, disease frequency is substantially greater, ranging from 1 in 1,000 to 1 in 10,000 individuals. Moreover, some microfoci reveal exceptionally elevated rates, with 5 cases

per 100,000 inhabitants reported in Majorca and 3.72 per 100,000 in Cyprus (42–45).

Beyond neuropathic forms, cardiac-predominant variants (ATTR-CM) contribute significantly to the disease burden. The Val122Ile mutation (c.424G>A, p.Val142Ile), in particular, represents one of the most frequent pathogenic alleles worldwide. This substitution is carried by 3–4% of individuals of African ancestry, while it remains rare among Caucasian and Latin American populations, where allele frequency is estimated below 0.5% (42,44). Such genetic and geographic heterogeneity underlines the need for region-specific strategies for early recognition and genetic counseling

On a global scale, early analyses suggested 5,000–10,000 living patients, yet more comprehensive modeling and registry accrual imply the burden is substantially higher ($\approx$ 30,000–40,000), as under-recognition of late-onset and cardiomyopathy-predominant cases steadily corrects prior underestimates (46).

2.1.1 Endemic foci and founder effects

From an epidemiological perspective, the distribution of ATTRv is strongly shaped by founder effects, giving rise to well-defined endemic foci.

In northern Portugal, particularly in the areas of Póvoa de Varzim and Vila do Conde, the **Val30Met** variant reaches an exceptionally high carrier frequency, up to 1 in 538 individuals. Patients here usually present with early-onset disease, characterized by small-fiber neuropathy, neuropathic pain, and prominent autonomic dysfunction such as gastrointestinal dysmotility and orthostatic hypotension (41,47).

A different scenario is observed in northern Sweden, where the same Val30Met mutation is associated with later onset, reduced penetrance, and a higher rate of cardiac involvement. The age-at-onset distribution is shifted compared with Portuguese kindreds, illustrating how identical mutations can generate divergent phenotypes depending on the genetic and environmental context (10,47).

In Japan, ATTRv is notable both for its heterogeneity and its geographical variability. While Val30Met remains the most common variant, both early- and late-onset cases are described, and a wide mutation spectrum has been documented with distinct regional clustering. These features underscore the complexity of the disease in Asian populations (44,48).

Beyond these classical foci, additional European hotspots have been identified. In the island of Mallorca, Spain, a hospital-based survey estimated a prevalence of approximately 5 cases per 100,000 inhabitants (43). Similarly,

in Cyprus, national evaluations reported a prevalence of 3.72 per 100,000 people, reflecting another localized founder effect (49).

Together, these examples illustrate how founder mutations have produced areas of markedly elevated ATTRv prevalence compared to the global average, while also highlighting the phenotypic heterogeneity that can emerge from the same genetic variant depending on population background.

<i>Region / Country</i>	<i>Predominant Variant(s)</i>	<i>Reported Prevalence or Carrier Frequency</i>	<i>Typical Age at Onset</i>	<i>Characteristic Phenotype</i>	<i>Reference</i>
<i>Northern Portugal (Póvoa de Varzim, Vila do Conde)</i>	Val30Met (p.Val50Met)	Carrier frequency up to 1:538	3rd–4th decade	Early neuropathy + dysautonomia	Andrade 1952; Conceição 2016
<i>Northern Sweden (Skellefteå, Piteå)</i>	Val30Met	Penetrance ~11% by age 50	>50 years	Late-onset, mixed neuro–cardiac	Hellman 2008; Conceição 2016
<i>Japan (multiple clusters)</i>	Val30Met ± others	Regional clustering	3rd–6th decade	Mixed early- and late-onset	Sekijima 2018; Ando 2013
<i>Mallorca (Spain)</i>	Val30Met	~5/100,000	Variable	Neuropathy – predominant	Reinés 2014
<i>Cyprus</i>	Val30Met	3.72/100,000	4th–6th decade	Neuropathy with variable autonomic signs	Parman 2016

Table 2. Endemic foci of ATTRv and key epidemiologic markers.

2.1.2 Population variants of special relevance

Among the many mutations in TTR gene, a few variants are of particular epidemiological and clinical importance because of their frequency in specific populations and their distinct phenotypic profiles.

One of the most relevant is the **Val122Ile (p.Val142Ile)** substitution, highly prevalent in individuals of West African ancestry. Population studies estimate a carrier rate of approximately 3–4% among African Americans, making it one of the most common amyloidogenic variants worldwide (12,50). Clinically, Val122Ile carriers usually present with a cardiomyopathy-predominant phenotype, characterized by restrictive physiology, conduction abnormalities, and progressive heart failure. Disease onset typically occurs in the seventh decade of life, and the variant is strongly enriched among elderly individuals with heart failure with preserved ejection fraction (HFpEF) (51,52). Importantly, despite its high prevalence, penetrance is incomplete, and many heterozygotes remain asymptomatic, highlighting the importance of genetic counseling and risk stratification.

Another well-characterized founder mutation is the **Thr60Ala (p.Thr80Ala)** variant, most frequently observed in individuals of Irish descent. In contrast to the cardiomyopathy-predominant phenotype of Val122Ile, Thr60Ala typically manifests as a mixed clinical picture, combining progressive peripheral neuropathy, autonomic dysfunction, and cardiomyopathy. The

disease usually becomes symptomatic after the age of 50, with gradual neurological decline accompanied by cardiac involvement (9,44). The heterogeneous presentation of this variant often complicates diagnosis and underscores the need for early recognition, particularly in patients with suggestive family histories or overlapping neurological and cardiac manifestations.

Together, these two population-specific variants illustrate how ancestral and regional genetic backgrounds shape the clinical expression of ATTR_v, with Val122Ile acting as a major driver of late-onset cardiac amyloidosis in African populations, and Thr60Ala representing a prototypical example of a mixed phenotype in Europe.

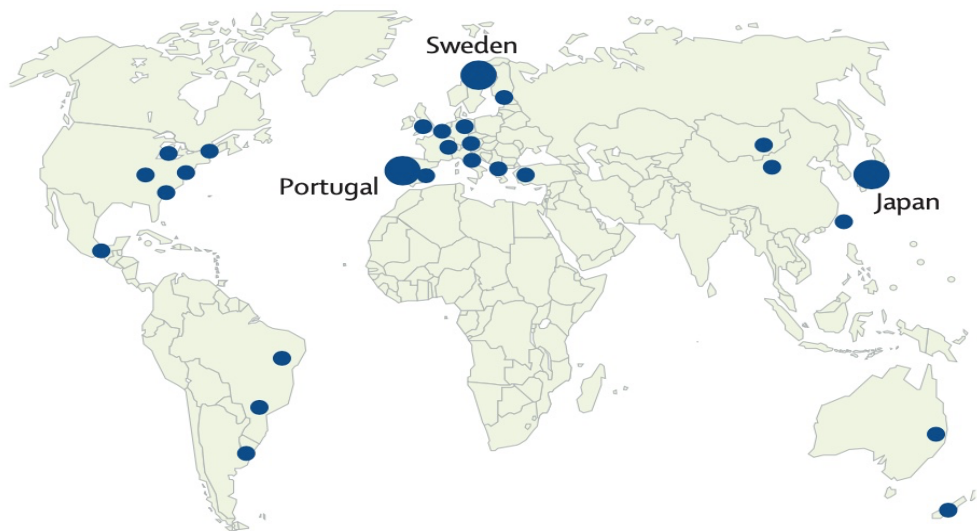


Figure 2. Geographic distribution of ATTR_v. From Planté-Bordeneuve et al. 2011(41).

2.2 Epidemiology in Italy: national picture and regional clusters

The Italian ATTRv landscape is strongly influenced by regional founder effects, which shape both the spectrum of circulating variants and the predominant clinical phenotypes.

In Tuscany, the Val30Met mutation remains the most frequent. While classically associated with early-onset neuropathy in endemic Portuguese and Japanese cohorts, in Italy this variant more often presents with a late-onset mixed phenotype combining neuropathy and cardiomyopathy. Importantly, diagnostic delays are common because late-onset forms may initially mimic other polyneuropathies or cardiac conditions (13).

The Lazio region has shown a higher prevalence than national averages, largely due to active case-finding strategies and systematic genetic screening. Here too, Val30Met and other mutations are observed, with mixed phenotypes predominating. The reported prevalence of ~17.2 per million underscores how regional epidemiology may be underestimated without targeted efforts (53).

In Sicily, Glu89Gln, Phe64Leu, and Thr49Ala are particularly relevant. Glu89Gln is typically associated with a rapidly progressive cardiac phenotype, while Phe64Leu more often leads to late-onset neuropathy, which

is sometimes overlooked because bone scintigraphy may yield false negatives (54). In contrast, Thr49Ala tends to present with autonomic symptoms as an early hallmark (20,55)

In Calabria and Apulia, Phe64Leu and other rare mutations occur, frequently identified through cascade family screening, reflecting the importance of genetic counseling in families with known probands (13).

Finally, the nationwide survey by Fumagalli et al. (2025) confirmed the heterogeneity of prevalence and mutation distribution, reporting regional hotspots but also highlighting significant under-recognition in areas without dedicated referral centers (56).

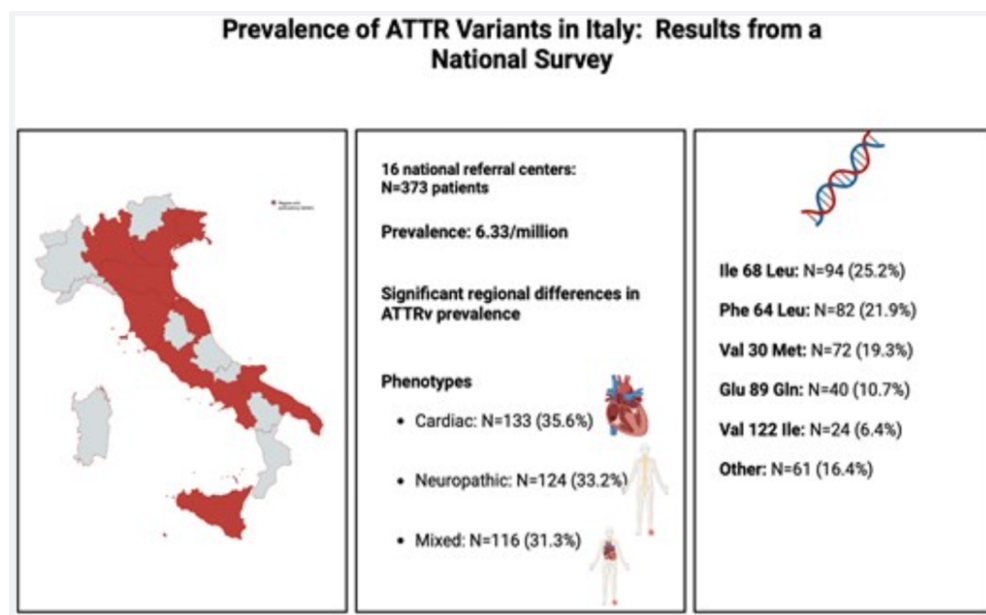


Figure 3. Prevalence and most common ATTRv mutations in the Italian regions. From Fumagalli et al. 2025 (56).

<i>Region</i>	<i>Predominant Variants</i>	<i>Phenotype Trend</i>	<i>Notable Diagnostic Issues</i>	<i>Reference</i>
<i>Tuscany</i>	Val30Met, mixed	Neuropathy and cardiomyopathy	Late-onset forms often underrecognized	Russo 2020
<i>Lazio</i>	Val30Met, various	Mixed	Higher prevalence captured with active screening (~17.2/million)	Luigetti 2021
<i>Sicily</i>	Glu89Gln, Phe64Leu, Thr49Ala	Glu89Gln: rapid cardiac course; Phe64Leu: late neuropathy; Thr49Ala: autonomic-first	Phe64Leu often missed on bone scintigraphy	Mazzeo 2015; Musumeci 2020; Gentile 2021
<i>Calabria & Apulia</i>	Phe64Leu, others	Mixed	Often detected through family-based cascade	Russo 2020

Table 3. Italy: regional clusters and recurrent variants

2.2.1 Sicily: three recurrent variants and founder signals

In addition to the variants with widespread demographic diffusion, some founder mutations markedly shape the epidemiology and clinical presentation of ATTRv in Italy and the Mediterranean.

Sicily illustrates how founder effects shape local genetics. Across family-based and clinic-based series, Glu89Gln, Phe64Leu and Thr49Ala are the most recurrent variants, with geographical micro-clusters on the island (20).

The Glu89Gln variant, particularly prevalent in Sicily and southern Italy, is associated with a mixed clinical course with progressive sensorimotor neuropathy and relatively early and aggressive cardiac involvement. Patients tend to present with weight loss, dysautonomia, and rapidly worsening cardio-neurological progression, requiring early treatment to avoid rapid functional decline. Regional series and multicenter reports have documented a worse cardiac profile compared to other locally endemic variants, reinforcing the need for close cardiac monitoring from the early stages (14,20).

Phe64Leu is concentrated in micro-clusters in Sicily and is typically associated with a late presentation with a predominantly neuropathic phenotype. Clinically, bilateral carpal tunnel syndrome and slowly progressive distal neuropathy are common; cardiac involvement may be present but is often less dramatic in the early stages. A practical concern is that bone scans may show reduced sensitivity in detecting amyloid cardiomyopathy in F64L carriers, a diagnostic caveat that dictates against ruling out ATTR based solely on a negative scan when clinical suspicion is high (20,54).

In familial lineages from southern Italy, Thr49Ala is often associated with early dysautonomia (orthostatic hypotension, gastrointestinal disturbances) that precedes overt sensorimotor neuropathy by years. This pattern may lead to delayed diagnosis if autonomic symptoms are not recognized as ATTRv

red flags. Penetrance appears high in middle age in published case series, supporting proactive carrier surveillance programs (20,44).

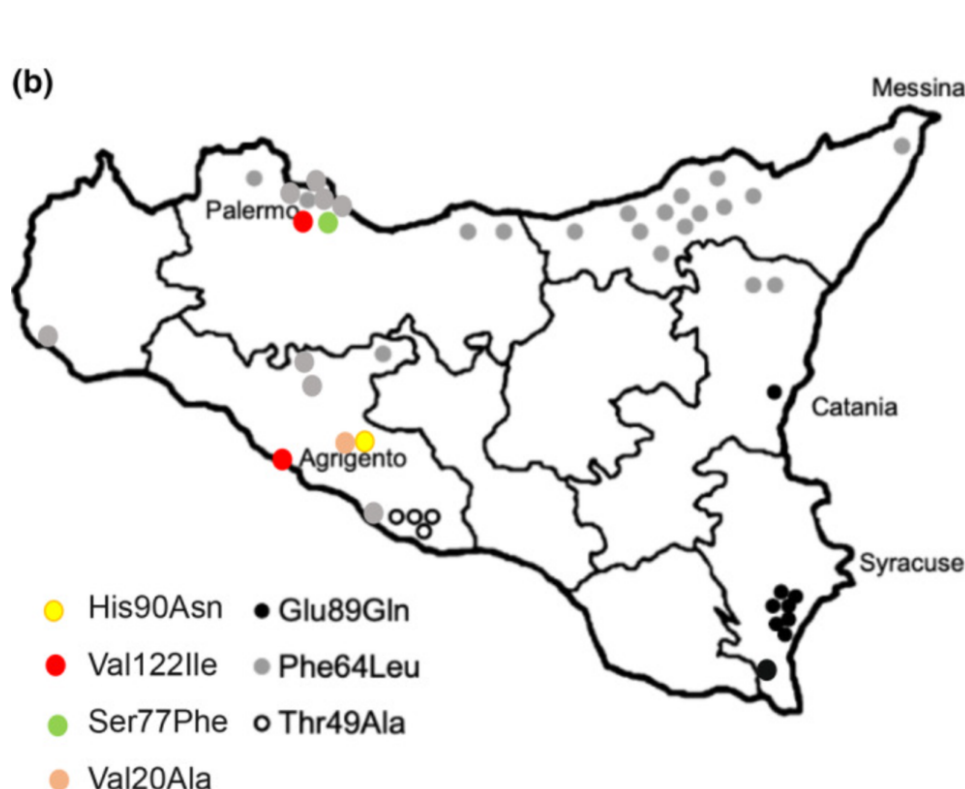


Figure 4. Geographical distribution of TTR mutations in Sicily. From Di Stefano et al. 2024 (15).

2.3 Clinical spectrum: three dominant phenotypes with organ-specific nuances

Although ATTRv is highly heterogeneous and patients frequently cross phenotypic boundaries, three dominant clinical patterns are usually recognized: a neuropathy-predominant form, a cardiomyopathy-predominant form, and a mixed or multisystem presentation.

2.3.1 Neuropathy-predominant disease

The neuropathic phenotype is classically a length-dependent axonal sensory-motor and autonomic polyneuropathy. Disease onset often involves small fibers, leading to pain, temperature loss, and burning feet, before spreading to large sensory and motor fibers. Autonomic dysfunction (manifesting as orthostatic hypotension, gastrointestinal dysmotility, and erectile dysfunction) is frequent, particularly in early-onset Val30Met cohorts from endemic areas such as Portugal and Japan (47,57). Pathological studies show early involvement of dorsal root ganglia and a proximal-to-distal spread of amyloid along nerves, which explains why distal nerve biopsies may be negative in early stages, leading to false-negative results (58).

Red flags that should prompt testing in neuropathic clinics include unexplained small-fiber symptoms in mid-life adults, disproportionate dysautonomia, bilateral CTS preceding symmetric neuropathy, family history of neuropathy/cardiomyopathy, and weight loss (47,48,59).

2.3.2 Cardiomyopathy-predominant disease

The cardiac phenotype of ATTRv is characterized by an infiltrative restrictive cardiomyopathy with concentric left ventricular thickening and preserved or mildly reduced ejection fraction. Hallmark features include diastolic dysfunction, atrial fibrillation, and conduction abnormalities, which frequently necessitate pacemaker implantation (19,25,60). Suspicion should

be heightened in cases of unexplained left ventricular hypertrophy, discordant low QRS voltages on ECG, HFpEF in older adults, or when cardiac disease coexists with bilateral carpal tunnel syndrome or lumbar spinal stenosis, increasingly recognized as sentinel signs of transthyretin amyloidosis (61,62). The Val122Ile variant, highly prevalent among individuals of West African ancestry, exemplifies this predominantly cardiac course, usually manifesting in the seventh decade of life. Studies have reported carrier rates of 3–4% among African Americans and up to 5% in elderly HFpEF cohorts, underlining its relevance as a public health issue in cardiology (12,52,63). By contrast, the Ile68Leu mutation, although rare, is associated with a particularly aggressive cardiac phenotype, with rapid disease progression and a median survival often less than five years without treatment, making timely identification and therapeutic intervention crucial (64).

2.3.3 Mixed and multisystem presentations

A considerable number of TTR variants produce a mixed clinical profile, combining neuropathy with early or parallel cardiac infiltration. Beyond these two major domains, extra-neurological involvement is increasingly recognized as part of the multisystemic nature of ATTRv.

Gastrointestinal manifestations are common and include alternating diarrhea and constipation, small intestinal bacterial overgrowth, and delayed gastric emptying, all of which contribute substantially to weight loss and impaired quality of life (65,66). Ocular involvement, such as vitreous opacities and

glaucoma, reflects local synthesis of TTR in the eye, which explains the persistence or progression of ocular disease even after hepatic-targeted therapies (67). Finally, renal involvement, ranging from proteinuria to overt chronic kidney disease, has been increasingly described in recent cohorts, underlining the need for systematic nephrological assessment in ATTRv (68,69).

In summary, while three major phenotypic patterns frame the clinical heterogeneity of ATTRv, the reality is one of frequent overlap and multisystem disease. This underlines the importance of a multidisciplinary approach to diagnosis and management, integrating neurology, cardiology, gastroenterology, ophthalmology, and nephrology for comprehensive patient care.

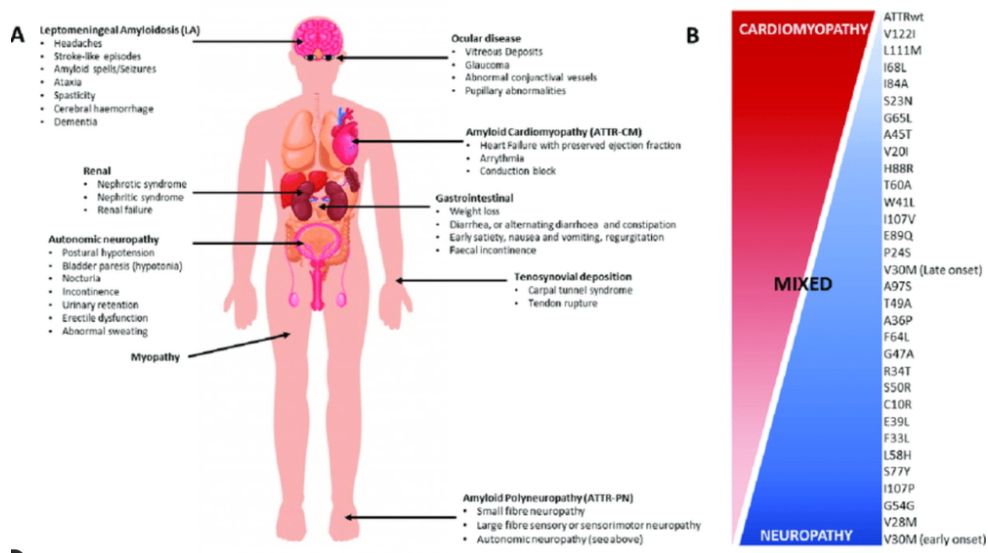


Figure 5. Genotype–phenotype correlation in ATTR amyloidosis: shows how some variants of the TTR gene are more often associated with neurological phenotypes, others with cardiac diseases or present mixed characteristics. From Carroll et al. 2022. (5)

2.4 Age at onset, penetrance, and expressivity

The age at onset, degree of penetrance, and clinical expressivity of ATTRv vary markedly according to the specific mutation and the population background in which it occurs.

The Val30Met variant illustrates this diversity particularly well. In the endemic Portuguese and Japanese clusters, onset typically occurs in the third to fourth decade of life, with high penetrance reaching approximately 80% by the age of 50 in classical Portuguese lineages. These early-onset forms are often characterized by severe dysautonomia, including orthostatic hypotension, gastrointestinal dysmotility, and erectile dysfunction (47,70). By contrast, in northern Sweden and other late-onset European cohorts, the same Val30Met mutation generally presents after the age of 50, with a markedly reduced penetrance, estimated at only ~11% by age 50. In these populations, cardiac manifestations are more frequent, and the clinical course differs substantially from the Portuguese pattern (10,47).

Other variants follow distinct trajectories. The Val122Ile mutation (p.Val142Ile), found in approximately 3–4% of individuals of West African ancestry, typically manifests as a late-life restrictive cardiomyopathy with minimal neuropathic involvement. This variant has been strongly associated with HFpEF in African American populations. The Ile68Leu mutation (p.Ile88Leu), although rare, is clinically important for its particularly

aggressive course. Multiple series have reported that patients with this variant develop rapidly progressive cardiomyopathy with a median survival of fewer than five years after diagnosis if untreated, highlighting the urgency of early recognition and intervention (12,51).

The wide variability in phenotype and penetrance cannot be explained by genotype alone. Modifying factors, including genetic background, epigenetic regulation, and environmental influences, play a crucial role. Furthermore, biochemical differences in amyloid fibril composition contribute to clinical heterogeneity: so-called type A fibrils (a mixture of full-length TTR and fragments) are strongly associated with late-onset and cardiac-dominant forms, whereas type B fibrils (composed mainly of full-length TTR) are typically found in early-onset neuropathic cases. These distinctions are not only of mechanistic interest but also correlate with imaging findings and disease course, reinforcing the importance of integrating molecular pathology into clinical assessment (57).

<i>Variant</i>	<i>Geographic/Population Context</i>	<i>Typical Age at Onset</i>	<i>Penetrance by Age 50</i>	<i>Predominant Phenotype</i>	<i>Reference</i>
<i>Val30Met Portugal</i>	Endemic	30–40 years	~80%	Early-onset neuropathy, dysautonomia	Coutinho 1980; Conceição 2016
<i>Val30Met Sweden / non-</i>	Late-onset	>50 years	~11%	Neuropathy with cardiac involvement	Hellman 2008; Conceição

<i>endemic Europe</i>					ção 2016
<i>Val122Ile</i> (<i>p.Val142Ile</i>)	African ancestry (3–4% carriers)	~70 years	Not well defined	Restrictive cardiomyop athy	Jacobs on 1997; Connor s 2009
<i>Thr60Ala</i>	Irish descent	>50 years	Moderat e	Mixed neuropathy– cardiac	Ando 2013; Koike 2012
<i>Glu89Gln</i>	Southern Italy (Sicily)	40s– 50s	High	Neuropathy → rapid cardiomyop athy, cachexia	Mazze o 2015; Gentile 2021
<i>Phe64Leu</i>	Southern Italy (Sicily)	>60 years	Moderat e	Neuropathy - predominan t; misdiagnosi s frequent	Musum eci 2020
<i>Ile68Leu</i>	Rare	40s– 50s	Very high	Aggressive cardiomyop athy, survival <5 years	Gagliar di 2018

Table 4. Age at onset and penetrance by genotype and geography.

2.5 Implications for surveillance and public health

Epidemiological data are crucial to defining both who should be tested and when testing should be performed. In endemic areas and founder communities, where the prevalence of ATTRv is high, cascade genetic testing combined with structured pre-symptomatic counseling has emerged as an essential strategy. This approach enables the timely identification of carriers

before irreversible organ damage occurs and provides an opportunity to initiate surveillance and treatment at the earliest stages of disease (42,71).

In contrast, in non-endemic regions, diagnosis remains challenging due to the rarity of the disease and its clinical overlap with more common neuropathic and cardiomyopathic conditions. Here, the implementation of red-flag indexes within neurology and cardiology clinics has been shown to improve detection rates, reduce the frequency of misdiagnoses, such as chronic inflammatory demyelinating CIDP, and accelerate access to disease-modifying therapies (1,72,73).

Furthermore, large-scale registries, most notably the Transthyretin Amyloidosis Outcomes Survey (THAOS), continue to play a pivotal role in refining prevalence estimates and elucidating genotype–phenotype correlations. These data have revealed a gradual shift in the clinical burden of ATTRv, with increasing recognition of late-onset and cardiomyopathy-predominant cases as awareness of the disease expands among clinicians. Such insights emphasize the importance of both population-specific surveillance strategies and global registry-based initiatives in shaping diagnostic and therapeutic pathways for ATTRv (14).

Chapter 3 – Diagnostic Pathways and Red Flags in ATTRv

3.1 Diagnostic Challenges

Italian multicenter evidence has highlighted how ATTRv is frequently misdiagnosed as CIDP. In a recent large-scale study, approximately 10% of patients initially labeled as CIDP but presenting with clinical **red flags** were subsequently found to carry a pathogenic TTR variant (74).

Diagnostic delay has profound implications not only for clinical outcomes but also for patient experience. Surveys reveal that fewer than 40% of patients receive a diagnosis within 6 months, while most see multiple physicians before the correct diagnosis is made (75). The frustration, unnecessary treatments, and psychological burden of misdiagnosis emphasize the need for both clinician education and supportive diagnostic tools. These delays are clinically significant, since they are consistently associated with poorer outcomes and reduced efficacy of disease-modifying therapies, as demonstrated in recent longitudinal analyses (24).

In clinical practice, the sources of these delays are multifactorial. First, red-flag symptoms such as early autonomic dysfunction, bilateral carpal tunnel syndrome, or rapidly progressive neuropathy are often overlooked or attributed to more common conditions. Second, overreliance on non-specific diagnostic tests, such as nerve conduction studies showing mild demyelination, frequently leads to an erroneous CIDP label. Finally, fragmented patient pathways, in which neurological and cardiological

assessments are pursued sequentially rather than in an integrated manner, further contribute to misdiagnosis and loss of critical therapeutic time (47,73).

3.2 Red Flags and initial clinical suspicion

Red flags are clinical or instrumental cues that, when clustered, substantially increase the pre-test probability of ATTRv and should trigger TTR sequencing. They fall into neurological, cardiac, and multisystem domains and are weighted differently across populations and genotypes (47).

3.2.1 Neurological red flags:

- *Length-dependent axonal sensorimotor polyneuropathy in non-diabetic adults*
- *Painful small-fiber neuropathy and early autonomic dysfunction disproportionate to neuropathic severity*
- *Bilateral carpal tunnel syndrome, often preceding overt neuropathy*
- *Unexplained progressive weakness, imbalance, or dysautonomia*

The recognition of early neurological **red flags** is crucial in shortening the diagnostic delay of ATTRv.

A small-fiber–predominant onset, manifesting as burning feet, lancinating pain, and thermal hypoesthesia in non-diabetic adults, should immediately raise suspicion. When these symptoms are accompanied by disproportionate

autonomic disturbances, such as orthostatic hypotension, erectile dysfunction, or gastrointestinal dysmotility, they strongly suggest the possibility of ATTRv even before overt motor impairment becomes evident (41,76,77). Importantly, autonomic dysfunction may precede motor disability by several years, underscoring its diagnostic value.

Another highly informative clue is bilateral CTS, often documented surgically. This manifestation can precede the development of generalized polyneuropathy by up to a decade in both hereditary and wild-type transthyretin amyloidosis. The recurrence or bilaterality of CTS, particularly when associated with additional systemic signs, significantly enhances its specificity as a marker of amyloidosis (59,78).

Less common, but diagnostically powerful, presentations include amyotrophic lateral sclerosis (ALS)-like phenotypes, such as fasciculations and rapidly progressive weakness or lingual atrophy with fasciculations. When such motor neuron disease-like features coexist with sensory or autonomic signs, they should redirect the diagnostic focus from motor neuron disease toward amyloid neuropathy (79,80).

These neurological warning signs have now been formally integrated into international expert consensus algorithms. Their goal is to prompt timely genetic testing and targeted diagnostic evaluations, thereby reducing misdiagnosis and facilitating earlier initiation of disease-modifying therapies (1,81).

Red-flag signs/symptoms and prior medical history raising suspicion of ATTRv amyloidosis in patients presenting with symptoms of neuropathy

- 🚩 **Rapid rate of polyneuropathy progression**
- 🚩 **Early autonomic dysfunction**
 - Erectile dysfunction
 - Lightheadedness from postural hypotension
 - Changes in bowel movements and GI symptoms (often dismissed or misdiagnosed as IBS)
- 🚩 **Bilateral CTS and/or prior surgery for CTS**
 - Recurring after release surgery
 - Present in other family members
- 🚩 **Accompanying or prior history of symptoms from other systems (Table 1)**
 - Cardiac: shortness of breath, arrhythmias, CHF with preserved EF, features of hypertrophic cardiomyopathy
 - Musculoskeletal: rotator cuff, biceps tendon
 - Ophthalmologic: vitreous opacities, periorbital hemorrhages
 - Renal: renal failure and proteinuria
 - GI: unexplained/unintentional weight loss, constipation, diarrhea
- 🚩 **Motor weakness**
 - Predominant or early in the course of neuropathy
- 🚩 **Family history of ATTRv amyloidosis**
- 🚩 **Prior family history of unexplained:**
 - Rapidly progressing polyneuropathy of unknown cause
 - Heart failure
 - Sudden cardiac death
 - Cardiac arrhythmia
- 🚩 **Lack of response to specific treatments for other neuropathies (i.e., IVIg for CIDP)**

Figure 6. Factors raising suspicion of ATTRv amyloidosis with polyneuropathy in the US. From Karam et al. 2024 (23).

3.2.2 Red Flags for Cardiac Involvement

- *Unexplained left ventricular hypertrophy with preserved ejection fraction*
- *Diastolic dysfunction and atrial fibrillation*

- *Low-voltage ECG despite increased wall thickness*
- *Poor tolerance to standard heart failure therapies (ACE inhibitors, β -blockers)*

Cardiac involvement in ATTR is characterized by a set of distinctive red flags that should prompt diagnostic consideration. Unexplained concentric left ventricular hypertrophy, especially when accompanied by discordantly low QRS voltages on ECG, represents a classic presentation. HFpEF in older adults is another frequent manifestation, with relevance in African-American populations, where the Val122Ile (p.Val142Ile) variant is highly prevalent and strongly associated with late-onset cardiomyopathy. In addition, conduction system disease often necessitating pacemaker implantation emerges as a recurrent hallmark of the cardiac phenotype (12,25,52,82).

An especially telling constellation arises when cardiac manifestations coexist with orthopedic **red flags**, including bilateral carpal tunnel syndrome, lumbar spinal stenosis, and spontaneous biceps tendon rupture. The presence of these extracardiac clues in association with hypertrophic or restrictive cardiomyopathy significantly increases the pre-test probability of ATTR and should raise strong suspicion during clinical evaluation (62,83).

Advances in imaging have revolutionized the diagnostic approach. Bone scintigraphy using ^{99m}Tc -labeled tracers such as DPD, PYP, or HMDP now provides a non-invasive diagnostic pathway, with Perugini grade 2–3 cardiac

uptake in the absence of monoclonal protein being diagnostic of ATTR cardiomyopathy without the need for biopsy (61). Complementary techniques further refine recognition: echocardiography frequently demonstrates a characteristic pattern of relative apical sparing on longitudinal strain imaging, while cardiac magnetic resonance (CMR) often reveals diffuse subendocardial late gadolinium enhancement together with abnormal T1 mapping and increased extracellular volume (84). Despite these powerful tools, genetic testing remains indispensable to differentiate hereditary (ATTRv) from wild-type (ATTRwt) disease and to enable appropriate family counseling and cascade screening.

3.2.3 Multisystemic red flags

- *Weight loss, cachexia, and gastrointestinal dysmotility*
- *Vitreous opacities or glaucoma*
- *Proteinuria or chronic kidney disease without alternative explanation*
- *Spinal stenosis or biceps tendon rupture as systemic clues*

3.3 Diagnostic Workflow

The diagnostic evaluation of ATTRv is best approached through a pragmatic three-step pathway encompassing clinical suspicion, confirmatory testing, and staging.

1. ***Clinical suspicion*** represents the cornerstone of diagnosis. Physicians should be alert to neurological and/or cardiac red flags such as progressive axonal polyneuropathy, unexplained HfpEF, bilateral CTS, or a family history of neuropathy or cardiomyopathy. Importantly, many late-onset patients may not display an evident pedigree, which underscores the need for heightened awareness even in apparently sporadic presentations (1,47).

2. ***Confirmatory testing*** relies primarily on genetic analysis. Sequencing of the TTR gene is the gold standard for establishing ATTRv and should be performed in all suspected cases (1,42). Tissue biopsy, although less central today, maintains a role in complex or ambiguous presentations. Biopsies from sural nerve, salivary gland, abdominal fat, or endomyocardium, examined with Congo-red staining and immunohistochemistry or mass spectrometry typing, can confirm amyloid deposits. Nevertheless, false negatives may occur in early disease, reflecting the proximal onset of deposition in dorsal root ganglia, which is not always detectable in distal nerve or fat tissue (58,85).

A critical step is the exclusion of *light-chain (AL) amyloidosis*, particularly in patients with cardiac uptake on bone scintigraphy or systemic manifestations. This requires serum and urine immunofixation alongside serum free light chain testing. Only after AL amyloidosis has been confidently ruled out can the non-biopsy diagnostic algorithm be safely applied, in which scintigraphy demonstrating grade 2–3 myocardial uptake without monoclonal proteins is considered diagnostic for ATTR cardiomyopathy (61).

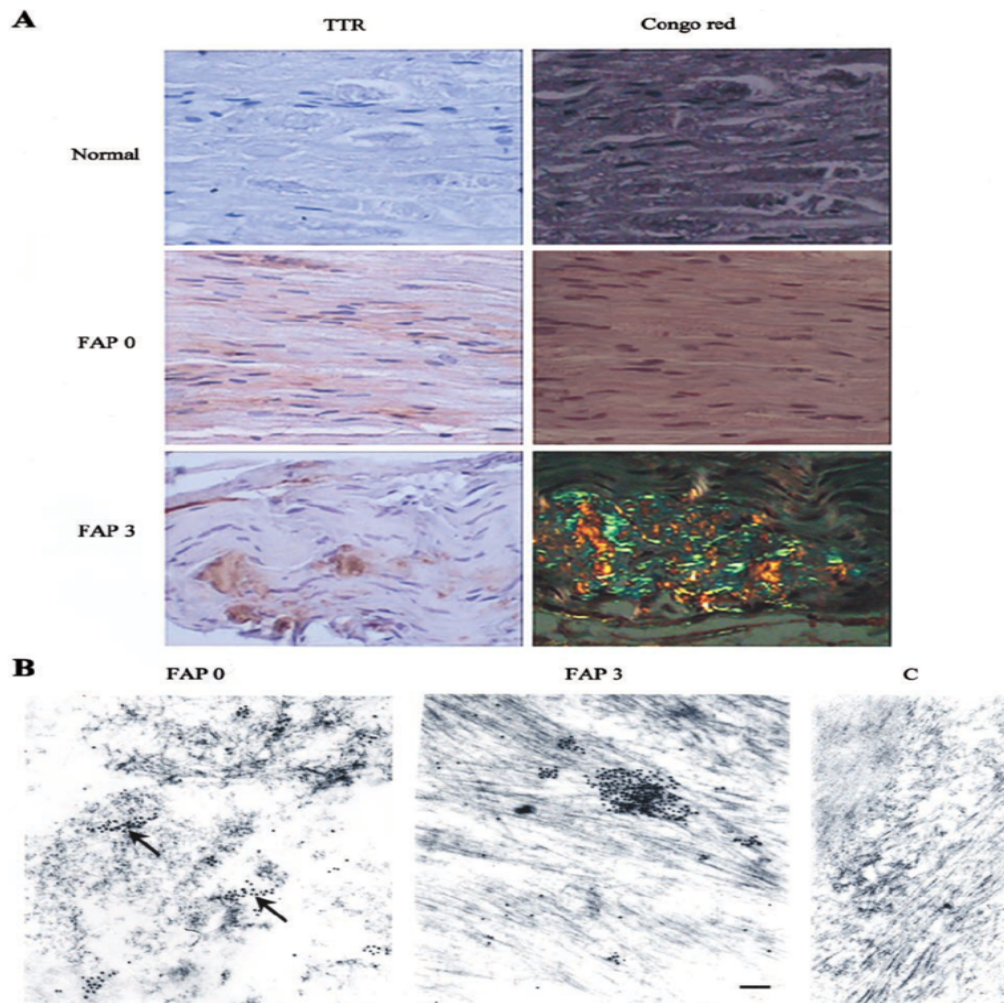


Figure 7. A: Immunohistochemistry with an anti-TTR antibody (left) and Congo Red staining (right) of normal nerves (top), FAP 0 patients (middle), and FAP 3 patients (bottom). B: TTR immunogold labeling and electron microscopy of nerves from a FAP 0 patient (left), a FAP 3 patient (middle), and a FAP 3 patient using preabsorbed anti-TTR (C, right). From Sousa et al. 2001 (86).

3.3.1 Organ involvement assessment

A comprehensive evaluation of organ involvement is fundamental in the diagnostic and staging process of ATTRv. From a neurological perspective, nerve *conduction studies (NCS)* and *electromyography (EMG)* typically reveal a length-dependent axonal sensory–motor polyneuropathy.

Complementary assessments such as quantitative sensory testing and autonomic evaluations, including tilt testing, beat-to-beat blood pressure monitoring, and sudomotor function studies, provide valuable insights, particularly in early disease where autonomic impairment may precede overt motor deficits (76).

The *cardiac work-up* integrates multiple modalities. ECG often demonstrates low voltage or conduction abnormalities, while echocardiography may show concentric left ventricular hypertrophy with strain imaging revealing relative apical sparing. CMR enhances tissue characterization by detecting diffuse subendocardial late gadolinium enhancement and abnormal T1/ECV values. Crucially, bone scintigraphy with technetium-labeled tracers (99mTc-DPD, PYP, or HMDP), interpreted using the Perugini grading system, enables non-invasive diagnosis of ATTR cardiomyopathy in the absence of monoclonal protein, thereby reducing reliance on endomyocardial biopsy (61,84).

Beyond the nervous and cardiac systems, other organs require systematic attention. Slit-lamp examination or ocular ultrasound can reveal vitreous opacities, a manifestation of local transthyretin production and deposition, while renal assessment with urinalysis, proteinuria quantification, and estimated glomerular filtration rate (eGFR) monitoring is essential given the risk of progressive renal dysfunction (67,69).

When this structured, multisystemic approach is applied in a prospective manner, diagnoses tend to be established earlier in the disease course. This,

in turn, allows for the timely initiation of disease-modifying therapies, which have been associated with measurable improvements in patient function, quality of life, and survival outcomes (1,72).

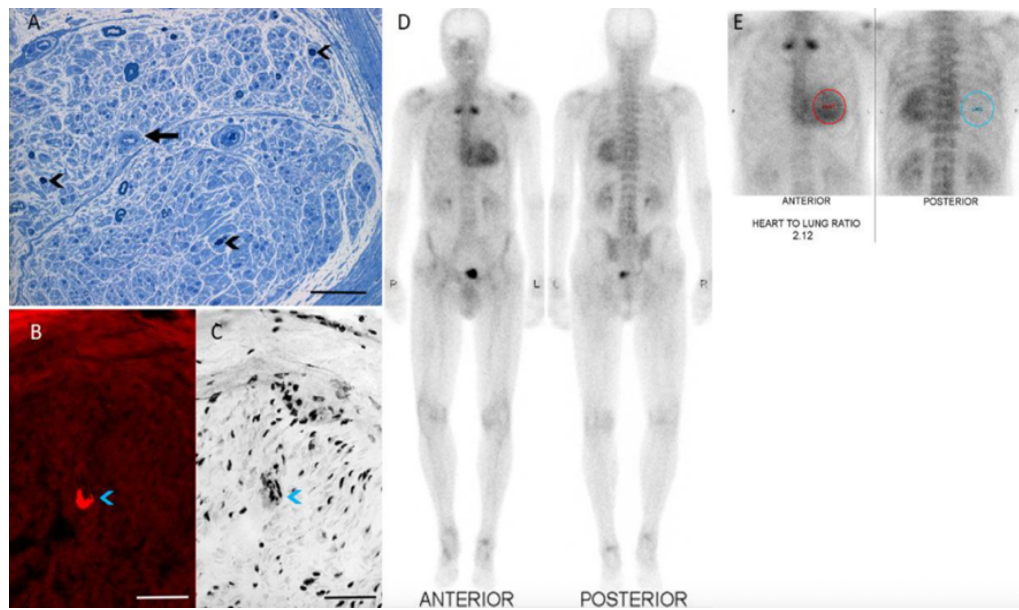


Figure 8. Sural nerve biopsy in ATTRv amyloidosis shows a marked loss of both myelinated and unmyelinated fibers, with signs of active degeneration. (A) Amyloid deposits are mainly localized within endoneurial blood vessel walls, causing vascular thickening. (B, C) Amyloid deposits in the endoneurial blood vessel wall (blue arrowheads), Congo red-stained frozen sections. (B) Immunofluorescence with Texas red filter and (C) transmitted light. (D, E) ^{99m}Tc -pyrophosphate scintigraphy demonstrates abnormal myocardial tracer uptake, with a heart-to-contralateral lung ratio >1.5 , supporting cardiac involvement in hereditary transthyretin amyloidosis. From Carroll et al. 2022 (5).

3.4 Diagnostic Pathways and Established Tools

Diagnosing ATTRv is difficult because the disease mimics common disorders and its phenotype varies by genotype, age at onset, and geography. Neuropathy-dominant forms are often labeled as CIDP, diabetic, or “idiopathic” axonal neuropathies; cardiac-dominant forms resemble

hypertensive or sarcomeric hypertrophic cardiomyopathy and HfpEF (1). In multiple series, the diagnostic delay is typically 5 years and longer delays correlate with worse functional outcomes despite modern therapies (24).

Clinical objective: compress time-to-diagnosis by recognizing red-flag clusters, deploying a stepwise testing pathway that integrates neurology and cardiology, and using genetic confirmation early once suspicion is credible (47,73,87).

A structured diagnostic cascade is essential to confirm ATTR:

3.4.1 Neurological work-up

- *Nerve conduction studies and electromyography*
- *Quantitative sensory testing and autonomic function assessment*
- *Tissue biopsy (sural nerve, salivary gland, abdominal fat) with Congo red staining and amyloid typing by immunohistochemistry or LC-MS*
- *Standardized outcome measures (mNIS+7, Norfolk QoL-DN)*

3.4.2 Cardiac work-up

- *ECG to detect low voltage and conduction abnormalities*
- *Echocardiography with longitudinal strain imaging*
- *CMR with diffuse subendocardial LGE, T1 mapping, and extracellular volume quantification*

- *Bone scintigraphy with 99mTc-labeled tracers (DPD, PYP, HMDP) , following Gillmore criteria for non-biopsy diagnosis (61).*

3.4.3 Genetic confirmation (the gold standard)

- *Genetic analysis of the TTR gene* is the gold standard to distinguish hereditary (ATTRv) from wild-type (ATTRwt) amyloidosis. It is mandatory in suspected cases to guide cascade screening and therapeutic decisions. Specially, it guides family cascade testing, and informs organ surveillance and therapy choice.

3.5 Biomarkers and what they add

Biomarkers provide crucial insights into disease burden, progression, and response to therapy in ATTR. While no single biomarker is definitive, certain cardiac and neurological markers have shown promise for monitoring disease trajectory, stratifying risk, and guiding therapeutic decisions.

3.5.1 Cardiac Biomarkers

- *NT-proBNP and Cardiac Troponins:* These markers are widely used to assess cardiac stress and amyloid burden. Elevated NT-proBNP and troponins correlate with worse cardiac involvement in ATTR, and they have prognostic value for mortality, hospitalizations, and functional cardiac deterioration (88). Although I did not locate a definitive 2019 study by Maurer or Brownrigg

explicitly proving the utility of screening in asymptomatic carriers with “cardiac tropism” genotypes, recent reviews (88) support the concept that these biomarkers can help detect subclinical cardiac involvement, especially in genotypes known to have cardiac predominance.

3.6 Neurological Biomarkers

- *Neurofilament Light Chain (NfL)* is emerging as a dynamic marker of axonal damage. Clinical trials, especially gene-silencing therapy trials (patisiran, vutrisiran) (89), have demonstrated that NfL levels decline with treatment, and these reductions correlate with improvements or stabilization in neurological function (90,91). Although NfL is not disease-specific, its responsiveness over time makes it useful for monitoring treatment effect and potentially in early recognition of disease. Furthermore, recent findings suggest that NfL may be a promising marker of symptom onset of peripheral nerve involvement in ATTRv and disease activity, though not of preclinical disease, and longitudinal studies are required to establish its role in monitoring progression and treatment response (92).
- *Variable echo time (vTE) MRI* has shown the ability to detect microstructural alterations of peripheral nerves even at early or mild disease stages, reliably distinguishing ATTRv patients from healthy controls. In particular, fascicular pseudo-T2 (pT2) emerges as a promising, novel, non-invasive radiological biomarker, with potential applications in both early diagnosis and longitudinal disease monitoring in ATTRv neuropathy (93).

3.7 Additional Investigational Biomarkers

- *TTR kinetic assays*: ex vivo assays of transthyretin tetramer stability—such as subunit exchange or denaturation methods—have been explored in carriers of pathogenic TTR variants to estimate protein instability (94). These tools can detect genotype-dependent differences in tetramer dissociation rates and have been used in early-phase trials of stabilizers as pharmacodynamic markers (95). However, they are not standardized and lack prospective validation for predicting disease onset in asymptomatic carriers, so they remain research tools (96).
- *Exploratory proteomics and metabolomics*: unbiased proteomic analyses in ATTRv cohorts and gene-silencing trials (e.g., APOLLO) have revealed broad shifts in circulating proteins, with neurofilament light chain (NfL) emerging as a dynamic biomarker that decreases under therapy and correlates with neurological improvement (89). Metabolomic studies in hereditary ATTR have also identified variant-specific metabolic signatures that may help in risk stratification of carriers (97). At present, these omics approaches remain investigational and require multicenter validation before clinical adoption.

3.8 Differential Diagnosis

A systematic differential prevents both over- and under-diagnosis:

- ***CIDP***: demyelinating features with conduction block respond to IVIg; lack of sustained response and prominent autonomic dysfunction argue against CIDP and favor ATTRv.
- ***Diabetic neuropathy***: prevalent and length-dependent but typically with proportional autonomic signs and diabetic stigmata; bilateral CTS alone is non-specific, but CTS + HfpEF + family history is atypical for diabetes.
- ***Small-fiber neuropathy of other causes***: Sjögren, Fabry, celiac disease—screen if clinical context suggests; absence of autonomic-cardiac-ocular triad reduces pretest probability of ATTRv.
- ***MGUS-related/AL amyloidosis***: mandatory to exclude with immunofixation/ κ - λ free light chains before ascribing scintigraphy uptake to ATTR.
- ***Sarcomeric HCM/ Left ventricular hypertrophy (LVH)***: wall thickness with high QRS voltages and family history of HCM, no extracardiac red flags.

<i>Domain</i>	<i>Differential Diagnosis</i>	<i>Key Distinguishing Features</i>
<i>Neurological</i>	Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP)	
	Diabetic Neuropathy	Associated with diabetes; distal symmetric neuropathy with autonomic features; no systemic amyloid
	Idiopathic Axonal Polyneuropathy	Slowly progressive, length-dependent; lacks multisystemic involvement

Cardiac	Charcot-Marie-Tooth Disease (CMT)	Early onset, hereditary neuropathy with foot deformities (pes cavus); confirmed by genetic testing
	AL Amyloidosis with Neuropathy	Peripheral neuropathy plus monoclonal gammopathy; confirmed by serum/urine immunofixation and bone marrow biopsy
	Hypertrophic Cardiomyopathy (HCM)	Asymmetric LV hypertrophy; sarcomeric mutations; no amyloid infiltration
	Hypertensive Heart Disease	LV hypertrophy secondary to long-standing hypertension; lacks neuropathy
	ATTRwt Amyloidosis (Wild-type)	Elderly males (>65); cardiac involvement without neuropathy or family history; no TTR mutation
Gastrointestinal	AL Amyloidosis (Cardiac)	Restrictive cardiomyopathy; monoclonal protein; rapid progression
	Celiac Disease	Malabsorption, diarrhea, positive anti-tTG antibodies, villous atrophy
	Inflammatory Bowel Disease (IBD)	Chronic diarrhea, abdominal pain, colonoscopic evidence; no neuropathy
	Irritable Bowel Syndrome (IBS)	Alternating diarrhea/constipation without systemic amyloid or neuropathy
Ocular	Diabetic Retinopathy	Microaneurysms, hemorrhages, neovascularization; associated with diabetes
	Vitreous Degeneration / Opacities (non-amyloid)	Age-related floaters; lacks systemic amyloid involvement
	Glaucoma	Progressive optic nerve damage; intraocular pressure elevation; no amyloid deposition
Renal	Diabetic Nephropathy	Albuminuria, progressive kidney

<i>Other Systemic</i>		dysfunction; associated with diabetes
	AL Amyloidosis (Renal)	Nephrotic syndrome, renal failure; monoclonal gammopathy; Congo red–positive renal biopsy
	Hypertensive Nephrosclerosis	Long-standing hypertension; progressive CKD without amyloid
	Parkinson’s Disease with Autonomic Dysfunction	Autonomic failure plus parkinsonism; no amyloid on biopsy
	Multiple System Atrophy (MSA)	Severe autonomic dysfunction with parkinsonism/cerebellar signs; MRI evidence of neurodegeneration

Table 5. Principal differential diagnosis.

Chapter 4 – Therapeutic Strategies in ATTRv Amyloidosis

4.1 Rationale and General Principles

ATTRv is a protein-misfolding disease in which pathogenic TTR variants destabilize the native tetramer, favoring dissociation into monomers that misfold and assemble into extracellular amyloid fibrils. Disease-modifying therapies therefore target three points along this cascade: tetramer stabilization, hepatic TTR suppression, and removal of residual deposits. Clinical management is multidisciplinary: neurology, cardiology, gastroenterology, nephrology, ophthalmology, genetics, and firmly time-sensitive: the earlier we treat, the greater the benefit, because approved agents slow (rather than reverse) damage already accrued in nerves and myocardium (98,99).

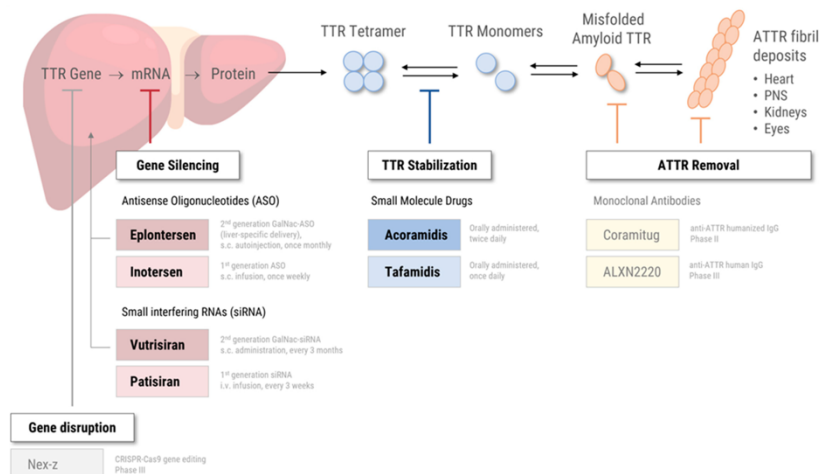


Figure 9. Mutation → tetramer destabilization → monomer misfolding → amyloid fibrils → organ dysfunction. Stabilizers act upstream; silencers reduce the source (liver); investigational antibodies/gene editing aim to clear or prevent deposits downstream. Tschöpe et al. 2025 (100).

4.2 Historical Disease Modification: Orthotopic Liver Transplantation (OLT)

Before pharmacotherapy, OLT was the only way to suppress the production of mutant TTR. Benefits were most evident in early-onset Val30Met (Portugal/Japan), with improved survival and slower neurologic progression (26). However, effectiveness proved genotype- and phenotype-dependent, with reduced benefit in late-onset Val30Met and many non-Val30Met mutations. TTR from the graft can continue to deposit in the heart, permitting cardiac progression (6). In addition, surgical risk and lifelong immunosuppression limit the indication. Contemporary practice reserves OLT to selected young Val30Met patients in expert centers, though pharmacologic disease modification has largely supplanted transplant in routine care (101).

<i>Item</i>	<i>Details</i>
<i>Best responders</i>	Early-onset Val30Met, low comorbidity, early-stage neuropathy
<i>Limitations</i>	No effect on existing deposits; ATTR-CM may progress due to wild-type TTR; immunosuppression
<i>Current role</i>	Highly selected cases in specialized centers; diminishing with effective pharmacotherapy

Table 6. OLT at a glance

4.3 TTR Tetramer Stabilizers

4.3.1 Tafamidis

Tafamidis acts by binding to the thyroxine-binding sites of TTR, thereby stabilizing the tetramer and preventing its dissociation into monomers prone to amyloidogenic aggregation. This mechanism reduces the formation of amyloid fibrils that are responsible for tissue damage in TTR-related amyloidosis.

The pivotal study published in *Neurology* in 2012, conducted in patients with familial amyloid polyneuropathy (FAP) due to the Val30Met mutation, demonstrated that tafamidis slows neuropathy progression and preserves quality of life compared to placebo (82,102). More recently, the ATTR-ACT trial, published in the *New England Journal of Medicine* in 2018, evaluated the efficacy of tafamidis in patients with TTR amyloid cardiomyopathy, including both wild-type and hereditary forms. Tafamidis treatment was associated with a significant reduction in all-cause mortality and cardiovascular hospitalizations, together with improvements in functional capacity and quality of life. These benefits were consistently observed across subgroups, including patients with NYHA functional class I–III (27).

From a practical perspective, tafamidis is administered orally, does not require complex titration, and is generally well tolerated. Clinical monitoring focuses primarily on functional and quality-of-life (QoL) outcomes, such as the modified neuropathy Impairment Score + 7 (mNIS+7) and Norfolk Quality of Life-Diabetic Neuropathy (Norfolk QoL-DN) in patients with

neuropathy, or NT-proBNP levels, six-minute walk test, and echocardiographic parameters (particularly ventricular strain) in those with cardiomyopathy.

4.3.2 Diflunisal

Diflunisal is a non-steroidal anti-inflammatory drug (NSAID) that has been shown to stabilize TTR in vitro, thereby reducing the dissociation of the tetramer into amyloidogenic monomers. A randomized clinical trial published in *JAMA* in 2013 evaluated the efficacy of diflunisal in patients with transthyretin familial amyloid polyneuropathy. In this study, treatment with diflunisal was associated with a significant slowing of neuropathy progression compared to placebo, highlighting its potential role in preserving neurological function (103). Nevertheless, the long-term use of diflunisal is constrained by adverse effects typical of NSAIDs, including nephrotoxicity, gastrointestinal complications, and fluid retention. These toxicities are particularly concerning in elderly patients and in those with concomitant cardiac disease. For this reason, diflunisal is generally considered when tafamidis is unavailable or contraindicated.

<i>Drug</i>	<i>Setting</i>	<i>Key outcomes</i>	<i>Common monitoring</i>	<i>Notes</i>
<i>Tafamidis</i>	Neuropathy (Val30Met, others); Cardiomyopathy (ATTR-CM)	Slows neuropathy; reduces mortality and CV hospitalizations in ATTR-CM	Clinical and functional, NT-proBNP, echo strain (CM)	Excellent tolerability

<i>Diffunis al</i>	Neuropathy	Slows neuropathy progression	Renal function, blood pressure, gastrointestin al adverse events	Consider risks in HF/elderl y
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Table 7. TTR stabilizers: indications, outcomes, safety

4.4 Gene-Silencing Therapies (TTR Suppression)

Gene-silencing therapies represent an innovative approach to the treatment of TTR amyloidosis, as they reduce total circulating TTR, both mutant and wild-type, thereby limiting the substrate available for fibrillogenesis. Two main platforms are currently in use: siRNA and antisense oligonucleotides (ASO).

4.4.1 Patisiran (siRNA, IV q3 weeks)

Patisiran is an siRNA administered intravenously every three weeks. The pivotal APOLLO trial, published in the *New England Journal of Medicine* in 2018, demonstrated that patisiran significantly improved mNIS+7 scores, quality of life, ambulation, and autonomic symptoms compared with placebo in patients with ATTRv polyneuropathy. Subgroup analyses in patients with cardiac involvement also revealed improvements in echocardiographic parameters (left ventricular strain) and reductions in NT-proBNP levels (30). Subsequent real-world studies confirmed the sustained neurological benefit and an overall favorable safety profile (104). Administration requires an

infusion center and premedication to mitigate infusion-related reactions, followed by routine clinical and autonomic monitoring.

4.4.2 Inotersen (ASO, SC weekly)

Inotersen, an ASO delivered by weekly subcutaneous injection, was evaluated in a randomized controlled trial published in the New England Journal of Medicine in 2018. Inotersen slowed neuropathy progression and improved quality of life compared with placebo (31). However, the pivotal program identified risks of thrombocytopenia and glomerulonephritis, necessitating close monitoring of platelet counts and renal function during therapy.

4.4.3 Vutrisiran (next-generation siRNA, SC q3 months)

Vutrisiran, a next-generation siRNA administered subcutaneously once every three months, was assessed in the HELIOS-A trial. Results, published in *Amyloid* in 2023, demonstrated non-inferior efficacy compared with patisiran in terms of neuropathy outcomes and quality-of-life measures, with a favorable safety profile and the convenience of quarterly dosing. Independent analyses further suggested consistent benefits across different baseline severity strata, supporting its use throughout the spectrum of neuropathic disease (105).

4.4.4 Eplontersen (next-generation ASO, SC monthly)

Eplontersen is a next-generation ASO conjugated with GalNAc, designed for targeted delivery to hepatocytes and administered subcutaneously on a monthly basis. By degrading hepatic TTR mRNA, it lowers both mutant and wild-type TTR production.

The NEURO-TTRansform trial (JAMA 2023) showed that Eplontersen significantly reduced serum TTR levels (by about 80%) and improved neuropathy scores and quality of life compared with placebo in patients with ATTRv polyneuropathy. The treatment was generally well tolerated, with a lower risk of thrombocytopenia and renal adverse effects than first-generation ASOs such as inotersen (32). Ongoing studies, including CARDIO-TTRansform, are evaluating its efficacy in patients with ATTR cardiomyopathy.

<i>Drug</i>	<i>Platform</i>	<i>Schedule</i>	<i>Pivotal Evidence</i>	<i>Key Monitoring</i>
<i>Patisiran</i>	siRNA (IV)	Every 3 weeks	<i>APOLLO</i> (NEJM 2018)	Infusion reactions; neurological and cardiac outcomes
<i>Inotersen</i>	ASO (SC)	Weekly	<i>NEJM 2018</i>	Platelets, renal function
<i>Vutrisiran</i>	siRNA (SC)	Every 3 months	<i>HELIOS-A</i> (Amyloid 2023)	Routine clinical monitoring
<i>Eplonterse n</i>	Next-generation ASO (GalNAc-SC)	Monthly	<i>NEURO-TTRansform</i> (JAMA 2023)	Routine labs; minimal thrombocytopenia risk

Table 8. TTR gene-silencers: platform, schedule, monitoring.
Abbreviations: siRNA, small interfering RNA, ASO, antisense oligonucleotide; GalNAc-SC, a next-generation N-acetylgalactosamine.

The choice of disease-modifying therapy in ATTR depends largely on the predominant clinical phenotype. In patients with neuropathy-predominant forms, or in mixed phenotypes with both neurological and cardiac involvement, gene-silencing agents, based on siRNA or antisense ASO, currently represent first-line therapy, owing to their proven ability to reduce circulating TTR levels and improve neurological outcomes. These include patisiran, vutrisiran, inotersen, and the next-generation ASO eplontersen, which offers monthly subcutaneous administration and an improved safety profile compared with earlier ASO platforms.

By contrast, in cardiac-predominant disease, tafamidis remains the cornerstone of therapy, supported by robust evidence of reduced mortality and cardiovascular hospitalizations, together with improvements in quality of life and functional capacity (27). However, in patients with concomitant neurological involvement or in cases where disease progression occurs despite Tafamidis treatment, the addition of a gene-silencing agent may be considered. The combined use of these therapeutic strategies nevertheless remains subject to regulatory approval and reimbursement policies across different healthcare systems.

4.5 Emerging and Investigational Strategies

4.5.1 In vivo gene editing (NTLA-2001)

A particularly innovative approach to the treatment of TTR is in vivo gene editing using *CRISPR/Cas9 technology*. The first-in-human clinical study,

published in 2021, demonstrated that a single intravenous administration of NTLA-2001 produced substantial and sustained reductions in serum TTR concentrations, providing proof of concept for a potential “one-and-done” therapy (34). More recently, the Nexiguran Ziclumeran (also called nex-z) gene editing platform was tested in patients with hereditary ATTR with polyneuropathy: in a phase 1 study, a single infusion of Nexiguran achieved mean TTR reductions of $\geq 90\%$ from baseline, which were durably sustained through 24 months, with an acceptable safety profile (35). Nevertheless, ongoing clinical trials are needed to clarify long-term durability, the risks of off-target effects, and the optimal criteria for patient selection.

4.5.2 Amyloid-directed antibodies

Another experimental strategy involves monoclonal antibodies targeting misfolded TTR epitopes, with the aim of promoting immune-mediated clearance of amyloid deposits. Agents such as *PRX004* (now referred to as *NNC6019*) have shown evidence of biological activity and acceptable safety in early-phase clinical trials. However, robust clinical outcome data are still lacking and will be required to establish their therapeutic role (106).

<i>Strategy</i>	<i>Mechanism</i>	<i>Examples</i>	<i>Notes</i>
<i>TTR Suppression</i>	Reduce TTR synthesis	LT, patisiran, inotersen, vutrisiran, eplontersen	Highly effective; LT limited by residual TTR production in select tissues

<i>TTR Stabilization</i>	Preserve functional tetramer	Tafamidis, diflunisal	Oral agents; delay progression, but don't remove existing amyloid
<i>Amyloid Clearance</i>	Remove existing deposits	Anti-amyloid antibodies (in development)	Add-on potential; currently under early evaluation

Table 9. Summary of Key Therapeutic Modalities.

4.5.3 Combination therapy

The combined use of stabilizers and gene-silencing therapies represents an attractive mechanistic approach. While silencers reduce the circulating substrate by lowering the production of both wild-type and mutant TTR, stabilizers prevent tetramer dissociation and the formation of amyloidogenic monomers. This synergistic action may also favor gradual regression of pre-existing fibrillar deposits. In real-world practice, such combination therapy has been shown to be feasible, although definitive evidence of long-term clinical benefit is still awaited (98).

4.6 Symptomatic and Multidisciplinary Care

Despite the availability of disease-modifying therapies, symptom control and preservation of function remain central goals in the management of patients with transthyretin amyloidosis.

- **Neuropathic pain:** management options include gabapentinoids, duloxetine, or low-dose tricyclic antidepressants. In selected cases, cautious

use of tramadol or short courses of opioids may be considered. Physiotherapy focused on improving balance and muscle strength is an essential adjunct.

- ***Cardiac involvement:*** Diuretics remain the cornerstone of therapy for congestion. ACE inhibitors and β -blockers are often poorly tolerated. Anticoagulation is recommended for atrial fibrillation. Pacemaker implantation may be indicated for conduction disease, and implantable cardioverter-defibrillators (ICDs) should be considered selectively (25).
- ***Orthostatic hypotension:*** salt and fluid loading, use of compression garments, and, when required, pharmacological support with midodrine or droxidopa.
- ***Gastrointestinal dysmotility and diarrhea:*** dietary measures such as meal fractionation, use of prokinetics (e.g., metoclopramide, domperidone), loperamide for diarrhea control, and treatment of small intestinal bacterial overgrowth (SIBO) with non-absorbable antibiotics such as rifaximin when suspected.
- ***Ocular manifestations:*** periodic ophthalmologic evaluation is recommended. Vitrectomy can be performed for vitreous opacities, while glaucoma requires ongoing surveillance (67).
- ***Renal involvement:*** management focuses on blood pressure control and the use of ACE inhibitors or ARBs in cases of proteinuria, with systematic nephrology follow-up (69).

Genetic counseling and cascade screening. In all index cases, genetic counseling and family cascade screening are essential to identify at-risk relatives and to enroll carriers into structured surveillance programs (107).

<i>Domain</i>	<i>First-line strategies</i>	<i>Escalation/alerts</i>
<i>Pain</i>	Gabapentinoids; SNRIs; TCAs	Refractory pain → pain specialist; fall-risk assessment
<i>Orthostatic hypotension</i>	Non-pharmacologic; midodrine/droxidopa	Syncope/bradyarrhythmias → cardiology
<i>GI</i>	Dietetic input; prokinetics; antidiarrheals; SIBO diagnosis	Weight loss >10%, malnutrition → nutrition/enteral options
<i>Cardiac</i>	Diuretics; rhythm/rate therapy; devices as indicated	Rapid decompensation, recurrent syncope → admit
<i>Ocular</i>	Vitrectomy if needed; glaucoma control	Sudden visual loss → ophthalmology
<i>Renal</i>	ACE-i/ARB; blood pressure; eGFR/albuminuria	Rapid eGFR drop or heavy proteinuria → nephrology

Table 10. Symptomatic/supportive therapy.

Abbreviations: SNRIs, serotonin-Norepinephrine Reuptake Inhibitors; TCAs, tricyclic antidepressants; SIBO, small intestinal bacterial overgrowth; ACE-i, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; eGFR, estimated glomerular filtration rate.

Chapter 5 – Machine Learning

5.1. Introduction

Artificial Intelligence (AI) and its subset, *Machine Learning (ML)*, are revolutionizing biomedical research and clinical medicine by enabling data-driven insights that go beyond the capabilities of traditional statistical approaches. The exponential growth of biomedical data, spanning genomics, imaging, electronic health records (EHRs), and wearable devices, necessitates advanced computational techniques for knowledge extraction and clinical decision support (108,109).

ML models excel at identifying complex, nonlinear relationships in high-dimensional datasets and can generate predictive models, stratify patients, and uncover biomarkers that enhance precision medicine. Recent studies highlight the transformative role of ML in domains such as radiology, pathology, genomics, drug discovery, and personalized therapy optimization (110,111).

5.2. Categories of Machine Learning Approaches

5.2.1 Supervised Learning

Supervised learning algorithms learn mappings between input features and output labels using annotated datasets. These methods are dominant in biomedical applications where diagnostic or prognostic ground truth is available.

Algorithms commonly applied: Support Vector Machines (SVMs), Decision Trees, Random Forests, Logistic Regression, and Deep Neural Networks (DNNs).

Applications: disease classification, image-based diagnostics (e.g., diabetic retinopathy detection), and prognosis prediction (108,111).

5.2.2 Unsupervised Learning

Unsupervised learning is applied when labeled data are unavailable. These methods uncover latent structures, clusters, or associations within complex datasets.

Algorithms commonly applied: k-means clustering, hierarchical clustering, Gaussian Mixture Models (GMM), Principal Component Analysis (PCA), Autoencoders.

Applications: disease subtype discovery, patient stratification, multi-omics feature extraction (108,110).

5.2.3 Reinforcement Learning

Reinforcement Learning (RL) is designed for sequential decision-making through interaction with an environment and optimization of cumulative rewards.

Algorithms commonly applied: Q-learning, Deep Q-Networks (DQN), Policy Gradient methods.

Applications: adaptive radiotherapy, optimization of drug dosing, dynamic treatment regimes in chronic diseases (108,112).

5.3 Machine Learning Methods in Biomedical Applications

5.3.1 Traditional Machine Learning Algorithms

Traditional ML algorithms remain essential due to their interpretability, computational efficiency, and ability to perform well with limited datasets.

Support Vector Machines (SVMs): Effective in high-dimensional spaces, widely applied in cancer classification and microarray data analysis (113).

Decision Trees and Random Forests: Provide interpretable models, extensively used in clinical decision support and risk prediction (108).

Naïve Bayes classifiers: Probabilistic models suitable for text mining in EHRs and gene expression analysis.

5.3.2 Ensemble Learning

Ensemble methods merge several models to enhance both robustness and predictive accuracy.

Bagging (e.g., Random Forests): Reduces variance by aggregating predictions from bootstrapped samples.

Boosting (e.g., XGBoost, AdaBoost): Improves weak learners sequentially by focusing on misclassified samples.

Applications: ensemble learning has shown superior performance in cancer outcome prediction, cardiovascular risk stratification, and image analysis tasks (114).

5.3.3 Deep Learning Approaches

Deep Learning (DL), a subset of ML, has demonstrated remarkable performance in biomedical data processing.

- *Convolutional Neural Networks (CNNs)*: Specialized for imaging tasks, used in radiology and pathology to achieve human-level performance (115).
- *Recurrent Neural Networks (RNNs) and Long Short-Term Memory (LSTM)*: Suited for sequential data such as physiological signals and clinical narratives.
- *Generative Adversarial Networks (GANs)*: Applied in medical image synthesis, augmentation, and denoising (116).
- *Transformers*: Emerging architectures that enable multimodal learning by integrating genomic, imaging, and clinical text data (117).

5.3.4 Hybrid and Multimodal Approaches

Biomedical problems often involve heterogeneous data sources (imaging, genomics, EHRs). Hybrid ML models integrate these modalities to capture complementary information.

Examples: combining CNN-based imaging features with genomic signatures to improve cancer prognosis prediction (118).

5.4. Workflow for ML Model Development in Healthcare

The ML pipeline in healthcare involves several critical steps (108):

- *Data Preprocessing:* cleaning, normalization, handling missing data, data augmentation.
- *Feature Selection and Dimensionality Reduction:* techniques such as recursive feature elimination, PCA, and autoencoders to reduce noise and enhance interpretability.
- *Model Training and Validation:* partitioning datasets into training, validation, and test sets, with hyperparameter optimization (Grid Search, Bayesian optimization).
- *Evaluation Metrics:* accuracy, sensitivity, specificity, precision, recall, F1-score, AUC-ROC.
- *External Validation:* testing models on independent cohorts to ensure generalizability.

5.5. Biomedical Applications of ML

5.5.1 Medical Imaging

CNNs and DL models are applied to radiology, histopathology, and ophthalmology, enabling early detection of cancers, neurological disorders, and retinal diseases (115,119).

5.5.2 Genomics and Precision Medicine

ML facilitates variant classification, biomarker discovery, and multi-omics integration for drug repurposing and personalized treatment (118,120).

5.5.3 Clinical Decision Support

Predictive ML models aid in risk stratification for sepsis, cardiovascular events, and ICU mortality, enhancing decision-making in real-time (121).

5.5.4 Remote Monitoring and Digital Health

Wearable sensors and IoT devices generate continuous physiological data analyzed by ML algorithms for early detection of deterioration and remote chronic disease management (122).

5.6. Challenges and Future Perspectives

Despite significant progress, ML in healthcare faces several challenges:

- *Data heterogeneity and integration* across healthcare systems
- *Algorithmic bias and fairness*, particularly for underrepresented groups

- *Interpretability and transparency*, essential for clinical adoption
- *Regulatory compliance and privacy* for patient data.
- *Scalability and workflow integration*, particularly in resource-limited settings (108,123)

Future directions include explainable AI (XAI), federated learning for privacy-preserving data sharing, and causal ML approaches to improve clinical interpretability.

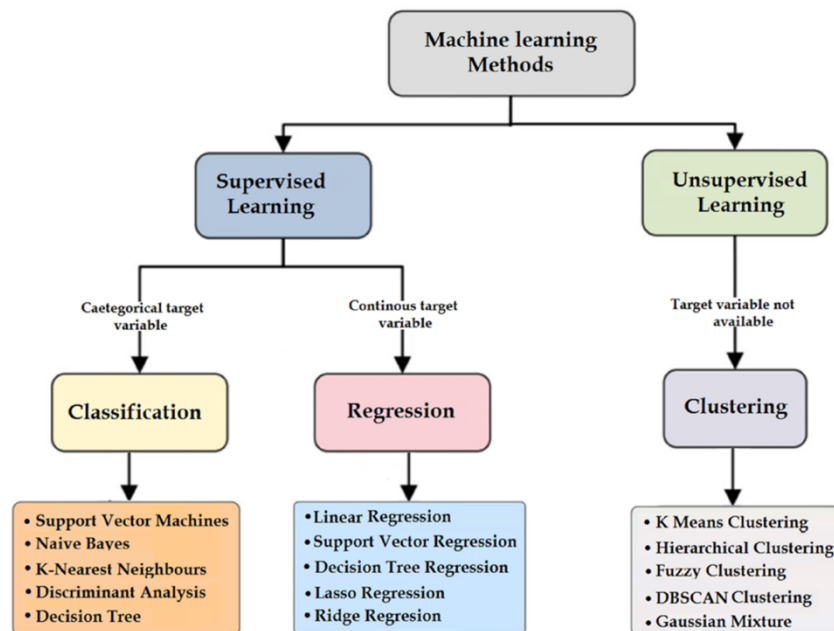


Figure 10. Machine learning techniques for biomedical data. From Binson et al. 2024 (108).

Chapter 6 – Machine Learning and Artificial Intelligence in ATTRv

6.1 Rationale: Why AI in a rare disease?

The application of ML to ATTR is a rapidly expanding field, reflecting the diagnostic complexity of this heterogeneous disorder. ATTR encompasses both hereditary (ATTRv) and wild-type (ATTRwt) forms, manifesting as progressive neuropathy, cardiomyopathy, or mixed phenotypes. Given the frequent diagnostic delays, the rarity of the condition, and the growing therapeutic arsenal that requires early initiation, data-driven tools such as ML are increasingly investigated to support clinicians in case identification, risk stratification, and differential diagnosis.

ATTRv is characterized by striking *clinical heterogeneity*, as the same pathogenic mutation can give rise to neuropathic, cardiac, or mixed phenotypes, with considerable variability in the age at disease onset. This diversity in presentation contributes significantly to the diagnostic challenge. ATTRv neuropathy is often initially misclassified as more common conditions, such as CIDP or diabetic polyneuropathy, while cardiac forms may mimic hypertensive or sarcomeric cardiomyopathy. Such overlap with prevalent disorders frequently results in *diagnostic delay*, which is typically estimated at 3–6 years and may be even longer in some cases. This delay has critical clinical implications, as it often leads to the loss of the therapeutic window during which disease-modifying treatments are most effective (1).

Traditional diagnostic tools (family history, red-flag checklists, imaging, biopsy) are essential but limited by subjectivity, low sensitivity in early disease, and reliance on expert centers.

ML and AI are particularly well-suited to address the diagnostic challenges of ATTRv. Unlike traditional statistical approaches, ML algorithms can capture *non-linear relationships* across multiple clinical variables, thereby identifying patterns that may escape conventional analyses. Moreover, these methods can *integrate multimodal datasets* (including clinical features, neurophysiological parameters, imaging findings, and circulating biomarkers) into unified predictive models. This ability to combine heterogeneous sources of information is especially valuable in a disease as phenotypically diverse as ATTRv. Finally, AI-based decision support systems hold particular promise in *non-endemic regions*, where disease awareness among clinicians is often low and diagnostic suspicion consequently delayed. By providing risk stratification and automated flagging of high-risk cases, such tools may contribute to earlier referral for genetic testing and specialist evaluation (124).

6.2 Clinical applications of ML in ATTRv

6.2.1 Neurology-focused models: red flags and neurophysiology

One of the most promising areas has been the use of ML to identify ATTRv among patients presenting with peripheral neuropathies. Multiple studies highlight the high rate of misdiagnosis and delay in ATTRv amyloidosis. It is estimated that about one-third to three-quarters of patients are initially misdiagnosed, often with more common neuropathies. The consequences of delayed or missed diagnosis are severe. To facilitate earlier diagnosis, researchers and experts have defined key *red flag* signs (clinical features that should raise suspicion of ATTRv) especially when they appear in combination. An Italian multicenter study applied a ML algorithm (XGBoost classifier) to 189 patients who were suspected to have ATTRv (had clinical features suggestive of amyloidosis) in non-endemic areas of Italy (36). The model was trained to distinguish those who ultimately tested positive for a pathogenic TTR mutation from those who tested negative, using the presence/absence of various symptoms as features. The ML analysis yielded some interesting predictors of a positive ATTRv genetic diagnosis: unexplained weight loss, gastrointestinal issues, and evidence of cardiomyopathy were strongly associated with having ATTRv, whereas patients who turned out negative were more likely to have features like bilateral CTS, diabetes, autoimmune disorders, ocular or renal involvement. These counterintuitive findings (e.g., CTS and diabetes correlating with negative tests) suggest that in a general neurology population, common comorbidities such as diabetes and CTS may lead to a false suspicion of ATTRv, whereas more systemic clues (weight loss, gastrointestinal, and cardiac signs) were more discriminative for true ATTRv cases. The authors

conclude that ML can help refine the clinical profile of ATTRv in non-endemic regions, improving the selection of patients for TTR gene testing.

A team in Belgium focused on using artificial intelligence to comb through electronic health records (EHRs) for red flags (125). They developed a natural language processing (NLP) algorithm to automatically extract occurrences of known ATTRv red flag symptoms from unstructured clinical notes. It flagged 128 patients who had ≥ 3 red-flag symptoms in their charts; upon expert clinical review, 69 of those patients (54%) were deemed appropriate candidates for TTR genetic testing. It flagged 128 patients who had ≥ 3 red-flag symptoms in their charts; upon expert clinical review, 69 of those patients (54%) were deemed appropriate candidates for TTR genetic testing. The algorithm demonstrated high accuracy in identifying documented red flags (with F1-scores between 0.88 and 0.98 for various symptom categories). Importantly, using this AI screening approach would have led to a 48.6% relative increase in genetic testing for ATTRv, presumably catching many patients who otherwise might not have been recognized. The authors conclude that an NLP-driven tool, once clinically validated, offers a powerful, efficient method for screening large populations for rare disease warning signs and can substantially aid earlier diagnosis and treatment of ATTRv amyloidosis. This study serves as a proof-of-concept demonstrating that AI can augment clinical vigilance. Instead of relying on individual physicians to recall every potential red flag, the algorithm can continuously surveil patient data and prompt providers when a concerning pattern emerges.

6.2.2 Cardiology-focused models

In cardiology, ML has been increasingly applied to bone scintigraphy, a cornerstone of the non-biopsy diagnostic pathway for ATTR cardiomyopathy. Halme et al. (2022) demonstrated the feasibility of convolutional neural networks (CNNs) in detecting and classifying scintigraphic uptake patterns compatible with ATTR, achieving high accuracy (126). Similarly, Delbarre et al. (2023) developed a deep-learning pipeline capable of automatically assigning Perugini scores, thereby reducing inter-observer variability and standardizing reporting (127). These advances highlight the potential of ML to automate and harmonize diagnostic interpretation, particularly in non-specialist centers.

Beyond bone scintigraphy, ML has also been applied to echocardiographic and CMR data. For example, Chang et al. (2024) demonstrated that a random forest model trained on echocardiographic parameters could identify cardiac amyloidosis with an area under the curve (AUC) of approximately 0.84, supporting the value of ML for pattern recognition in imaging-derived variables (128). Complementary evidence comes from routine laboratory data: Pan et al. (2024) developed a machine learning prediction model for cardiac amyloidosis in patients with left ventricular hypertrophy using standard blood test parameters (129). Their model achieved robust discrimination between affected and non-affected individuals, underscoring that even non-imaging clinical data can contribute to early detection.

6.2.3 Multimodal integration

Taken together, the literature supports the feasibility and potential clinical value of ML in ATTR. In neuropathy-focused cohorts, ML can refine suspicion indexes and prioritize genetic testing, while in cardiology, AI-driven imaging analysis is poised to improve accuracy and reduce diagnostic variability. The translation into routine practice, however, requires validation on large, multicenter, and multi-ethnic datasets, as well as integration within explainable frameworks to ensure clinical trust. As therapeutic options expand, the timely application of ML tools may help reduce diagnostic delays and improve patient outcomes.

The future direction is multimodal AI, integrating:

- *clinical red flags,*
- *serum biomarkers (NT-proBNP, troponin, NfL),*
- *electrophysiology,*
- *imaging (echo/CMR/scintigraphy).*

Pilot studies show that multimodal ML classifiers outperform single-domain models, suggesting that integrated pipelines could be the first step toward clinical-grade decision-support.

Chapter 7 – Materials and Methods

7.1 Aims and objectives

This study was conducted on a cohort of subjects aged between 19 and 86 from Western Sicily, including patients from Palermo, Trapani, and Agrigento, and from Eastern Sicily, comprising patients from Catania, Enna, and Syracuse, presenting at least one significant clinical manifestation of the disease (defined as a red flag). The objective is twofold: first, to evaluate the performance of the most consolidated classification algorithms in the literature for this type of problem, focusing on those based on trees; second, to employ XAI methodologies to identify which of the red flags are most associated with the presence of the genetic mutation triggering the disease.

7.2 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 CTS, 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. Age <18 years
3. Positive history for: a) hematological pathology or possible cause or responsible for secondary amyloidosis; b) secondary amyloidosis; 4) 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 to the expected clinical onset) (18).

7.3 Genetic screening of ATTRv

The genetic analyses included in this study were conducted between June 2019 and August 2024 in Western Sicily, including patients from Palermo, Trapani, and Agrigento, and Eastern Sicily, comprising patients from Catania, Enna, and Syracuse.

A Genilam swab genetic testing for TTR mutations was performed for each patient. This test employed Polymerase Chain Reaction (PCR) targeting exons 2, 3, and 4 of the TTR gene (NM_000371.4), followed by automated

sequencing using the SeqStudio Flex Genetic Analyzer. This method enabled the detection of small point mutations, deletions, or insertions (<20 bp) in the specified gene regions.

Demographic data were collected for each patient who underwent genetic testing for TTR gene mutations, including age, sex, and the presence or absence of a TTR mutation (along with the specific mutation, if present). Additionally, past medical history was gathered, including the presence of autoimmune diseases, prior diagnoses of CIDP, or ALS, diabetes, and any previous misdiagnoses. Clinical data were recorded, such as the presence or absence of symptoms. Furthermore, red flags were systematically documented, including unexplained weight loss, bilateral CTS, sensory neuropathy, lumbar canal stenosis, ataxia, gastrointestinal disturbances, autonomic dysfunction, cardiomyopathy, positive bone or cardiac scintigraphy, renal dysfunction, vitreous opacities, positive tissue biopsy, family history of cardiomyopathy, family history of neuropathy, and family history of ATTRv. Each item from the past medical history, clinical data, and red flag checklist was encoded in a binary format, with '0' indicating absence and '1' indicating presence. All available data, including demographic information, medical history, clinical features, and red flags, were systematically collected and integrated into a structured dataset for subsequent analysis. Then, the total cohort was divided into two subcohorts: the Western Sicily subcohort, including patients from Palermo, Trapani, and Agrigento, and the Eastern Sicily subcohort, comprising patients from Catania, Enna, and Syracuse.

7.4 Algorithmic Modeling and Feature Attribution Analysis

To investigate which red flags are most strongly associated with the pathogenic gene mutation, XAI techniques were employed. After an initial data preprocessing phase, several algorithms were implemented, including both linear and non-linear models suitable for categorical variables. ***Multiple GridSearch cycles*** were performed using different feature combinations. The variable family history of ATTRv was excluded, given the autosomal dominant inheritance of the disease, while age was not considered, as it only reflects the time of diagnosis. For each model, hyperparameter tuning was conducted based on the available dataset and the red flag variables. Once the evaluation metrics were computed for each model, selected XAI methods were applied. These techniques enable the interpretation and understanding of ML model behavior, allowing the explanation of predictions and the identification of the decision-making processes underlying the models.

7.5 Model Training

The selected classification models are based on supervised learning algorithms, which require a labeled dataset for training. These models operate as binary classifiers, in line with the binary structure of the output variable. The training and evaluation pipeline included the following phases:

1. ***Data Preprocessing.*** Data cleaning and preprocessing were performed based on the insights derived from the previous exploratory data analysis.

This step ensured the removal of inconsistencies and the transformation of variables into appropriate formats for model training.

2. **Feature Selection.** In the initial training phase, all variables in the dataset were considered except for age and family history of ATTRv, which were excluded due to their limited informative value or potential redundancy. Feature selection was then performed iteratively to assess the impact of specific predictors related to a family history of neurological and/or cardiovascular disorders.

3. **Train-Test Split:** The dataset was split into a training set and a test set. Specifically, data from facilities located in Western Sicily were used for training, while data from Eastern Sicily were used for testing. This approach allows for the evaluation of the model's generalization capability on a population with potentially distinct demographic and clinical profiles distinct from those used for training, thereby providing a robust measure of its performance on unseen data. Such a clear separation ensures that a model trained on a cohort with specific characteristics is validated on another, effectively simulating its application in a real-world scenario different from the original one.

4. **Hyperparameter Tuning:** Hyperparameter optimization was performed through a two-phase strategy using GridSearchCV with Repeated Stratified 10-fold Cross-Validation (20 repeats). In the preliminary screening phase, a wide range of algorithms from different families were compared, including Logistic Regression, SVC, K-Nearest Neighbors, Naive Bayes, Random

Forest, Gradient Boosting, XGBoost, and MLP. Tree-based models and their ensemble variants consistently outperformed the others across different feature combinations. The second phase focused on an in-depth evaluation of decision tree-based algorithms (Random Forest, ExtraTrees, Gradient Boosting, XGBoost, LightGBM, CatBoost, and Decision Tree), benchmarked against linear classifiers. Hyperparameters were tuned to maximize the F1-score, chosen for its robustness under class imbalance. This rigorous validation strategy mitigated biases from random data partitioning, improved reproducibility, and ensured that model selection relied on a multi-objective assessment rather than a single performance metric.

5. ***Model Evaluation:*** After training, model performance was evaluated on the test set. Evaluation metrics included accuracy, precision, recall, F1-score, and the confusion matrix, allowing for a comprehensive assessment of classification performance. The final goal was not only to evaluate the model's performance, but also to explore its decision-making process through global XAI methods.

7.6 Permutation Importance

Permutation Importance is a model-agnostic technique used to assess the relevance of input features in a predictive model. This approach can be applied to any machine learning algorithm and offers an intuitive way to

interpret the contribution of individual variables to the model's predictions.

The procedure involves the following steps:

1. **Baseline Performance Calculation:** The model's predictive performance is first evaluated using the original test dataset.
2. **Feature Permutation:** For each feature, its values are randomly shuffled across the test set, effectively disrupting the relationship between that feature and the target variable.
3. **Performance Recalculation:** The model's performance is then re-evaluated using the permuted dataset.
4. **Importance Estimation:** The importance of each feature is determined by computing the difference between the original and the permuted performance scores. A substantial drop in performance suggests that the feature plays a crucial role in the model's predictions.

7.7 SHAP (SHapley Additive exPlanations)

SHAP (Shapley Additive Explanations) is an interpretability technique for predictive models based on cooperative game theory. It assigns each feature a value (the Shapley value) that quantifies the average marginal contribution of that feature to the model's prediction, considering all possible combinations of features. SHAP quantifies the contribution of each feature to the prediction by calculating how much the prediction changes when the feature is included versus when it is excluded, considering all possible

combinations of features. This approach, grounded in game theory, ensures that the attribution of importance is both fair and consistent across all features. The individual contributions, known as Shapley values, add up to the final prediction, enabling a clear decomposition of the model's output. This makes it possible to understand not only the overall influence of each feature across the entire dataset but also how each feature impacts specific individual predictions. This dual capability makes SHAP especially useful in clinical settings where understanding both the global behavior and local decision-making of the model is essential.

7.8 Statistical Analysis

The final dataset consisted of *452 subjects* and *21 variables*. Among these, 20 potential features and one output variable, represented by the ATTR mutation, were identified.

During the feature selection phase, two variables were excluded from the analysis for sound methodological reasons:

1. The variable "*family history of transthyretin amyloidosis*" was removed to avoid logical confounding. Since ATTRv is an autosomal dominant inherited disease, family history acts as a strong proxy for the target variable (the presence of the genetic mutation). Its inclusion would therefore have masked the predictive contribution of clinical manifestations (red flags), which are the true focus of the analysis.

2. The variable "*age*" encodes the patient's age at diagnosis, not at symptom onset, and therefore is not a parameter available during the initial patient assessment, thus compromising the applicability of the model in a real-world predictive context.

Quantitative variables are reported in raw numbers and by calculating the mean, standard deviation, and percentage.

Chapter 8 – Results

8.1 Cohort characteristics

In this study, a total of **452 subjects** were evaluated across two Sicilian cohorts, *Western Sicily*, including patients from Palermo, Trapani, and Agrigento, and in *Eastern Sicily*, comprising patients from Catania, Enna, and Syracuse recruited from referral neuromuscular clinics between June 2019 and August 2024. The cohort included 210 males (46.5%) and 242 females (53.5%), with a mean age of 61 (range 19–86, SD 14.7). There were no missing data in the dataset, and aside from age (treated as a continuous variable), all features were binary (presence/absence of specific signs or history). The negative (mutation-free) group was substantially larger than the positive group: 330 patients (73%) tested negative for the pathogenic TTR mutation, versus 122 (27%) who were ATTRv mutation-positive, reflecting class imbalance.

Among them, **68 individuals (15.0%)** were confirmed carriers of pathogenic TTR mutations, either symptomatic at the time of testing or identified during family screening. Importantly, more than half of mutation carriers (51.5%) already exhibited overt clinical manifestations at the time of genetic confirmation, reflecting a still substantial diagnostic delay in real-world practice.

When stratifying by age (quantiles: 19–55, 56–64, 65–72, 73–86 years), mutation-positive patients clustered predominantly in the intermediate age

groups (56–72 years), while extremes of the age spectrum (73-86) were less represented (Figure 11).

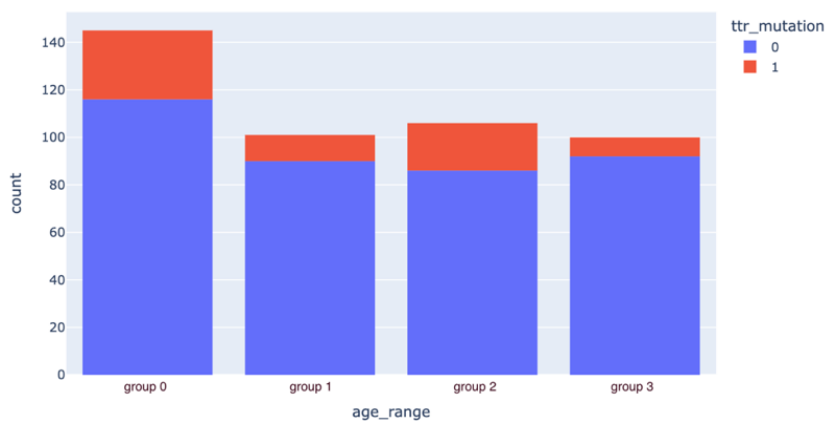


Figure 11. Histograms representing age distribution of the total cohort and mutation-positive subgroup. Group 0: 19 to 55 age range; Group 1: 56 to 64 age range; Group 2: 65 to 72 age range; Group 3: 73 to 86 age range

Gender distribution revealed a slight predominance of males overall. However, no significant difference emerged when correlating gender with TTR mutation positivity, suggesting that sex does not represent a strong discriminative factor in this population (Figure 12).

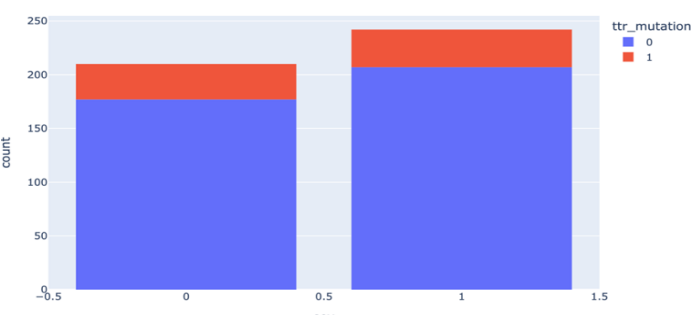


Figure 12. Histograms representing individuals with a positive ATTRv test divided by gender. Group 0: female subjects; Group 1: male subjects

8.2 Distribution of clinical red flags

The prevalence of key clinical features (red flags) in the cohort is summarized here. The most frequent red flag was **sensory peripheral neuropathy**, present in 283 patients (62.6% of the cohort). A **family history of cardiopathy** was reported by about half of the patients (234/452, 51.8%), and nearly as many had documented **bilateral carpal tunnel syndrome (CTS)** (209/452, 46.2%). Other common features included **unexplained weight loss** (33.6%), **autonomic dysfunction** (36.7%), and **ataxia** (31.4%). In contrast, some red flags were relatively rare in this cohort – for example, only 45 patients (10.0%) had **renal dysfunction**, and 78 (17.3%) had **lumbar spinal canal stenosis**. As expected in a hereditary disease, a considerable subset had a **family history of neuropathy** (166 patients, 36.7%), while 123 patients (27.2%) reported a family history of known ATTRv amyloidosis (though this specific variable was later excluded from modeling, see below).

Class Feature	Total		Western Sicily		Eastern Sicily	
	0	1	0	1	0	1
ataxia	310 (68.58%)	142 (31.42%)	215 (65.15%)	115 (34.85%)	95 (77.87%)	27 (22.13%)
autonomic_dysfunction	286 (63.27%)	166 (36.73%)	203 (61.52%)	127 (38.48%)	83 (68.03%)	39 (31.97%)
bilateral_cts	243 (53.76%)	209 (46.24%)	198 (60.00%)	132 (40.00%)	45 (36.89%)	77 (63.11%)
biopsy	441 (97.57%)	11 (2.43%)	319 (96.67%)	11 (3.33%)	122 (100.00%)	0 (0.00%)
bone_scintigraphy	421 (93.14%)	31 (6.86%)	299 (90.61%)	31 (9.39%)	122 (100.00%)	0 (0.00%)
cardiac_uptake	424 (93.81%)	28 (6.19%)	302 (91.52%)	28 (8.48%)	122 (100.00%)	0 (0.00%)
cardiopathy	336 (74.34%)	116 (25.66%)	238 (72.12%)	92 (27.88%)	98 (80.33%)	24 (19.67%)
family_history_of_att_rv	329 (72.79%)	123 (27.21%)	239 (72.42%)	91 (27.58%)	90 (73.77%)	32 (26.23%)
family_history_of_cardiopathy	218 (48.23%)	234 (51.77%)	169 (51.21%)	161 (48.79%)	49 (40.16%)	73 (59.84%)
family_history_of_neuropathy	286 (63.27%)	166 (36.73%)	204 (61.82%)	126 (38.18%)	82 (67.21%)	40 (32.79%)
gastrointestinal_disturbances	312 (69.03%)	140 (30.97%)	223 (67.58%)	107 (32.42%)	89 (72.95%)	33 (27.05%)
lumbar_canal_stenosis	374 (82.74%)	78 (17.26%)	270 (81.82%)	60 (18.18%)	104 (85.25%)	18 (14.75%)
ocular_disorder	364 (80.53%)	88 (19.47%)	252 (76.36%)	78 (23.64%)	112 (91.80%)	10 (8.20%)
positive_biopsy	442 (97.79%)	10 (2.21%)	320 (96.97%)	10 (3.03%)	122 (100.00%)	0 (0.00%)
renal_dysfunction	407 (90.04%)	45 (9.96%)	298 (90.30%)	32 (9.70%)	109 (89.34%)	13 (10.66%)
sensory_neuropathy	169 (37.39%)	283 (62.61%)	96 (29.09%)	234 (70.91%)	73 (59.84%)	49 (40.16%)
sex	210 (46.46%)	242 (53.54%)	142 (43.03%)	188 (56.97%)	68 (55.74%)	54 (44.26%)
unexplained_weight_loss	300 (66.37%)	152 (33.63%)	249 (75.45%)	81 (24.55%)	51 (41.80%)	71 (58.20%)

Table 11. Shows the percentages of each red flag observed in the total population and in the relief populations of eastern and western Sicily.

8.3 Distribution of TTR Variants in Mutation-Positive Patients

Among the 452 individuals screened, 68 (15.0%) were identified as carriers of a pathogenic TTR mutation. Within this mutation-positive subgroup, the distribution of variants was markedly heterogeneous, although dominated by a single genotype. The **F64L (Phe64Leu)** variant was by far the most prevalent, accounting for 70.6% of cases. The E89Q and V122I substitutions each represented 7.4% of cases. The following were found in decreasing percentages: H90A (5.9% of carriers), F84L (2.9%), E61A (2.9%), S97F (1.5%), and V20A (approximately 1.5% each).

Figure 13 (a pie chart of mutation frequencies) illustrates this stark predominance of the F64L variant, with all other genotypes comprising only a small fraction of the mutation-positive subgroup. In summary, while a wide mutational spectrum was observed, the ***F64L mutation clearly predominated*** in both Sicilian cohorts – a finding of practical importance for regional genetic screening strategies.

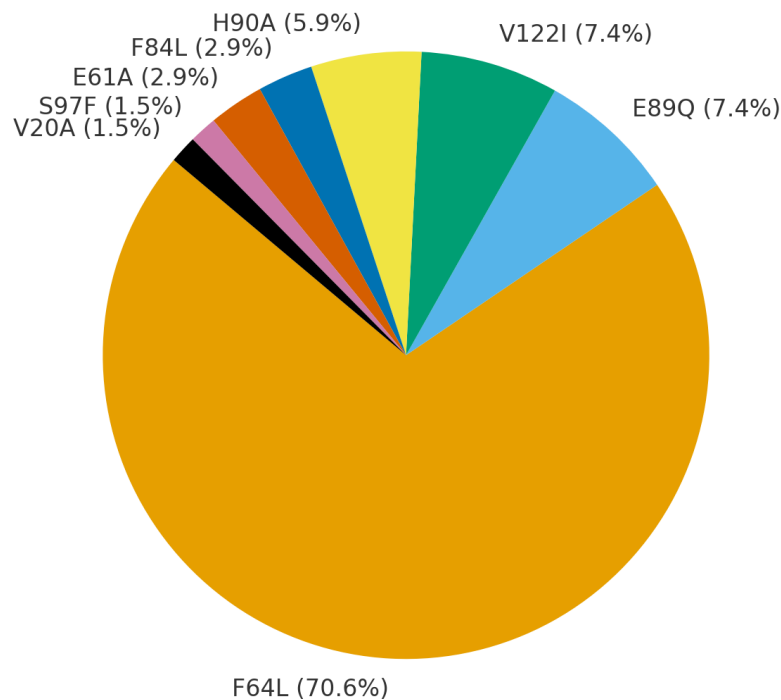


Figure 13. Distribution of TTR Variants Among Mutation-Positive Patients. The pie chart illustrates the relative frequency of TTR variants in the mutation-positive subgroup (n = 68). The most common variant was F64L (70.6%), followed by E89Q (7.4%), V122I (7.4%), H90A (5.9%), F84L (2.9%), E61A (2.9%), and single cases of S97F (1.5%) and V20A (1.5%)

8.4 Misdiagnosis

Notably, more than half of the mutation carriers (51.5%) already exhibited overt neuropathic or cardiac manifestations by the time of genetic confirmation.

A significant portion of individuals (n. 16, 3.5%) initially received an incorrect diagnosis, often leading to inappropriate or delayed treatment. The most common misclassification was an ALS-like presentation (5 cases, 31.3%), characterized by tongue fasciculations, atrophy, or generalized weakness.

CIDP was the second most common (3 cases, 18.8%), underlining the overlap between ATTRv neuropathy and acquired immune-mediated polyneuropathies. Other erroneous diagnoses included anorexia nervosa (13 cases, 8.8%), chronic idiopathic axonal polyneuropathy (CIAP) (2 cases, 12.5%), diabetic neuropathy (1 case, 6.3%), lumbar spinal stenosis (1 case, 6.3%), and dilated cardiomyopathy (1 case, 6.3%),

These findings underscore the protean presentation of ATTRv and the need for systematic suspicion indexes to reduce diagnostic delay.

8.5 Univariate Analysis of Clinical Predictors (Chi-square Tests)

We first examined each clinical feature (red flag) in isolation for association with the presence of the pathogenic TTR mutation. Figure 14 summarizes the Chi-square tests for independence between each predictor and the outcome. Several features emerged as significant univariate predictors of ATTRv, while many others showed no significant association:

- **Family history of neuropathy:** This was the strongest individual predictor (**Chi-square = 69.4, $p < 0.0000000001$**). Over two-thirds of mutation-positive patients had a family history of neuropathy, compared to only roughly one-quarter of mutation-negative patients – a striking difference indicating that neuropathy in relatives is a major red flag for ATTRv in our cohort.

- **Family history of cardiopathy:** Also significantly associated with genotype status (**Chi-square = 14.2, p = 0.0001670412**). This variable was common in both groups but still enriched among those with ATTRv (approximately 60% of gene-positive vs. ~45% of gene-negative patients had a history of cardiomyopathy in the family).
- **Sensory neuropathy:** Present in a majority of patients overall, this feature showed a trend toward association with the mutation, but just missed statistical significance (Chi-square = 3.70, p = 0.0543623700). In absolute terms, nearly 70% of ATTRv carriers had peripheral sensory neuropathy, compared to about 60% of non-carriers, indicating only a modest difference. The high prevalence of neuropathy among mutation-negative individuals (many of whom likely have other causes of neuropathy) reduced its discriminative value as a lone predictor.
- **Renal dysfunction:** Similarly to neuropathy, renal impairment was more frequent in mutation-positive patients ($\approx 10.7\%$ vs. 9.7%), but this difference did not reach significance (Chi-square = 3.52, p = 0.0606136700).
- **Bilateral CTS:** Perhaps surprisingly, the occurrence of bilateral CTS was not significantly different between the groups on its own (Chi-square = 0.65, p = 0.4197656000). Approximately 46% of both ATTRv-positive and -negative patients had bilateral CTS. This suggests that, in univariate context, CTS (even if bilateral) is a common but nonspecific finding in an amyloidosis referral population.

- **Ataxia:** The presence of ataxia alone did not show a significant univariate association (Chi-square = 1.90, $p = 0.1681028000$). Only about one-third of patients had ataxia, and it was nearly equally distributed between mutation-positive and -negative subgroups in simple comparisons.
- **Other features:** A number of red flags – including unexplained weight loss, gastrointestinal disturbances, autonomic dysfunction, ocular disorders, and lumbar canal stenosis – showed no significant correlation with genotype (p values ranging from 0.30 to 0.90). For example, the frequency of gastrointestinal symptoms was similar in both groups (~31% in positives vs. 27% in negatives, $p = 0.6824029000$), and lumbar stenosis tended to be slightly more common in mutation-negative patients ($p = 0.4365608000$). Cardiomyopathy (clinical evidence of cardiac involvement) notably had no discriminatory power by itself (Chi-square = 0.00, $p = 1.0000000000$) – reflecting that cardiac manifestations were present in roughly 25% of both ATTRv carriers and non-carriers in this cohort.

In addition, the explicitly hereditary indicator “**family history of ATTRv**” (known TTR amyloidosis in the family) was, as expected, highly associated with mutation status (Chi-square = 95.1, $p < 0.0000000001$). In fact, virtually all patients with a known ATTRv family history turned out positive for the mutation. Because this particular variable is essentially tautological (ATTRv is autosomal dominant), it was excluded from further modeling to avoid biasing the results. Overall, the univariate analysis identified family neuropathy history (and to a lesser extent family cardiopathy history) as the most significant red-flag predictors of ATTRv. Traditional red flags like

neuropathy, ataxia, and CTS, while prevalent in cases, did not alone differentiate cases from controls with statistical significance – underlining the need for a multivariate predictive approach.

<i>Feature</i>	<i>Chi-squared</i>	<i>P-value</i>
<i>cardiopathy</i>	0.000000	1.0000000000
<i>positive_biopsy</i>	0.000000	1.0000000000
<i>autonomic_dysfunction</i>	0.016698	0.8971845000
<i>sex</i>	0.057257	0.8108845000
<i>gastrointestinal_disturbances</i>	0.167434	0.6824029000
<i>unexplained_weight_loss</i>	0.434611	0.5097349000
<i>lumbar_canal_stenosis</i>	0.605306	0.4365608000
<i>bilateral_cts</i>	0.650971	0.4197656000
<i>ocular_disorder</i>	0.828239	0.3627823000
<i>ataxia</i>	1.899781	0.1681028000
<i>cardiac_uptake</i>	2.191570	0.1387678000
<i>renal_dysfunction</i>	3.520513	0.0606136700
<i>sensory_neuropathy</i>	3.701537	0.0543623700
<i>family_history_of_cardiopathy</i>	14.169674	0.0001670412
<i>family_history_of_neuropathy</i>	69.415500	0.0000000001
<i>family_history_of_att_rv</i>	95.144911	0.0000000001

Table 12. Chi-square test results for clinical red-flag features in relation to TTR mutation status. The table reports chi-square statistics and corresponding p-values for each variable. P-values are shown in decimal format, with statistically significant associations ($p < 0.05$) highlighted in bold. Family history of neuropathy, family history of cardiopathy, and family history of ATTRv emerged as the strongest predictors of mutation positivity.

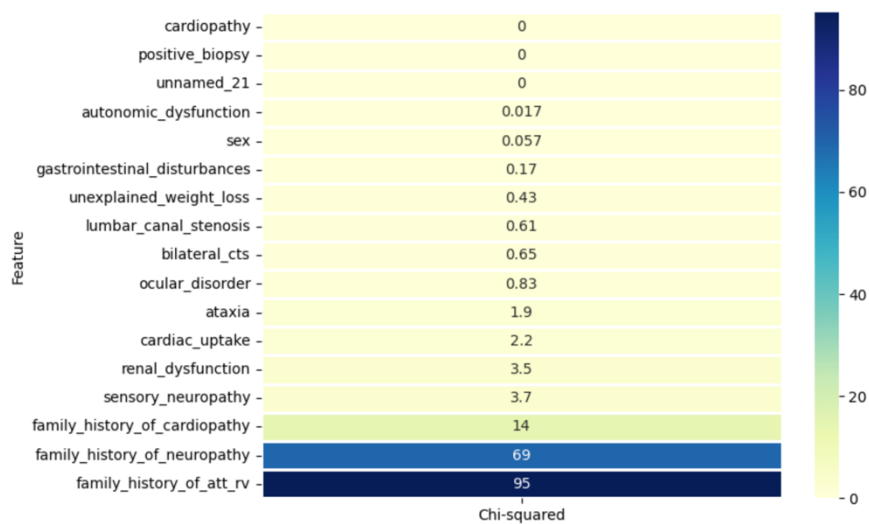


Figure 14. Chi-square test for selecting variables useful for model training.

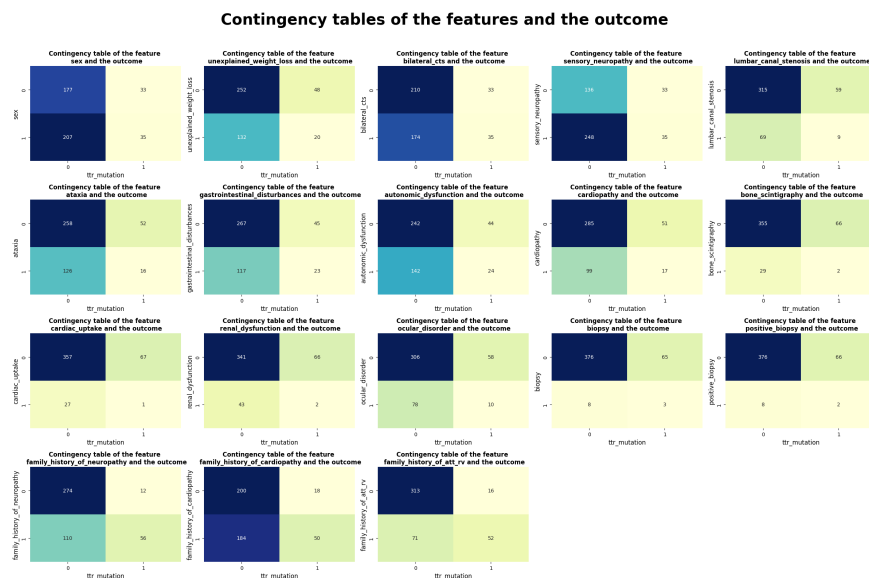


Figure 15. Contingency table between the target variable and each other variables.

Figure 15 further examines the contingency tables for key predictors, illustrating the outcome proportions with vs. without each red flag. For

instance, among patients with a family history of neuropathy, a large fraction (roughly two-thirds) have the TTR mutation, whereas among those without such a history, only ~22% were carriers. A similar (though less extreme) pattern is seen for family history of cardiomyopathy (where the mutation was present in ~34% of those with a positive family history vs ~19% of those without). In contrast, for features like bilateral CTS, the proportion of mutation carriers was essentially the same whether CTS was present or absent (~27% in both subsets), consistent with the non-significant chi-square result. These contingency analyses confirm which clinical features act as red flags (significantly shifting the probability of an ATTRv diagnosis) and which are more background features in this referred population.

8.6 Model training and evaluation

Given the above findings, we developed and evaluated multiple machine learning classifiers to predict ATTRv mutation status from the combination of clinical features. We partitioned the data by center (patients from Palermo formed the training set, and those from Catania the independent test set) to ensure geographic and temporal generalizability. A variety of classification algorithms were tested, including logistic regression, support vector machines, k -nearest neighbors, Gaussian naïve Bayes, and ensemble tree-based methods (Random Forest, XGBoost, LightGBM, etc.). Model hyperparameters and feature subsets were optimized via grid search and cross-validation on the training data.

Overall, tree-based classifiers achieved the highest accuracy and robustness, substantially outperforming non-tree models. This trend held across different feature selections and tuning iterations, suggesting an intrinsic advantage of decision-tree ensembles for this problem (possibly due to the categorical nature of the predictors and complex feature interactions). In contrast, simpler classifiers often struggled with the imbalanced data.

After hyperparameter optimization, the best performance was attained with ensemble tree models, particularly Random Forest and XGBoost. Table 1 summarizes the performance of these models on the independent test cohort of 122 patients:

- **Random Forest:** Achieved an accuracy of $\sim 81.9\%$, with sensitivity (recall) $\approx 88.9\%$ and precision $\approx 44.4\%$, resulting in an F1-score of 0.593. In practical terms, the Random Forest correctly identified almost 9 out of 10 ATTRv-positive patients, while producing some false positives (about one false alert for each true positive identified). Notably, it did not miss many mutation carriers (low false-negative rate), aligning with the clinical priority of maximizing case detection.
- **XGBoost:** Achieved a similar accuracy ($\sim 77.9\%$) but with an even higher recall of 100% – capturing all true positive cases in the test set – at the expense of a lower precision ($\sim 40.0\%$). The model flagged many more patients as positive, some of whom were ultimately mutation-negative, yielding an F1-score of 0.57. This reflects a more aggressive approach: XGBoost effectively prioritized sensitivity (no false negatives) but generated

more false positives (lower precision). Such a model might be useful as an initial screener, albeit with the trade-off of potential overdiagnosis.

	<i>accuracy</i>	<i>precision</i>	<i>recall</i>	<i>F1 score</i>	<i>ROC AUC</i>
<i>Logistic Regression</i>	0.5327 87	0.2253 52	0.8888 89	0.3595 51	0.6800 21
<i>LGBMClassifier</i>	0.6967 21	0.3137 25	0.8888 89	0.4637 68	0.7761 75
<i>XGBClassifier</i>	0.7786 89	0.4000 00	1.0000 00	0.5714 29	0.8701 92
<i>RandomForestClassifier</i>	0.8196 72	0.4444 44	0.8888 89	0.5925 93	0.8482 91
<i>GradientBoostingClassifier</i>	0.7868 85	0.1000 00	0.0555 56	0.0714 29	0.4845 09
<i>DecisionTreeClassifier</i>	0.7786 89	0.3902 44	0.8888 89	0.5423 73	0.8242 52
<i>ExtraTreesClassifier</i>	0.7868 85	0.3947 37	0.8333 33	0.5357 14	0.8060 90
<i>CatBoostClassifier</i>	0.7540 98	0.3500 00	0.7777 78	0.4827 59	0.7638 89

Table 13. Achieved performance of the Random Forest Classifier, Gradient Boosting Classifier, Logistic Regression, Multi-layer Perceptron (MLP) Classifier, Support Vector Classification (SVC), Extreme Gradient Boosting (XG) Boost Classifier, K-Nearest Neighbors (KNN) algorithms computed considering the 20-Repeated 10-Fold Cross Validation. Accuracy, Precision score, Recall score, F1 score, Area Under Curve Receiver Operating Characteristic (AUC-ROC) score, Confusion Matrix: Total true negatives (TTN), Total false positives (TFP), Total false negatives (TFN), Total true positives(TTP) were computed as metrics to evaluate model performance, reporting mean and standard deviation.

Across all models, we evaluated performance with multiple metrics (accuracy, precision, recall, F1, and ROC-AUC) to ensure a comprehensive assessment. Emphasis was placed on **maximizing recall (sensitivity)** – i.e. catching as many true ATTRv cases as possible – since missing a case (false

negative) could delay a critical diagnosis. The Random Forest provided the best **balance** between sensitivity and precision, achieving high recall while keeping precision at an acceptable level (roughly 0.44). In contrast, XGBoost’s perfect sensitivity came with a precision drop to 0.40, indicating a higher false-positive rate. In a clinical context, this means Random Forest would miss slightly more cases than XGBoost, but issue fewer false alarms; XGBoost would flag every true patient at the cost of flagging many extras. Both approaches may be valid depending on the clinical tolerance for false positives. For our dataset, we selected the Random Forest model as the **final classifier** given its strong F1-score and more tempered false-positive rate, ensuring both robust case detection and reasonable specificity. This final model achieved a **test-set accuracy** of ~82% and an **area under the ROC curve (AUC)** of approximately 0.85–0.90 (by cross-validation estimates), indicating excellent overall discrimination.

8.7 Feature Importance and Key Predictors of ATTRv

To understand the model’s predictions and identify which clinical factors were most informative, we applied explainable AI techniques to the final Random Forest. A **SHAP (Shapley Additive Explanations)** analysis was conducted to compute the contribution of each feature to the model’s output. The global feature importance ranking (derived from SHAP summary plots and permutation importance) revealed that a narrow set of clinical variables predominantly drives the model’s decisions. In fact, the model’s output was largely determined by just three top predictors (in descending order of

importance): *(1) family history of neuropathy, (2) ataxia, and (3) bilateral CTS*. These three features showed the highest information gain and consistently had the greatest influence on risk predictions for ATTRv.

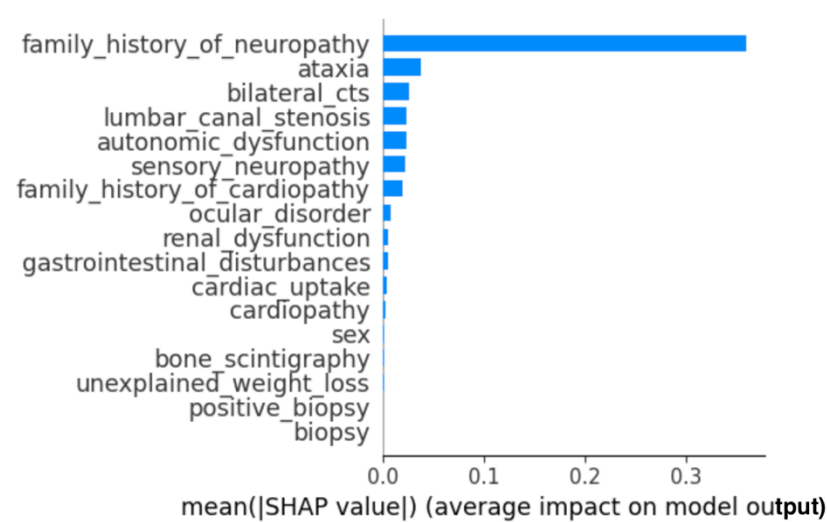


Figure 16. SHAP (SHapley Additive exPlanations) bar charts.

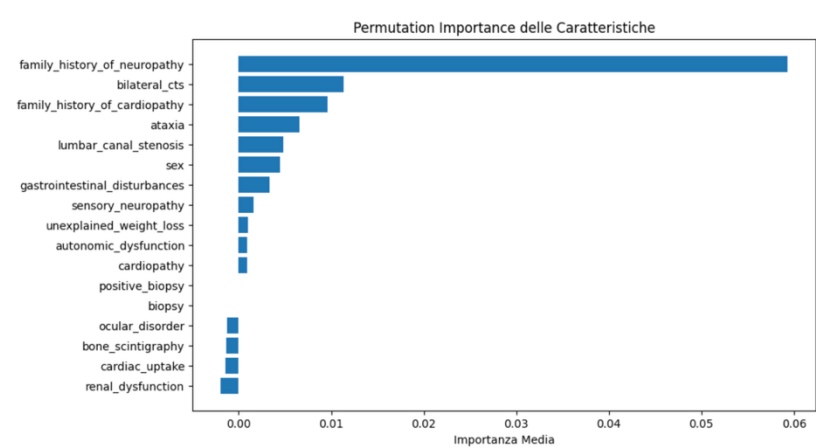


Figure 17: Bar chart derived from the SHAP (SHapley Additive exPlanations) method of chi-square values for each variable.

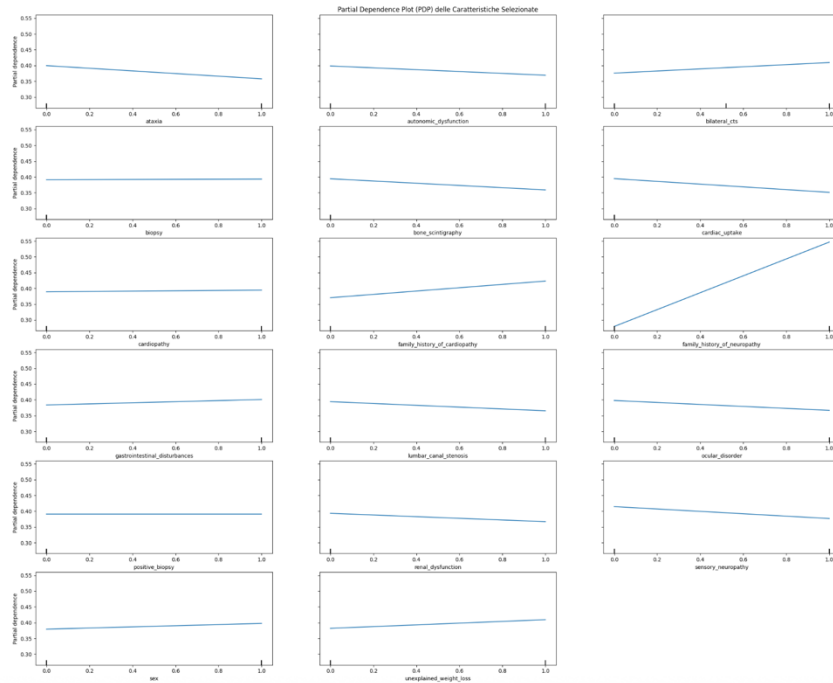


Figure 18. The Partial Dependence Plot (PDP) analyzes the effect of one or more characteristics on a model's predictions. Looking specifically at the slope of the line, one can see how the family history of neuropathy is the greatest influence on the model, followed by the family history of cardiopathy and bilateral CTS.

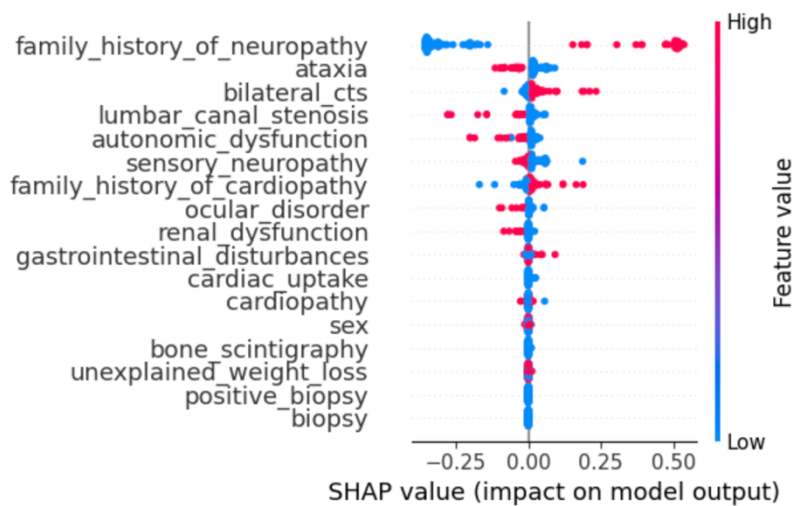


Figure 19. SHAP plot. The red dots indicate the presence of the red flag, the blue dots indicate the absence of the red flag. The red dots on the left of the graph indicate that the presence of the red flag is predictive of no TTR mutation, while those on the right are predictive of TTR mutation (same for blue dots).

Importantly, the model's interpretive plots indicate that the presence of each of these top features tends to push the prediction toward the ATTRv-positive class (i.e. they have positive SHAP values when the feature is true). In other words, patients with these clinical characteristics were assigned a higher probability of carrying the TTR mutation by the model. For example, having a family history of neuropathy or bilateral CTS strongly and positively contributed to the model's output for being mutation positive. These two factors emerged as the most powerful predictors, showing a robust and direct relationship with the outcome: patients with either familial neuropathy or bilateral CTS were much more likely to be correctly identified as ATTRv-positive by the model. This finding aligns with clinical intuition: a neuropathy running in the family suggests a heritable neuropathic condition, and bilateral carpal tunnel syndrome (especially in older adults) is a known red flag for systemic amyloid deposition, reinforcing that the model appropriately leveraged these clues.

Ataxia was also identified as a strong predictor, but interestingly, its role was more complex. Unlike the first two features, the presence of ataxia was associated with a reduced predicted probability of ATTRv in the model. In the SHAP analysis, ataxia had a high importance but negative directionality: cases with ataxia tended (on average) to be classified as less likely to have the TTR mutation. This counterintuitive result suggests that, within our dataset, ataxia often co-occurred with alternative diagnoses (i.e. many ataxic patients

turned out mutation-negative). Thus, while ataxia was informative to the model, it functioned as a sort of negative predictor – its presence shifted the model toward predicting “no mutation,” perhaps because isolated ataxia without other red flags was more characteristic of false-positive referrals. Clinically, this underscores that not all red flags contribute to the same direction: some features (like family neuropathy or CTS) raise suspicion of ATTRv, whereas others (like ataxia) may, in context, point away from ATTRv if they appear in certain patterns. The model was able to discern these nuances.

Beyond the top three, the model did incorporate other features, but with substantially lower influence. For instance, family history of cardiomyopathy and autonomic dysfunction had some predictive value, yet their contributions were about 5–6 times smaller than that of family neuropathy. Similarly, features such as ocular involvement or positive cardiac uptake on bone scintigraphy, while present in some ATTRv patients, provided only modest incremental information (their absence/presence did not markedly change the risk prediction). The dominance of the three key variables suggests that the model (and possibly the clinical syndrome) is driven by a few critical red flags. Notably, sensory neuropathy, despite being very common in ATTRv, did not appear among the top contributors in the multivariate model, likely because it was ubiquitous in both mutation-positive and -negative groups and therefore less useful for discrimination when other features are considered.

In summary, our results indicate that a ML approach can successfully stratify patients for ATTRv genetic risk using clinical data. The Random Forest model demonstrated robust performance, identifying ~90% of mutation carriers with moderate false positives, outperforming other methods. Crucially, the model's interpretability analyses highlight which clinical features carry the most weight in predicting ATTRv: a family history of neuropathy, bilateral carpal tunnel syndrome, and ataxia. The first two features positively flagged high-risk individuals, whereas ataxia (somewhat unexpectedly) emerged as an important inverse indicator in this cohort. These findings provide an evidence-based ranking of clinical red flags (Figures 14 and 15) in terms of their predictive power for hereditary TTR amyloidosis. They reinforce the clinical intuition that certain history elements (neuropathy in family) and exam findings (bilateral CTS) are meaningful risk markers, while also offering new insights (for example, that ataxia may differentiate subgroups), which will be further considered in the Discussion. Overall, the integration of machine learning and XAI has allowed us to quantify the diagnostic relevance of each feature, pointing to a focused set of clinical predictors that could improve early identification of ATTRv in practice.

Chapter 9 – Discussion

9.1 Main findings

This study represents one of the first systematic applications of ML to the diagnostic pathway of ATTRv. Unlike traditional approaches, which often rely on univariate analysis or regression-based models, ML offers the ability to capture non-linear interactions and multidimensional relationships among clinical features. This is particularly relevant in ATTRv, a disease characterized by marked phenotypic heterogeneity and frequent overlap with other more common disorders (98).

By analyzing two independent Sicilian cohorts, we sought to identify the most informative clinical red flags associated with TTR mutation positivity and to evaluate the performance of different ML algorithms in predicting diagnostic outcomes.

The results demonstrate that three variables, *family history of neuropathy*, *bilateral CTS*, and *ataxia*, emerged as the strongest predictors of mutation positivity within our dataset. Among these, family history of neuropathy showed the highest discriminatory power, while bilateral CTS confirmed its relevance as an early manifestation of ATTRv neuropathy. Ataxia, although not statistically significant in univariate analysis, was retained in the multivariate ML models, reflecting its role in complex clinical phenotypes.

From a computational standpoint, tree-based ensemble models, particularly ***Random Forest***, outperformed both linear classifiers and neural networks. Random Forest achieved the best compromise between sensitivity (recall 0.89) and specificity (precision 0.44), with an AUC of 0.85. By contrast, XGBoost reached perfect sensitivity but at the cost of markedly reduced precision, suggesting possible overfitting. These findings underscore the robustness and clinical applicability of tree-based models in capturing categorical and hierarchical relationships among clinical variables.

9.2 Comparison with Existing Literature

Our findings are consistent with the well-documented diagnostic delay in ATTRv, often ranging from 3 to 6 years, largely due to misclassification as CIDP, diabetic neuropathy, or idiopathic axonal neuropathy (21,24,130). This delay is clinically deleterious, as irreversible neurological or cardiac damage may occur before correct diagnosis.

Previous works have proposed structured ***red flag*** clusters to guide suspicion. Conceição et al. (2016) emphasized systemic features such as weight loss, gastrointestinal symptoms, autonomic dysfunction, and cardiomyopathy as major diagnostic triggers (131). Di Stefano et al. (2023), applying ML in Italian non-endemic regions, also found systemic manifestations (weight loss, cardiomyopathy, gastrointestinal disturbances) as top predictors, while CTS was less discriminative (36). By contrast, Warendorf et al. (2023), in a Dutch

cohort, highlighted bilateral CTS and family history as strong indicators of ATTRv, in close agreement with our findings (132).

Taken together, these studies suggest that while systemic features (weight loss, autonomic dysfunction, cardiomyopathy) are critical in large and heterogeneous populations, hereditary and neuromuscular features (family history, bilateral CTS, ataxia) may carry greater discriminative power in endemic or genetically clustered settings such as our Sicilian cohort.

Our analysis identified *family history of neuropathy, bilateral CTS, and ataxia* as the most relevant red flags for predicting ATTRv in our cohort. This finding shows both overlap and divergence from previously published studies.

This discrepancy likely reflects differences in population genetics and epidemiological context. In our cohort, dominated by the F64L mutation (70.6%), hereditary and neurologic red flags carried greater weight, while systemic features had less predictive value. Thus, our results extend previous literature by showing that the relative importance of red flags may vary across populations and that ML can recalibrate their weight according to local genetic epidemiology.

The prominence of family history in our model underscores the importance of cascade screening and genetic counseling, while bilateral CTS confirms its role as an early manifestation of ATTRv neuropathy.

Another interesting finding concerns ataxia. Although ataxia is less frequently cited as a classic red flag, its contribution in our analysis may reflect phenotypic variability or advanced disease stages, warranting further investigation. While clinically relevant in some variants (e.g., leptomeningeal ATTR or late-onset Val30Met), in our ML model it was paradoxically associated with a lower probability of genetic positivity. This discrepancy likely stems from the non-specific nature of gait impairment, which can arise from multiple acquired neuropathies, spinal disease, or systemic conditions. The model correctly captured this reduced specificity, underlining the advantage of data-driven weighting over purely clinical heuristics.

In summary, our results complement existing literature by highlighting the contextual variability of red flags across populations and by demonstrating that ML can help recalibrate their relative importance according to local epidemiology.

In our cohort, the most frequent genotype was L64L, detected in 70.6% of patients. This result is consistent with previous reports from Sicily, where the F64L mutation has been identified as the predominant variant. In a single-center study, Russo et al. (2021) reported that less than half of ATTRv cases carried this mutation, while more recent real-world data confirmed a prevalence around 49–64% in the same region (55). By contrast, in central Italy (Lazio), F64L accounted for only about one-quarter of patients, highlighting regional founder effects and differences in genetic epidemiology

across the Italian peninsula (53). From a phenotypic perspective, the F64L variant is typically associated with a late-onset, neuropathy-predominant presentation, often with only mild or mixed cardiac involvement. This pattern was first described in Sicilian series and subsequently confirmed in international data from the THAOS registry, where F64L carriers presented predominantly with neurological symptoms, and cardiac-only forms were rare (133). Other variants were detected at substantially lower frequencies, in particular E89Q and V122I, two well-described mutation in southern Italy with mixed neuropathic and cardiomyopathic features (134), and the latter a predominantly cardiogenic variant most frequently reported in African American populations but also occasionally found in Mediterranean cohorts (12). The H90A variant was observed in 5.9% of carriers, confirming its rare but recurrent presence in Italian ATTRv cases. Less frequent variants included F84L and E61A, both associated with variable phenotypes and reported only sporadically in the Italian registry. Finally, single cases of S97F and V20A were detected, highlighting the wide mutational spectrum of TTR despite strong founder effects in specific regions.

Therefore, these findings indicate that the predominance of F64L in our study reflects both the genetic background of the Sicilian population and the neurology-driven recruitment pathway of our cohort, where neuropathic phenotypes are overrepresented. Importantly, these observations emphasize the need to consider regional genetic backgrounds when interpreting red-flag patterns and designing diagnostic algorithms for ATTRv.

9.3 Clinical Interpretation

The identification of *family history of neuropathy* as the single most predictive feature is particularly noteworthy. Historically, this variable has been underestimated, partly due to underdiagnosis in previous generations. Our analysis demonstrates that systematic incorporation of family history into diagnostic workflows could substantially improve early recognition of ATTRv, reinforcing the need for genetic counseling and cascade screening.

The role of *bilateral CTS* was also confirmed. Although it did not reach significance in univariate analysis, its importance emerged within ML models, consistent with CTS being an early neuropathic manifestation of ATTRv, often preceding polyneuropathy.

The finding regarding *ataxia* was more complex. While not significant as a standalone variable, it contributed to ML predictions, albeit sometimes with paradoxical effects (reduced likelihood of positivity). This may reflect the overlap between amyloid neuropathy and other causes of gait ataxia, such as CIDP or idiopathic neuropathies. Nevertheless, its presence in ATTRv carriers supports its consideration within composite diagnostic indices, particularly in advanced disease stages

9.4 Robustness and Reproducibility

One of the strengths of this study lies in the use of two independent cohorts from distinct regions of Sicily (Western and Eastern). Despite differences in demographic composition and referral patterns, the predictive performance of tree-based models remained stable, suggesting good reproducibility and external validity.

This aspect is particularly important, as many prior ML applications in rare diseases suffered from small, single-center cohorts with limited generalizability. By validating our findings across geographically distinct populations, we provide stronger evidence for the robustness of ML-based diagnostic approaches in ATTRv.

Nevertheless, larger multi-ethnic studies will be required to test whether the observed superiority of tree-based methods is specific to Sicilian founder mutations or generalizable across broader genetic and phenotypic landscapes.

9.5 Clinical Implications

First, the identification of family history of neuropathy, bilateral CTS, and ataxia as the strongest predictors supports their integration into diagnostic suspicion indexes. Practical algorithms for primary care physicians, neurologists, and cardiologists could leverage these red flags to prioritize genetic testing, thereby reducing diagnostic delays.

Second, the use of ML-based decision support tools could transform clinical workflows. For example, in regions with low disease awareness, electronic

health records (EHRs) could be scanned for combinations of red flags, automatically flagging high-risk patients for referral to specialized centers.

Third, the findings carry translational implications. Earlier diagnosis enables timely initiation of disease-modifying therapies, tafamidis, patisiran, inotersen, or vutrisiran, which are most effective before irreversible organ damage occurs (30,31,105,135). Furthermore, ML-driven early detection may facilitate multidisciplinary care integration, promoting coordinated evaluation by neurologists, cardiologists, ophthalmologists, and genetic counselors.

Finally, our results suggest that family screening protocols should be reinforced. The recognition of family history as a strong predictive marker justifies more aggressive cascade genetic testing, even in relatives presenting with subtle or atypical features such as CTS or cardiomyopathy.

9.6 Limitations and Future Directions

This study has several limitations. First, the analysis was restricted to Sicilian populations, which may limit generalizability. Second, only categorical variables were included; quantitative data such as neurophysiological scores, cardiac or neurological biomarkers (NT-proBNP, NfL), or imaging parameters (pT2) might improve model performance. Third, most patients were recruited through neurology-driven pathways, potentially underrepresenting cardiac presentations. Fourth, red flags variability,

considering that some features (e.g., erectile dysfunction, gastrointestinal symptoms) are subject to underreporting, while instrumental findings may be influenced by selection bias. Finally, although explainable AI tools were applied, ML models remain probabilistic and should complement, not replace, clinical judgment.

Future research should focus on validating these findings in larger, multi-ethnic cohorts, integrating multimodal data (clinical, biomarkers, imaging, genetics), and deploying ML models in real-world workflows. Explainable AI and federated learning approaches may enhance generalizability and clinical trust.

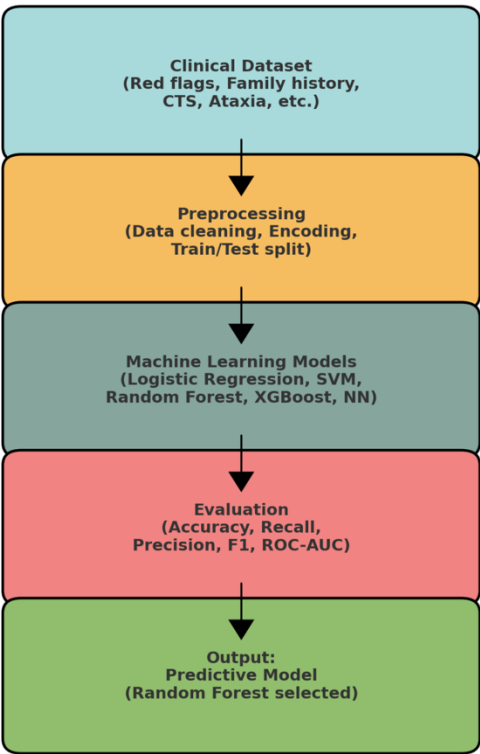


Figure 20. Workflow of ML applied to ATTRv diagnostic prediction.

Chapter 10 – Conclusions

10.1 Summary of Main Findings

ATTRv remains a paradigmatic example of a rare yet increasingly treatable disease in which timely recognition is essential. The findings of this thesis confirm that diagnostic delay is frequent and often driven by misdiagnosis, despite the presence of recognizable red flags. This doctoral work demonstrates the feasibility and clinical utility of integrating ML into the diagnostic pathway of ATTRv. Through the systematic analysis of two large Sicilian cohorts, the study pursued two complementary objectives:

- *To identify the clinical red flags most strongly associated with a positive TTR genetic test.*
- *To develop and validate a robust predictive model capable of supporting clinicians in the early recognition of high-risk patients.*

The findings highlight three variables (family history of neuropathy, bilateral CTS, and ataxia) are key predictor with the highest informative power. Importantly, the recognition of family history as a discriminative factor represents a key advance, as this variable has historically been underappreciated in diagnostic suspicion indexes.

From a computational standpoint, tree-based algorithms, particularly Random Forest and XGBoost, consistently outperformed linear models and neural

networks. Random Forest emerged as the most clinically applicable solution, offering a balanced compromise between sensitivity (recall) and specificity (precision). The application of explainable AI (XAI) confirmed that the model relied on clinically plausible predictors, reinforcing its interpretability and real-world applicability.

10.2 Translational Implications

The study carries important translational implications for the management of ATTRv:

- *Diagnostic acceleration:* By prioritizing key red flags, the model supports earlier genetic testing and reduces diagnostic delay, which in some series exceeds 5 years (24,73).
- *Therapeutic relevance:* Timely diagnosis is crucial, since current therapies (tafamidis, patisiran, inotersen, vutrisiran) are most effective when initiated before irreversible organ damage (27,30,105).
- *Multidisciplinary care:* ML-driven risk stratification can facilitate earlier referral to specialized centers, promoting integrated management by neurologists, cardiologists, gastroenterologists, ophthalmologists, and genetic counselors (99,107).

- *Family screening*: The recognition of family history as a top predictor supports the implementation of cascade genetic testing strategies, ensuring earlier identification of at-risk relatives and expanding opportunities for preventive interventions (136).

10.3 Methodological Contributions

This work also contributes methodologically by demonstrating that:

- *Tree-based ML models* are particularly suited to rare disease diagnostics, where categorical variables and feature interactions dominate.
- *Explainable AI tools* (*permutation importance*, *PDP*, *SHAP*) can overcome the “black box” problem, enhancing clinical trust in computational predictions.
- *Validation* across two independent regional cohorts strengthens external validity and supports the reproducibility of findings, which has often been a limitation of prior ML studies in rare diseases.

These methodological advances may be extended beyond ATTRv to other rare or heterogeneous disorders, where early diagnosis remains challenging.

10.4 Future Perspectives

Our work, though consistent with international data, is limited by the regional nature of the cohort and the absence of external validation. Furthermore, our ML models require calibration in diverse populations to ensure fairness across age, sex, and genotype. A validation beyond Sicilian populations, with further studies involving multi-ethnic cohorts, is necessary to confirm the generalizability of results.

Moving forward, integrating clinical red flags with biomarkers (NT-proBNP, troponins, NfL) and AI tools may create robust diagnostic pathways that shorten time to recognition. Explainable AI and federated learning approaches (108) could enhance generalizability and trust, while patient-centered education may empower earlier presentation to specialists.

A multimodal approach, linking red flags, biomarkers, imaging, and ML decision support, represents the most promising strategy to transform the diagnostic landscape in ATTRv. Embedding ML algorithms into electronic health record systems could provide real-time diagnostic support, automatically flagging patients at risk. Future models may incorporate genotype–phenotype correlations, thereby guiding not only diagnosis but also therapeutic stratification.

Ultimately, longitudinal data would be valuable to evaluate whether red flag–based models can predict not only diagnosis but also disease trajectory.

10.5 Final Remarks

In conclusion, this study illustrates how AI and ML can reshape clinical pathways in ATTRv amyloidosis. By combining the identification of high-yield red flags with the construction of a reliable predictive model, the work provides both theoretical insights into disease patterns and practical tools for diagnostic optimization.

Ultimately, the integration of AI into clinical workflows has the potential to shorten diagnostic delays, improve resource allocation, enable earlier treatment initiation, and enhance outcomes for patients and families affected by this devastating disease.

This dual computational and clinical contribution underscores the importance of multidisciplinary, technology-driven innovation in rare diseases, paving the way for faster, more accurate, and more equitable diagnosis in ATTRv and beyond.

<i>Domain</i>	<i>Main Findings</i>	<i>Translational Implications</i>
<i>Clinical features</i>	Family history of neuropathy, bilateral carpal tunnel syndrome (CTS), and ataxia emerged as the most informative red flags.	These variables should be prioritized in suspicion indexes and clinical workflows to accelerate genetic testing.
<i>Computational analysis</i>	Tree-based algorithms (Random Forest, XGBoost) consistently outperformed linear and neural network models.	Tree-based ML models are particularly suitable for diagnostic tasks in rare diseases characterized by

	Random Forest provided the best balance between recall and precision.	categorical and heterogeneous features.
<i>Explainable AI (XAI)</i>	SHAP, PDP, and permutation importance confirmed that the model relied on clinically plausible predictors.	Enhances interpretability and fosters trust in the integration of AI tools into routine clinical practice.
<i>Reproducibility</i>	Validation across two independent Sicilian cohorts demonstrated robust and consistent performance.	Supports external validity and highlights the potential for broader application in multi-center and multi-ethnic settings.
<i>Diagnostic challenges</i>	Misdiagnoses included ALS, CIDP, and diabetic neuropathy, leading to significant diagnostic delays.	AI-driven prioritization could reduce diagnostic errors, shorten time to diagnosis, and improve early treatment initiation.
<i>Therapeutic context</i>	Timely diagnosis is critical, as current disease-modifying therapies (tafamidis, patisiran, inotersen, vutrisiran, eplontersen) are most effective before irreversible damage.	Reinforces the clinical need to embed AI-assisted decision tools in early patient evaluation pathways.
<i>Future directions</i>	Integration of multimodal data (genetics, imaging, biomarkers) and validation in larger, multi-ethnic cohorts.	Paves the way toward precision medicine and AI-enabled real-world diagnostic support in ATTRv amyloidosis.

Table 14. Key Messages of the Thesis

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