NEW PERSPECTIVES IN CARDIAC AMYLOIDOSIS

PhD thesis

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Abbreviations

AA amyloidosis: reactive systemic amyloidosis

ACE: angiotensin-converting enzyme

AL amyloidosis: immunoglobulin light chain amyloidosis

AS: aortic stenosis

ATTR: transthyretin amyloidosis

ATTR-FAP: transthyretin-related familial amyloid polyneuropathy

ATTRv: variant transthyretin amyloidosis

ATTRwt amyloidosis: wild-type transthyretin amyloidosis

AVA: aortic valve area

AVR: aortic valve repair

BSA: body surface area

CA: cardiac amyloidosis

CMR: cardiac MRI

CTS: carpal tunnel syndrome

CW: continuous-wave

ECG: electrocardiogram

ECV: extracellular volume

EF: ejection fraction

ESC: European Society of Cardiology

HF: heart failure

HFpEF: heart failure with a preserved ejection fraction

HFrEF: heart failure with a reduced ejection fraction

HTX: heart transplantation

ISA: International Society of Amyloidosis

LS: longitudinal strain

LV: left ventricle, left ventricular

LVEF: left ventricular ejection fraction

MGUS: monoclonal gammopathy of undetermined significance

MM: myeloma multiplex

mRNA: messenger RNA

NT-proBNP: N-terminal pro b-type natriuretic peptide

NYHA: New York Heart Association

PYP: pyrophosphate

PW: pulsed-wave

siRNA: small interfering RNA

TAVI: transcatheter aortic valve implantation

THAOS: Transthyretin Amyloidosis Outcomes Survey

TTE: transthoracic echocardiography

TTR: transthyretin

UK: United Kingdom

US: United States

1. Introduction

1.1. Brief history

The term amyloid was first used in 1838 by a German botanist to describe the amylaceous structure in plants. Later, Rudolf Wirchof used the same expression for the corpora amylacea's reaction to iodine in 1854. He thought at the time that it consisted of starch. For years, it was debated whether amyloid deposits contain fatty or carbohydrate elements. In 1859 it was shown that it consisted of albuminoid components after analyzing a "waxy spleen" [1]. Amyloid was regarded as an extracellular material that showed high variation in distribution, properties, and clinical manifestations [2]. The early classification distinguished primary, secondary, tumor-forming, and myeloma-associated forms of amyloidosis [3].

Samuel Wilks described the first primary amyloidosis. The patient did not have obvious secondary causes like syphilis or osteomyelitis. The kidneys were primarily affected, leading to nephrotic syndrome. The first case of amyloidosis due to multiple myeloma was probably described by Weber in 1967, although he did not know about it at the time. The patient had pathologic fractures, and bone tissue was replaced by greyish-red tissue containing small nucleated cells. The kidneys and the spleen contained amyloid, and the patient had left ventricular (LV) hypertrophy on autopsy [1].

Using aniline dyes to identify amyloid was reported in 1875. These metachromatic stains were replaced by the Congo red aniline dye later. Since then, amyloids have been generally examined using their ability to bind Congo red [4].

In the last century, especially in the previous couple of decades, our understanding of amyloid-caused diseases improved exponentially. Analysis of amyloid showed fibrillary structure, appearing as amorph material histologically. The fibrils consist of protofilaments formed of protein layers organized in a beta-sheet structure. These structures are capable of binding dyes like Congo red. X-ray diffraction analysis showed the cross-beta structure of amyloid fibrils [5]. In 2018, a new definition for amyloid was proposed: amyloid fibril refers to any cross-beta-sheet fibril. However, when talking about "amyloid," its origin and nature should be clarified [6]. Previously, amyloid was regarded as abnormal, which changed after the concept of functioning amyloid. During amyloidosis, the deposition of amyloid fibrils formed by different proteins after a conformational change leads to distinct clinical manifestations based on the affected

tissues [7]. The fibrils consist of subunits of a large diversity of proteins with low molecular weight, in the range of 5 to 25 kDa on average.

1.2. Types of amyloid

More than 30 proteins can form amyloid in the human body and damage the affected tissues [2], and with the help of modern mass spectrometry, even more will be identified in the future. The extracellular deposition can be due to abnormal proteins (immunoglobulin light chain [AL] amyloidosis – which is acquired, hereditary amyloidosis like variant transthyretin [ATTRv] amyloidosis), the abundance of normal proteins (reactive systemic [AA] amyloidosis) and can be the result of the aging process (wild-type transthyretin amyloidosis [ATTRwt]) as well. The mechanism of the latter is unknown. In Table 1., MD Benson et al. summarized the amyloid fibrils known to cause human diseases. Amyloidosis can be systemic, leading to potentially lethal systemic diseases like transthyretin amyloidosis (ATTRv and ATTRwt), AL, or AA amyloidosis, or it can be localized, like in some cases of AL amyloidosis. There are other conditions with characteristic amyloids or amyloid protein aggregate like Alzheimer's disease (amyloid protein: A-beta) or type 2 diabetes (islet amyloid polypeptide), but the term amyloidosis or organ-specific amyloidosis is rarely used in their cases. Amyloid may not play an essential role in the pathogenesis of these diseases [2].

The terms suggested by the latest recommendations of the International Society of Amyloidosis (ISA) Nomenclature Committee published in 2020 are used in the thesis [2].

Table 1 *Amyloid fibril proteins and their precursors in human[a] – MD Benson et al., with permission [2]*

Fibril protein	Precursor protein	Systemic and/or localised	Acquired or hereditary	Target organs
AL	Immunoglobulin light chain	S, L	A, H	All organs, usually except CNS
AH	Immunoglobulin heavy chain	S, L	A	All organs except CNS
AA	(Apo) serum amyloid A	S	A	All organs except CNS
ATTR	Transthyretin, wild type	S	A	Heart mainly in males, lung, ligaments, tenosynovium
	Transthyretin, variants	S	H	PNS, ANS, heart, eye, leptomeninges
Aβ2M	β2-microglobulin, wild type	S	A	Musculoskeletal system
	β2-microglobulin, variants	S	H	ANS
AApoAI	Apolipoprotein A I, variants	S	H	Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants)
AApoAII	Apolipoprotein A II, variants	S	H	Kidney
AApoAIV	Apolipoprotein A IV, wild type	S	A	Kidney medulla and systemic
AApoCII	Apolipoprotein C II, variants	S	H	Kidney
AApoCIII	Apolipoprotein C III, variants	S	H	Kidney
AGel	Gelsolin, variants	S	H	Kidney PNS, cornea
ALys	Lysozyme, variants	S	H	Kidney
ALECT2	Leukocyte chemotactic factor-2	S	A	Kidney, primarily
AFib	Fibrinogen α, variants	S	H	Kidney, primarily
ACys	Cystatin C, variants	S	H	CNS, PNS, skin
ABri	ABriPP, variants	S	H	CNS
ADan[b]	ADanPP, variants	L	H	CNS
Aβ	Aβ protein precursor, wild type	L	A	CNS
	Aβ protein precursor, variant	L	H	CNS
AαSyn	α-Synuclein	L	A	CNS
ATau	Tau	L	A	CNS
APrP	Prion protein, wild type	L	A	CJD, fatal insomnia
	Prion protein variants	L	H	CJD, GSS syndrome, fatal insomnia
	Prion protein variant	S	H	PNS
ACal	(Pro)calcitonin	L	A	C-cell thyroid tumours
		S	A	Kidney
AIAPP	Islet amyloid polypeptide[c]	L	A	Islets of Langerhans, insulinomas
AANF	Atrial natriuretic factor	L	A	Cardiac atria
APro	Prolactin	L	A	Pituitary prolactinomas, aging pituitary
AIns	Insulin	L	A	Iatrogenic, local injection
ASPC[d]	Lung surfactant protein	L	A	Lung
ACor	Corneodesmosin	L	A	Cornified epithelia, hair follicles
AMed	Lactadherin	L	A	Senile aortic, media
AKer	Kerato-epithelin	L	A	Cornea, hereditary
ALac	Lactoferrin	L	A	Cornea
AOAAP	Odontogenic ameloblast-associated protein	L	A	Odontogenic tumours
ASem1	Semenogelin 1	L	A	Vesicula seminalis
AEnf	Enfurvitide	L	A	Iatrogenic
ACatK[e]	Cathepsin K	L	A	Tumour associated
AEFEMP1[e]	EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1	L	A	Portal veins Aging associated

[a]Proteins are listed, when possible, according to relationship. Thus, apolipoproteins are grouped together, as are polypeptide hormones.
[b]ADan is the product of the same gene as ABri.
[c]Also called amylin.
[d]Not proven by amino acid sequence analysis.
[e]Full amino acid sequence to be established.

1.3. Epidemiology of amyloidosis

Systemic amyloidosis is a rare disease, except in some endemic areas. However, it causes global epidemics with profound health, social and economic implications and was without a cure until recently [8]. For instance, in the United States (US), the incidence of AL amyloidosis is around 9-14 cases per million person-years [9]. In the United Kingdom (UK), the available data suggested that the incidence exceeds 0.8/100.000 in the English population [10]. The National Amyloidosis Centre in the UK summarized the data of 5100 patients with amyloidosis from 1987 to 2012 [11]. In their review, Wechalekar et al. presented the data, shown with permission in Table 2.

Table 2 *Characteristics of the common types of amyloidosis – Wechalekar et al., with permission [11]*

	Acquired or hereditary	Patients seen at UK-NAC (%; n=5100)	Underlying disorder	Precursor protein	Organ involvement					Treatment	Treatment target
					Heart	Kidneys	Liver	PN (AN)	Other		
AL	Acquired	4067 (68%)	Plasma cell dyscrasia	Monoclonal immunoglobulin light chain	+++	+++	++	+(+)	Soft tissue gastrointestinal	Chemotherapy or ASCT	dFLC <40 mg/L
AA	Acquired	633 (12%)	Inflammatory disorders (RA, JIA, IVDU, FPS)	SAA	–/+ (late)	+++	+(late)	–	Gastrointestinal (late)	Suppression of inflammation	SAA <4 mg/L
ATTR	Acquired	168 (3·2%)	..	Wild-type TTR	+++	–	–	–	Carpal tunnel syndrome	Supportive	Optimum control of heart failure
	Hereditary	339 (6·6%)	Mutations in TTR gene	Abnormal TTR	++	–	–	+++ (+++)	–	Liver transplant (younger patients with V30M-related ATTR), diflunisal, (doxycycline/ TUDCA) Supportive	Optimum control of congestive heart failure and symptoms of PN/AN
AFib	Hereditary	87 (1·7%)	Mutations in fibrinogen α-chain gene	Abnormal fibrinogen	–	+++	–/+	–	–	Supportive, organ transplant	Preserve renal function
ALect2	Acquired	16 (0·3%)	Uncertain	Lect2	–	+++	++	–	–	Supportive	Preserve renal function
AApoA1	Hereditary	40 (0·8%)	Mutations in apolipoprotein A1 gene	Abnormal ApoA1	+	++	++	+/–(–)	Testis	Supportive, organ transplant	Preserve renal function
ALys	Hereditary	17 (0·3%)	Mutations in lysozyme gene	Abnormal lysozyme	–	+	++	–	Gastrointestinal or skin	Supportive	..
AGel	Hereditary	4 (0·1%)	Mutations in gelsolin gene	Abnormal gelsolin	–	-/+	–	++(–) cranial	–	Supportive	..
Aβ2M	Acquired or hereditary	93 (1·8%)	Long-term dialysis	Aβ2M	–	–	–	–(+*)	Carpal tunnel syndrome, arthropathy	Supportive, renal transplant	..

Aβ2M=β2-microglobulin-related. AFib=fibrinogen A α-chain. AGel=gelsolin amyloid. AL=amyloid light chain. ALect2=leucocyte cell-derived chemotaxin 2. ALys=lysozyme amyloid. AN=autonomic neuropathy. ASCT=autologous stem cell transplant. ATTR=amyloid transthyretin. dFLC=difference between involved and uninvolved free light chain. FPS=familial periodic fever syndromes. IVDU=intravenous drug abuse. JIA=juvenile inflammatory arthritis. PN=peripheral neuropathy. RA=rheumatoid arthritis. SAA=serum amyloid A. TTR=transthyretin. TUDCA=tauro-ursodeoxycholic acid. UK-NAC=UK National Amyloidosis Centre. *AN only in familial Aβ2M amyloidosis.[8] + indicates relative frequency: +++ very common, ++ common; + less common; –/+ rare; – not applicable or does not occur in this condition. (drug)=undergoing clinical trials. AA=amyloid A. AApoA1= apolipoprotein A1 amyloid.

AL, AA, and ATTR (ATTRv and ATTRwt) were responsible for around 90% of cases. After the era of this data recovery, there were some changes in epidemiologic tendencies. The incidence of AA amyloidosis caused by chronic inflammatory diseases such as tuberculosis, osteomyelitis, untreated rheumatic diseases (chronic inflammatory arthritides, vasculitides), and periodic fevers decreased. The reason behind this partially lies in the therapeutic advances in the treatment of infectious diseases and rheumatic diseases. On the other hand, the incidence of ATTR amyloidosis, mainly manifesting as heart failure and polyneuropathy, increased in recent decades due to new diagnostic approaches (cardiac MRI [CMR], cardiac scintigraphy) and the increased attention because of newly available therapies in certain cases.

The epidemiologic data of ATTRv patients from the US, Western Europe, South America, and some Asian countries are provided in the Transthyretin Amyloidosis Outcomes Survey (THAOS) registry. Until a recent publication of Austrian data, apart from Bulgaria, there was scarce information about the epidemiology of ATTRv in Eastern

and Central Europe [12]. Presenting the epidemiologic data of Hungarian ATTRv patients is one of the objectives of this work.

1.4. Etiology and clinical features of amyloidosis

While the deposited material is called amyloid, the disease caused by amyloid fibers is called amyloidosis. Systemic amyloidosis is a rare protein misfolding and deposition disorder causing tissue damage and leading to various organ dysfunction and disease manifestations [14]. The amyloids may affect almost any organ, leading to diverse clinical manifestations, which are rarely specific to one kind of amyloidosis. This also explains the diagnostic difficulties and the usually delayed diagnosis.

The most commonly encountered forms of systemic amyloidosis are AL, AA, and ATTR.

AL amyloidosis is a potential complication of any plasma cell dyscrasia: myeloma multiplex (MM), monoclonal gammopathy of undetermined significance (MGUS), non-Hodgkin lymphoma, and chronic lymphocytic leukemia. The produced immunoglobulin light chains in these diseases deposit in tissues [15]. Amyloid may also derive from immunoglobulin heavy chains, called immunoglobulin heavy-chain (AH) amyloidosis. As a systematic disease, it can manifest with different signs and symptoms: nephrotic range proteinuria, hepatosplenomegaly, edema, unexplained heart failure, carpal tunnel syndrome (CTS), etc. Commonly, the dysfunction of one organ dominates the clinical picture. In the case of AL amyloidosis, the affected organ is most commonly the heart, the kidneys, the liver, and occasionally the peripheral nervous system. Macroglossia and periorbital purpura is thought to be pathognomonic for AL amyloidosis if detected in the same patient.

AA amyloidosis (previously named secondary amyloidosis) is the consequence of chronic or re-occurring inflammation during which fibrils are formed from fragments of serum amyloid A protein, an acute phase reactant. Around 50 conditions are associated with AA amyloidosis [16]. Historically AA amyloidosis was primarily related to chronic infections like osteomyelitis, and to this day, in developing countries, this etiology is not negligible. Inflammatory arthritides became a relatively more common cause, although, with current therapeutic options, the incidence is declining in western countries. In endemic areas like the Mediterraneum, familiar periodic fever syndromes are behind significantly more amyloidosis cases [17]. In the case of AA amyloidosis, the kidneys are

the most commonly affected organs, leading to nephrotic syndrome mostly. Cardiac involvement may also be seen rarely.

ATTR amyloidosis is divided into ATTRv and ATTRwt amyloidosis.

In ATTRv amyloidosis (previously called hereditary or familial amyloidosis because of its autosomal dominant inheritance), modified transthyretin (TTR), also called prealbumin, is responsible for amyloid deposition. It has an autosomal dominant inheritance and a variable penetrance. The mainly affected organs are the heart and the peripheral nervous system. CTS is also a common manifestation. It may also involve other organs like the eyes, intestines, central nervous system, etc. The clinical picture and the disease's course largely depend on the mutation affecting ATTR. Some cause mostly progressive polyneuropathy, formerly called transthyretin-related familial amyloid polyneuropathy (ATTR-FAP), which is endemic in certain regions, like Sweden, Portugal, and Japan, with early-onset ATTRVal30Met mutation [18]. Like in the case of ATTRVal122Ile, others cause primarily cardiac amyloidosis (CA), manifesting as heart failure with preserved ejection fraction (HFpEF) endemically in West Africa [19]. The TTR gene is located on chromosome 18q12.1. It has 4 exons and 5 introns. More than 120 relevant TTR gene mutations have been found so far. Most of them are rare except for some mutations in endemic regions. Some relevant mutations are shown in Figure 1 (Rapezzi et al. with permission – modified). The later presented important Hungarian mutations are incorporated with a red margin.

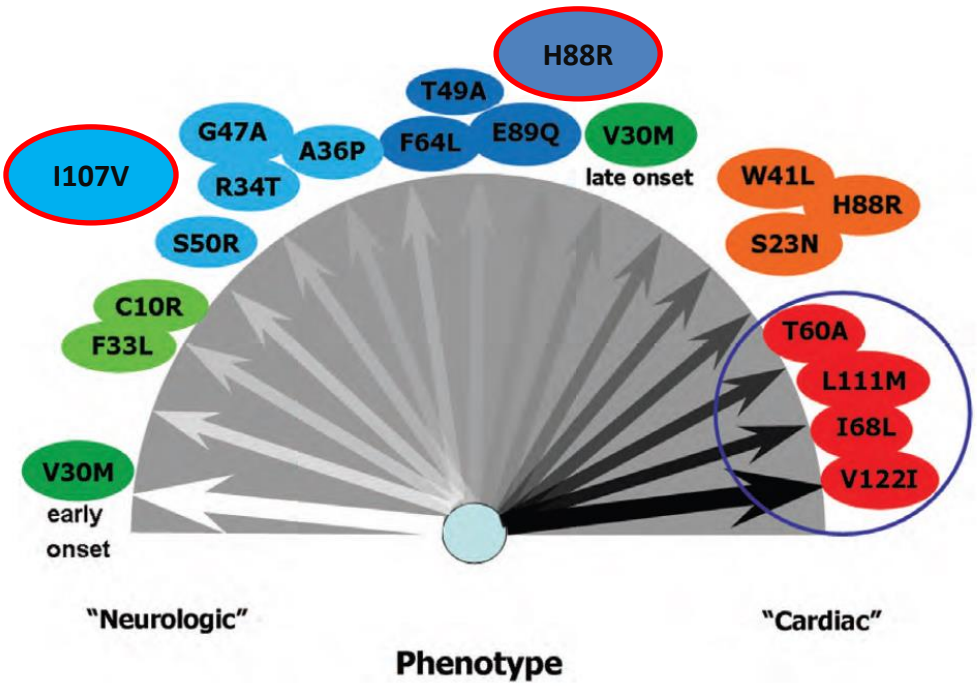

Figure 1 *Possible spectrum of genotype-phenotype correlations in transthyretin-related amyloidosis. Phenotypic expression of transthyretin-related amyloidosis varies widely from an almost exclusively neurological involvement (Val30Met mutation with early-onset disease) to a predominant or exclusively cardiac involvement (Thr60Ala, Leu111Met, Ile68Leu, and Val122Ile mutations). Several transthyretin-related amyloidosis mutations are associated with variable degrees of neurological and cardiological involvement, including Val30Met with the late-onset disease – Rapezzi et al. with permission, modified to show the later presented important Hungarian mutations, as well.*

In ATTRwt amyloidosis, previously called senile ATTR amyloidosis, because it manifests in the elderly, transthyretin forms the beta-sheet structure that deposits in the tissues. Cardiac involvement leading to heart failure is one of the critical manifestations of ATTRwt amyloidosis. CTS is the other. This form manifests later than ATTRv, in the 6^{th}-7^{th} decade of life. In patients older than 85 years, autopsy shows ATTRwt CA in 25% of cases [20]. Also, in older adults having surgery because of CTS, ATTwt deposits can be identified on histology in one-third of patients [21]. Also, ATTRvt has a significant male predominance – man:woman ratio is 25-50:1 [22].

1.5. Cardiac amyloidosis (CA)

Cardiac infiltration by amyloids, leading to restrictive HFpEF cardiomyopathy, is a characteristic feature of AL and ATTR amyloidosis. The involvement of the heart is the primary cause of morbidity and mortality, independently of the etiology of systemic amyloidosis [23]. The amyloid deposits in the extracellular matrix cause wall thickening, remodeling of the ventricles, and low cardiac output in the end. Atrial dilation is the consequence of locally increased pressure. The coronary arteries can also be affected, interrupting myocardial perfusion [24]. Arrhythmias like atrial tachycardia or fibrillation and conduction abnormalities are common complications of systemic amyloidosis affecting the heart.

Thus, CA has several clinical presentations. Heart failure (HF) is the leading manifestation, presenting with signs and symptoms like dyspnea, peripheral edema, elevated jugular venous pressure, hepatic congestion, etc. These are caused by restrictive cardiomyopathy and primarily right-sided heart failure. Angina is uncommon. As CA progresses, signs and symptoms of low cardiac output appear, such as filiform pulse, long capillary refill time, fatigue, etc. Patients having HFpEF over 60 years, 6-13% were diagnosed with ATTR CA after screening [25, 26]. Syncope and presyncope can also be a symptom in CA. In ATTR amyloidosis (ATTRv and ATTRwt), progressive conduction system abnormalities are common, leading to pacemaker implantation. On the other hand, a high-degree atrioventricular block is rare in AL CA [27]. Atrial fibrillation is another common consequence of CA, with the possible complication of cardiac thromboembolism, especially in AL CA [28].

In the case of ATTRwt CA, nearly all patients are above the age of 60 when the diagnosis is made, most of them becoming symptomatic above the age of 70 or 80. The mechanism by which the cardiac involvement of amyloid formed of genetically normal TTR increases over time is not well understood. Although CA can be diagnosed in 25% of patients by autopsy, cardiac dysfunction only manifests in a fracture in these patients [22].

In ATTRv CA, the onset of symptoms is relatively early compared with ATTRwt, as it may happen in the 3^{rd} – 4^{th} decade of life [23] – although it is mutation dependent and some have late-onset cardiac manifestations. Influenced by the mutations affecting the TTR gene, cardiac and neurologic manifestations usually overlap, just as shown in Figure 1, and some mutations have primarily cardiac manifestations like Val122Ile,

Thr60Ala, Ile58Leu, and Leu111Met [29]. Studying the genotype-phenotype correlation in Hungarian ATTRv patients is another objective of this work [13].

In the case of AL CA, it is usually part of the multisystem deposition of amyloids, but in some cases, the heart is the only clinically affected organ. It is involved in about 50-70% of cases [23]. Polyneuropathy and autonomic neuropathy are common in patients with AL amyloidosis. In these cases, diagnosing early CA is a challenge.

The diagnostic evaluation and diagnosis of CA changed a lot in recent years. Although the gold standard for the diagnosis is still the cardiac biopsy, ATTR CA diagnosis can now be established without biopsy [30-32]. As stated in the guidelines, the first and most crucial step in the diagnosis of CA is the arising of suspicion upon coming across red flags like seeing low voltage on an electrocardiogram (ECG) despite thickening of the septum/posterior wall (12mm); unexplained LV hypertrophy; intolerance of beta-blockers or angiotensin-converting enzyme (ACE) inhibitors; history of bilateral carpal tunnel syndrome, etc. Some of these are more typically seen in AL CA, such as macroglossia and/or periorbital edema. Others, like the new diagnosis of hypertrophic cardiomyopathy or low-flow, low-gradient AS in elderly patients, are typical in ATTR CA.

The well-known low voltage on ECG is only present in 30% of AL CA patients and 50% in ATTR CA patients [30], meaning its diagnostic value is limited. Other possible findings are the pseudoinfarct pattern and conduction anomalies which are more prevalently seen in ATTR CA.

Laboratory parameters like N-terminal pro b-type natriuretic peptide (NT-proBNP) or high sensitivity Troponin-T have their role in staging and prognostic evaluation of CA. Their diagnostic role is primarily seen in AL CA in the case of extracardiac histological proof of amyloidosis. The elevated NT-proBNP level (>332ng/L) in this scenario has 99% sensitivity for CA [33].

Histology was deemed necessary for diagnosing CA and remains the gold standard. With adequate sampling and processing, its accuracy is nearly 100% [34]. Amyloid fibrils are detected by Congo Red staining. Further characterization is done by immunohistochemistry or mass spectrometry. Following current guidelines [31, 32] for the diagnosis of AL CA, amyloid presence should be proven by histology (fat, rectum, lip, etc.). However, the sensitivity of these biopsies is far from 100%, meaning that a negative result does not rule out amyloidosis. On the other hand, ATTR CA can be

diagnosed without biopsy in case of positive scintigraphy, absence of clonal plasma cell process, and typical cardiac imaging features.

Echocardiography has a primary role in not just the diagnosis of CA but in recognizing complications (like intraventricular thrombus), differential diagnosis, and follow-up. The term "restrictive cardiomyopathy" is often used in connection with CA because it describes the hemodynamic changes well. From the morphologic point of view, concentric LV hypertrophy is the most characteristic anomaly. Above a wall thickness of 12 mm, the suspicion of CA should appear. Besides LV hypertrophy, the typical picture is: enlarged atria, increased atrial pressure, thickened mitral-, tricuspid-, and sometimes aortic valve, thickened right ventricular free wall, diastolic dysfunction, increased left atrial filling pressure, normal systolic LV ejection fraction (EF) (which decreases by time), decreased systolic right ventricle EF, increased pulmonary pressure, wide inferior vena cava, commonly pleural effusion, rarely pericardiac effusion [31]. Left ventricular endocardiac strain analysis shows the characteristic "cherry on cake" picture in advanced stages, as shown later in Figure 2, part B. This is a very sensitive and specific sign in CA [35]. For prognosis, stroke volume is the best echocardiographic marker in AL CA [36]. There is no difference with this imaging method between the types of CA. They all look the same.

Although even cardiac MR (CMR) does not have 100% sensitivity, it has an important role in diagnosing CA. And because of its excellent tissue characterization and anatomical imaging, it is also crucial in differential diagnosis [31]. Diffuse, often transmural late-type contrast-enhancing, independently of vascular anatomy and LV hypertrophy, are the two most specific abnormalities in CA. With MR imaging technics like T1 mapping, if we know the patient's hematocrit, we can calculate the cardiac muscle's extracellular volume (ECV). Diffuse and significant elevation of the ECV is specific for CA. Although CMR can detect CA with good sensitivity, it cannot distinguish between the different forms of CA [32]. It also has some relative or absolute contraindications like implanted metal, implanted pacemaker, atrial fibrillation, claustrophobia, and renal insufficiency.

Previous observations revealed that scintigraphy with a bone tracer enhances the heart in ATTR CA. After the publication of a multicenter study in 2016 [37], scintigraphy became the first-line diagnostics for ATTR CA. Three kinds of radiopharmacons can be used, of which only 99mTc labeled pyrophosphate (PYP) is available in Hungary. If the heart enhancement equals or is greater than the bone uptake, the diagnosis of ATTR CA

is almost certain. In this scenario, the diagnosis of ATTR CA can be made without a biopsy. However, serum light chain measurement and immunofixation are needed to rule out AL amyloidosis. Without enhancement in the heart, ATTR CA is ruled out. Further evaluation is necessary if there is some enhancement in the heart but to a lesser extent than the bone uptake. Scintigraphies to diagnose CA are more and more commonly done in Wester-Europian Centers.

Previously, definitive treatment of CA meant heart transplantation (HTX) if the underlying disease was treatable, meaning bone marrow transplantation in case of AL amyloidosis or successful pharmacological therapy in case of ATTR amyloidosis. Before novel therapeutic options, liver transplantation was done to cure ATTRv amyloidosis, stopping the production of the harmful protein. CA came to the spotlight partially because of novel therapeutic options for treating ATTR amyloidosis in the last decades. A previously more or less untreatable disease has become successfully treatable in many cases and improved the patients' life expectancy.

Independently of the treatment itself, it is of utmost importance that treatment is started as early in the course of the disease as possible. AL CA treatment is oriented against the underlying condition, primarily monoclonal gammopathy of undetermined significance (MGUS) or myeloma multiplex. In combination with glucocorticoid and cyclophosphamide, the proteasome inhibitor bortezomib is a typical starting therapy in these patients [30]. Newer agents like revlimide (angiogenesis inhibitor), daratumumab (CD38 monoclonal antibody), and venetoclax (anti-apoptotic protein B-cell lymphoma 2 inhibitor) are also used. It was an exciting observation that doxycycline helps dissolve the deposited amyloid and improves outcomes in these patients [38].

The drug tafamidis was first used to treat patients with ATTRVal30Met having polyneuropathy in the endemic region of Portugal. This mutation usually does not lead to cardiac involvement. It was registered to treat ATTRv amyloidosis causing stage I. neuropathy (patient can walk without help) with a daily dose of 20 mg. The drug stabilizes the amyloid tetramers, preventing monomer formation and deposition. After studying its effectiveness in ATTRv and ATTRwt CA, it got a label for treating ATTR CA (both etiologies) with a daily dose of 61 mg [39]. It improves not just the quality of life but survival as well.

Patisiran is a small interfering RNA (siRNA). It inhibits the TTR protein transcription by forming a complex with the messenger RNA (mRNA) and a protein that slices the mRNA. The process may repeat a thousand times, significantly decreasing the

amount of the TTR mRNA, thus decreasing the protein production and its concentration in the serum. The molecule is packed in vesicles that attach to liver cells, so it is explicitly delivered to the site of protein production [40]. It is administered intravenously every three weeks in stage I and stage II (patient requires a walking aid) ATTRv polyneuropathy. There are studies on the way for the application in CA.

Inotersen is an antisense oligonucleotide also inhibiting TTR transcription in liver cells by attaching to mRNA. However, the drug and mRNA rate is 1:1 in this case. It also has a label for treating ATTRv-caused stages I. and II. polyneuropathy. The drug is administered subcutaneously. One major side-effect is thrombocytopenia, warranting blood count controls [41].

The prognosis of CA depends on many factors. The etiology itself is one of the most important ones. AL CA has the worst prognosis without treatment, with overall median survival of only six months [42]. With treatment, survival improves significantly [43]. Decades later, an extensive follow-up study of CA comparing AL ATTRv and ATTRwt CA also found that AL CA has the worst prognosis with the rapid progression of HF [44]. Hemodynamic impairment was more common in AL CA despite the less outstanding morphological abnormalities like wall thickness. It may be attributed to the direct toxicity of immunoglobulin light chains in AL amyloidosis.

On the other hand, in ATTR CA, there is time for compensatory mechanisms to take effect because of the gradual nature of the amyloid deposition. In the case of ATTRwt CA, the median survival is better, 3.5 years without treatment [45]. In ATTRv amyloidosis, the clinical phenotype and prognosis vary among the patients with different mutations. Primarily cardiac involvement determines the prognosis. The median survival is around 4-5 years if CA is present [46].

There is no universally used prognostic score for CA. In everyday practice, the etiology, New York Heart Association (NYHA) stages for HF, NT-proBNP, high sensitivity Troponin T, stroke volume, and wall thickness are used to estimate prognosis. There has been significant progress in treating amyloidosis affecting the heart in the last couple of decades. Still, the prognosis remains poor for those who present with advanced heart involvement.

1.6. Cardiac amyloidosis (CA) and aortic stenosis (AS)

As a common valvular disease, AS reduces life expectancy when it becomes significant. Patients with ATTRwt CA or AL CA and those with AS have similar

demographic [47] and echocardiographic features like left ventricular hypertrophy, diastolic dysfunction, or elevated left ventricular filling pressure. CA may not be diagnosed when the patient also has AS because the abnormalities detected by echocardiography are readily explained by AS, and so the diagnosis of CA is overlooked. Recently, case studies reported ATTRwt CA and AS co-existence, emphasizing the diagnostic challenges of these cases [47, 48]. Studies examining the prevalence of ATTR CA in AS used different diagnostic methods and examined different populations [49-53]. Some included more than 100 patients with AS. Among patients having AS, the incidence of ATTR CA was found to be 6 to 16%. If we examine the situation the other way around, we will find that among patients with ATTR CA, the prevalence of moderate/severe AS is 27% [54]. The prevalence of AS in consecutive CA patients – including those with ATTR and AL – to our knowledge, has not been examined yet. This marks another objective of the thesis [55].

Patients with concomitant AS and CA are more frequently present with a low-flow, low-gradient pattern (AVA<1.0 cm^2, mean gradient<40 mm Hg, and stroke volume index < 35 ml/m^2) compared to patients without CA (30% to 80% vs. <30%) [56]. This may be due to severe left ventricular remodeling, diastolic dysfunction, right ventricular dysfunction, and impaired left ventricular longitudinal systolic function. Assessing the severity of AS is harder in patients with low-flow, low-gradient AS. Using dobutamine stress echocardiography in the diagnostic work-up of patients with concomitant CA and low-flow, low-gradient AS was the last objective of the thesis [55].

2. Objectives

Assessing the co-existence of CA and AS in consecutive CA patients

1) Study the prevalence, severity, and type of AS in consecutive patients with CA
2) Evaluate the potential use of stress echocardiography in the diagnostic process

Assessing Hungarian patients with ATTRv

1) Investigate the prevalence, regional distribution, and genotypes of Hungarian patients with ATTRv
2) Study the phenotype-genotype correlation in Hungarian patients with ATTRv

3. Methods

Methods already published with my authorship are only briefly described following the guidance of the Doctoral School. For details, I refer to the cited publications [13, 55].

3.1. Assessing the co-existence of AS and CA

We retrospectively interpreted and analyzed the available echocardiographic and clinical data of 55 consecutive CA patients between January 2009 and January 2019. CMR was routinely done, if not contraindicated, for differential diagnosis and the confirmation of CA. Left ventricular longitudinal strain analysis was done in all patients with clinical suspicion of CA after 2015. We examined segmental differences looking for "apical sparing." Also, the routine measurements for AS were done. In cases where less than 0.6 cm^2 was measured as the indexed aortic valve area, but resting echocardiography showed low-flow, low-gradient AS (LFLG AS), dobutamine stress echo was performed regardless of the LV EF to separate pseudo- and true-severe AS [55].

3.2. Assessment of Hungarian patients with ATTRv

With the coordinated help of Hungarian cardiology, neurology, and rare disease centers, all genetically confirmed ATTRv patients were identified in the country retrospectively. Available clinical and epidemiological data were collected and interpreted. Epidemiologic data consisted of family history, sex, date of birth and death, residence, and the type of ATTRv mutation. Clinical data covered age at the time of clinical diagnosis, time from the first disease-specific symptom to the clinical diagnosis of ATTRv, and the initial clinical presentation. The primary cause of death was also processed when available. Also, it was noted if the patient was already symptomatic at the time of the genetic diagnosis or was found asymptomatic during family screening. Latter patients were only included in the epidemiologic analysis. A search for publications was also performed in PubMed ((Hungary) OR (Hungarian)) AND ((TTR) OR (transthyretin)) to find further patients with ATTRv [13].

We established CA in the ATTRv patients according to recent recommendations based on broad consensus during our retrospective analysis. The neurologic involvement of the patients was diagnosed during complex neurologic evaluation following actual guidelines, involving the electrophysiological assessment of peripheral nerves. We did not consider CTS a primary neurologic manifestation.

PubMed search identified one family with ATTRAsp18Gly, published decades ago in several articles [57-60]. Their genetic test was done in New York, NY, USA. The method is described in the original article. The genetic tests of all other patients were performed in Hungary (Department of Internal Medicine, University of Szeged or at the Department of Internal Medicine and Hematology, Semmelweis University, Budapest).

4. Results

4.1. Results from our assessment of the co-existence of AS and CA [55]

We performed transthoracic echocardiography at rest on 55 consecutive patients with CA between January 2009 and January 2019. Among them, 45 had AL amyloidosis, 9 had ATTR amyloidosis (6 ATTRv, 3 ATTRwt), and one had AA amyloidosis. The median age was 65 years. Reviewing these patients, we identified 5 (9%) with moderate or severe AS. The crucial characteristics (clinical data, biomarker, and echocardiographic results) of these patients are shown in Table 3.

Table 3 *Clinical characteristics of the 55 CA patients grouped according to the presence or absence of aortic valve stenosis [55]. Values are presented as medians with interquartile ranges (IQR) or as percentages. We calculated the strength of the associations with the nonparametric Mann-Whitney test or the chi-square test.*

	Patients without AS (n=50)	Patients with AS (n=5)	p-value
Clinical data			
Age (years)	63.5 (58-73)	69 (68-82)	p=0.055
Male (n, %)	26 (52%)	2 (40%)	p=0.553
ATTRwt CA (n, %)	2 (4%)	1 (20%)	p=0.391
ATTRv CA (n, %)	6 (12%)	0 (0%)	p=0.485
AL amyloidosis (n, %)	41 (82%)	4 (80%)	p=0.741
AA amyloidosis (n, %)	1 (2%)	0 (0%)	p=0.909
NYHA III-IV stage (n, %)	32 (64%)	5 (100%)	p=0.278
Atrial fibrillation (n, %)	11 (22%)	1 (20%)	p=0.312
Laboratory data			
B-type natriuretic peptide (pg/ml)	606 (234-1240)	341 (77-657)	p=0.303
Troponin T (ng/L)	66 (39-104)	134 (47-215)	p=0.464
Echocardiography			
Left ventricular ejection fraction (%)	56 (43-63)	59 (51-60)	p=0.823
Septal wall thickness (mm)	16 (13-18)	17 (13-20)	p=0.578
Inferior wall thickness (mm)	15 (13-17)	15 (13-16)	p=0.780
Left ventricular end-diastolic diameter (mm)	42 (36-45)	41 (37-42)	p=0.776
E/e' (Average of lateral and septal e')	20.6 (16-24)	18.1 (16.9-20.6)	p=0.241
Lateral S'	5.5 (4-7)	6.25 (4,25-8.3)	p=0.588

Each patient with AS had severe HF with NYHA stage III or IV. There was no significant difference between the patient's clinical characteristics with AS and CA or CA alone. However, this may be because of the relatively small number of patients. Patients having AS and CA simultaneously tended to be older, but the p-value of difference was

only 0.055. The calculated aortic valve area (AVA) during baseline echo was less than 0.6cm^2/body surface area (BSA) in 3 cases. In the other 2 cases, AVA/BSA was 0.65 and 0.63 cm^2/m^2, indicating moderate AS. In the 3 cases with AVA/BSA below 0.6 cm^2/m^2, we performed stress echocardiography with dobutamine to separate true severe AS from pseudo-severe AS. In one patient with an LVEF of 48%, true-severe AS was diagnosed. Echocardiography and ECG also suggested CA. CMR showed characteristic abnormalities for CA. Finally, a diagnosis of AL CA was made in this case. The patient died two months after diagnosis. In the other two cases, dobutamine stress echo revealed a significant increase in AVA, meaning these patients had pseudo-severe AS with LVEF of 51% and 59%. One of them was an 89-year-old male, initially diagnosed with colon cancer. During the preoperative evaluation, HF was diagnosed, and low-flow, low-gradient AS was seen during the echo. There was severe LV hypertrophy and concurrent relative low voltage on ECG. The stress echo showed pseudo-severe AS, and the PYP isotope scintigraphy showed typical results for TTR CA. A hemicolectomy was performed without complications. We present the cardiac images of this patient in Figure 2. The other patient with low-flow, low-gradient AS had AL CA, and therapy was started for the diagnosed plasma cell dyscrasia. Patient characteristics who had AS and also CA are summarized in Table 4.

Apical sparing of the LV was obvious during visual assessment in all cases. Still, the average apical longitudinal strain (LS)/(average basal LS + average mid-LS) ratio did not reach 1.

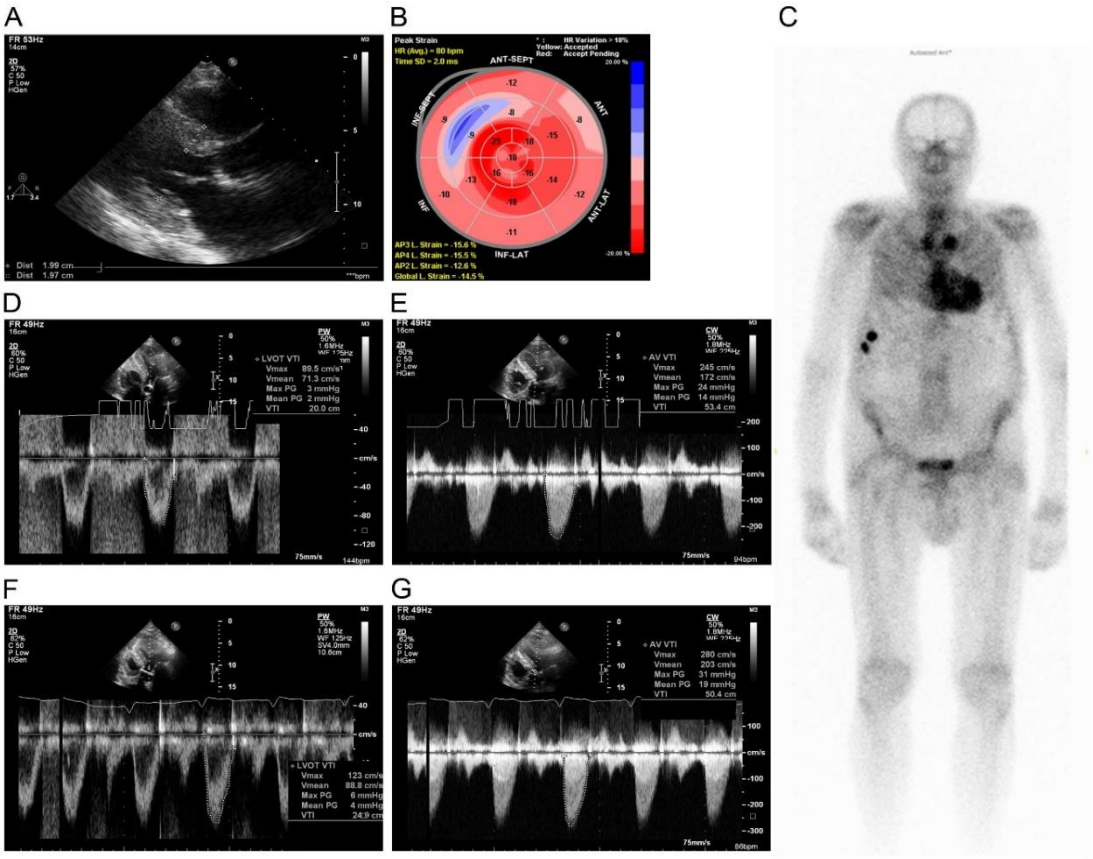

Figure 2 *Images of a patient with ATTRwt cardiac amyloidosis and low-flow, low-gradient, pseudo-severe aortic valve stenosis. LVEF was 51%. A: Transthoracic echocardiography, parasternal long-axis view. The septum and inferior wall are 20mm thick at the end-diastole. B: Bull's s eye image of the left ventricular longitudinal strain. Typical apical sparing. C: Pyrophosphate isotope scan, with significant take-up of the tracer in the heart: Perugini score 3. D-G: Pulsed-wave (PW) and continuous-wave (CV) Doppler images of the left ventricular outflow tract at rest and at low dose dobutamine stress test. D: Resting PW Doppler, E: Resting CW Doppler. Resting calculated AVA: 0.54 cm^2/BSA. F: PW Doppler at dobutamine test. G: CW Doppler at dobutamine test. Significant elevation in systolic volume and AVA at the dobutamine test. Calculated AVA at dobutamine test: 0.76 cm^2/BSA [55]*

Table 4 *Characteristics of patients with CA and AS [55]. LS: longitudinal strain, TTE: transthoracic echocardiography, NA: not applicable, DNP: did not perform.*

Patient's number, age (years) and sex	Type of CA	LV wall thickness (septum/posterior wall, mm) measured with echo, left ventricular ejection fraction (%), stroke volume index (ml/m^2)	Presence of typical LGE in CMR or semiquantitative score>1 at PYP isotope scan in TTR amyloid	Cardiac biopsy positive for amyloid	Apical sparing at strain analysis. (Average apical LS/(average basal LS + mid-LS))	AVA/BSA (cm^2/m^2) and mean aortic valve gradient at rest, measured by TTE echo	AVA/BSA (cm^2/m^2) and aortic valve gradient during dobutamine stress echo	Final diagnosis of the type of AS
1. 66, male	AL	17/16, 48, 20	yes	DNP	yes 0.75	0.45/BSA, 13	0.45/BSA, 22	True-severe AS
2. 68, female	AL	13/13, 60, 40	DNP	DNP	yes 0.77	0.65/14	DNP	Moderate AS
3. 89, male	ATTRwt	20/20, 51, 22	yes	DNP	yes 0.81	0.54, 12	0.76, 19	Pseudo-severe AS
4. 69, female	AL	20/15, 61, 31	DNP	yes	yes 0.77	0.63, 22	DNP	Moderate AS
5. 83, female	AL	12/12, 59, 38	yes	DNP	yes 0.77	0.58, 19	0.86, 25	Pseudo-severe AS

4.2. Results from the assessment of Hungarian patients with ATTRv [13]

4.2.1. Epidemiology of ATTRv patients in Hungary

We identified 36 individuals from 22 families with known pathogenic TTR mutations. Twelve were asymptomatic, and twenty-four were symptomatic at the time of the diagnosis. We found seven different pathogenic mutations: ATTRHis88Arg (9 families, 13 individuals), ATTRIle107Val (8 families, 11 individuals), ATTRVal30Met (2 families, 5 individuals), ATTRVal122del (1 family, 1 individual), ATTRPhe33Leu (1 family, 1 individual), ATTRIle84Ser (1 family, 5 individuals) — data summarized in Figure 3. Figure 4 shows the geographical distribution of these patients. All patients were native Hungarians, born in Hungary. Even though we contacted all cardiology, neurology, and rare disease centers, most patients originated from two regions, as shown in Figure 3.

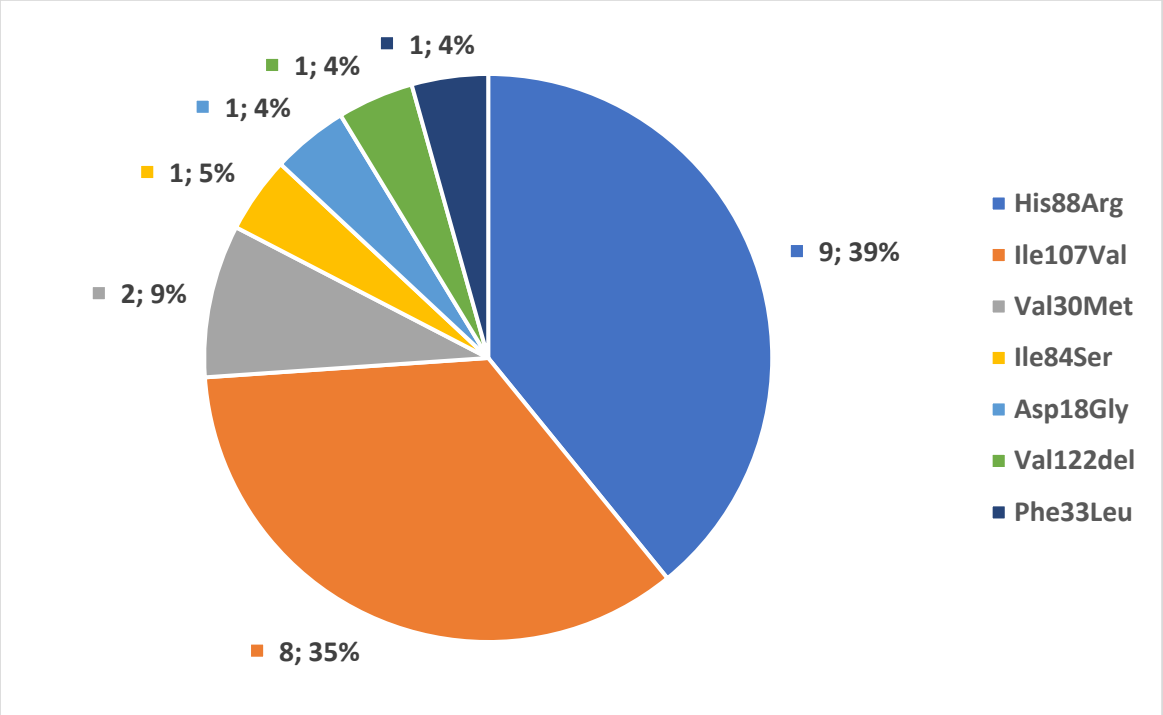

Figure 3 *Genetic spectrum of TTR mutations in Hungarian families, expressed in number and percentage of the affected families (n=23) [13].*

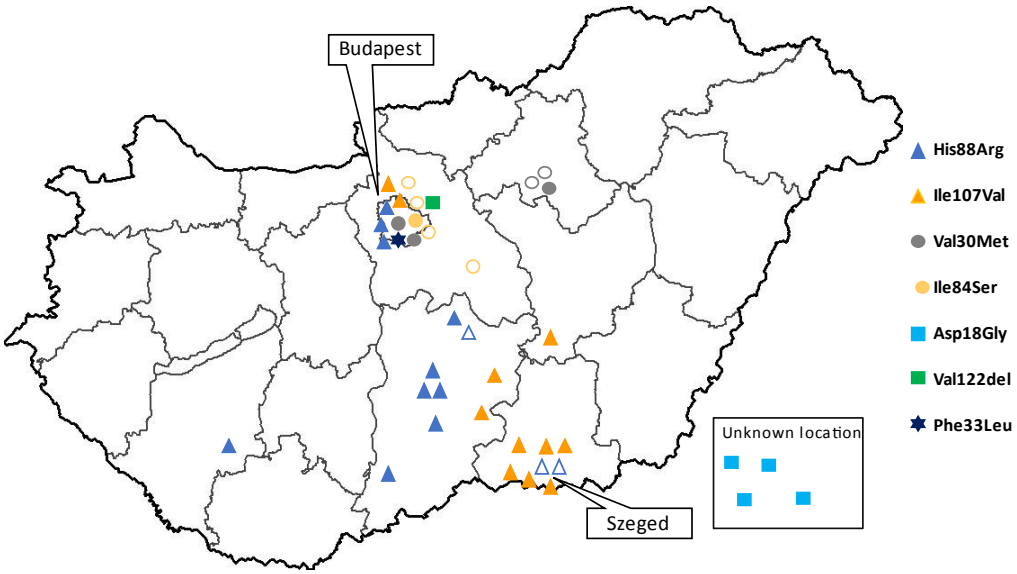

Figure 4 *Map of Hungary with the geographic distribution of different genetic subtypes of ATTRv patients (n=40). Full symbols represent symptomatic individuals, while empty symbols represent asymptomatic individuals. The four patients with unknown locations are the patients found during the literature search. We found one additional Hungarian family with four affected individuals during the PubMed search. They had ATTRAsp18Gly mutation, and the cases were published more than 20 years ago. The authors did not describe any clinical signs of polyneuropathy or HF. The leading symptoms were associated with central nervous system involvement. Detailed clinical data are not available about these patients. Thus, we included them exclusively in the epidemiologic analysis [13].*

4.2.2. Clinical characteristics of ATTRv patients in Hungary

We summarized the clinical findings of all 24 symptomatic patients in Table 5. The twelve asymptomatic individuals were considered carriers. Four had CTS, and eight were without any clinical manifestation. These asymptomatic carriers were identified during family screening. They have regular follow-ups. At the time of genetic testing, their median age was 29 years. On the other hand, the median age of the other 24 symptomatic patients was 65 years at the time of clinical diagnosis. From the first clinical symptoms to the clinical diagnosis, the median time was 54.8 months. Every symptomatic patient had cardiac involvement at the time of the clinical diagnosis. Twenty of them had neurologic involvement as well. Seventeen out of the 24 patients had CTS in their medical history. Cardiac symptoms were dominant clinically in eight cases – three had no neurologic signs of the disease. In eleven cases, the neurologic symptoms were more severe than the cardiac symptoms – although all patients had some level of HF as well.

The burden of HF and polyneuropathy was equally prominent in three cases. Data were insufficient in two cases. Three, eight, eight, and two patients were in NYHA I, II, III, and IV functional stages, respectively, at the time of clinical diagnosis. Data were missing in four cases. We found NT-proBNP values at the time of clinical diagnosis in 15 patients. Their median value is 3511 pg/mL. Eight, eight, three, and zero patients had modified polyneuropathy disability (PND) scores of I, II, III, and IV, respectively, at the time of the diagnosis. They all had late-onset length-dependent sensorimotor axonal polyneuropathy. Three patients had no polyneuropathy, data were unavailable in one case, and data were insufficient to determine the PND stage in one case. Twelve patients were already dead at the time of the analysis, and one had HTX. Causes of HTX and death were the progression of heart failure (6 cases), arrhythmia (1 case), other (3 cases), or unknown (3 cases). None of the patients died because of progressive neuropathy. When the analysis was performed, ten patients were receiving targeted therapy for ATTRv or had received treatment earlier. The clinical data of ATTRv patients are summarized in Table 5. Figure 5 shows the timeline of when families with ATTRv were identified.

ATTRHis88Arg was the most commonly identified mutation with 10 cases. At the time of clinical diagnosis, the median age was 62 years. 43.8 months passed between symptom onset (median 58.35 months, if we exclude CTS) to clinical diagnosis. The median NYHA stage was II; the median PND stage was I. With eight cases, ATTRIle107Val was the second most common mutation. Patients with this mutation had a median age of 73 years at the diagnosis. The first disease-specific symptoms started at the median age of 67.44 years. The median time to clinical diagnosis from this point was 66.7 months. The median NYHA stage was III, and the median PND stage was II in these patients. The clinical phenotypes of the two most common Hungarian mutations are summarized and compared in Table 6.

Table 5 *Clinical characteristics of patients with symptomatic ATTRv (n=24) [55]. NYHA: New York Heart Association – functional classification of heart failure; PND: modified polyneuropathy disability score; CTS: carpal tunnel syndrome; N/A: not available; HF: heart failure; * 1 additional patient participates in a placebo-controlled clinical trial*

Sex		Male 18 / Female 6
Age at clinical diagnosis (median)		65 years
Time to clinical diagnosis (median)		54.8 months
Initial presentation (at the time of onset of the first symptom)	cardiac	33.33% (n=8)
	neurologic	45.83% (n=11)
	mixed	12.5% (n=3)
	N/A	8.33% (n=2)
Stage of heart failure according to NYHA functional class at the time of clinical diagnosis	I.	12.5% (n=3)
	II.	33.33% (n=8)
	III.	33.33% (n=7)
	IV	8.33% (n=2)
	N/A	12.5% (n=3)
The median value of NT-proBNP at the time of clinical diagnosis		3511 pg/ml (n=15)
PND at the time of clinical diagnosis	no polyneuropathy	12.5% (n=3)
	I.	33.33% (n=8)
	II.	33.33% (n=8)
	III.	12.5% (n=3)
	IV	0% (n=0)
	N/A	8.33% (n=2)
CTS in medical history	positive history	75% (n=18)
	negative history	20.83% (n=5)
	N/A	4.16% (n=1)
Total number of patients receiving targeted pharmacological therapy		41.6% (n=10)*

Table 6 *Genotype-phenotype correlation in symptomatic Hungarian ATTRv patients with the two common mutations (ATTRHis88Arg and ATTRIle107Val) [13]. NYHA: New York Heart Association – functional classification of heart failure; PND: modified polyneuropathy disability score; * All patients with ATTRIle107Val and ATTRHis88Arg had mixed phenotypes, except one patient with only cardiac symptoms*

		ATTRIle107Val n=8	**ATTRHis88Arg n=10**
Number of patients with	heart failure as leading symptom	2	4*
	equally severe cardiac and neurologic symptoms	1	2
	polyneuropathy as leading symptom	5	4
Median age at clinical diagnosis		73 years	62 years
Median time from first symptoms to diagnosis		66.7 months	43.8 months
Median age at first symptoms		67.44 years	58.35 years
Median NYHA stage		III	II
Median PND stage		II	I

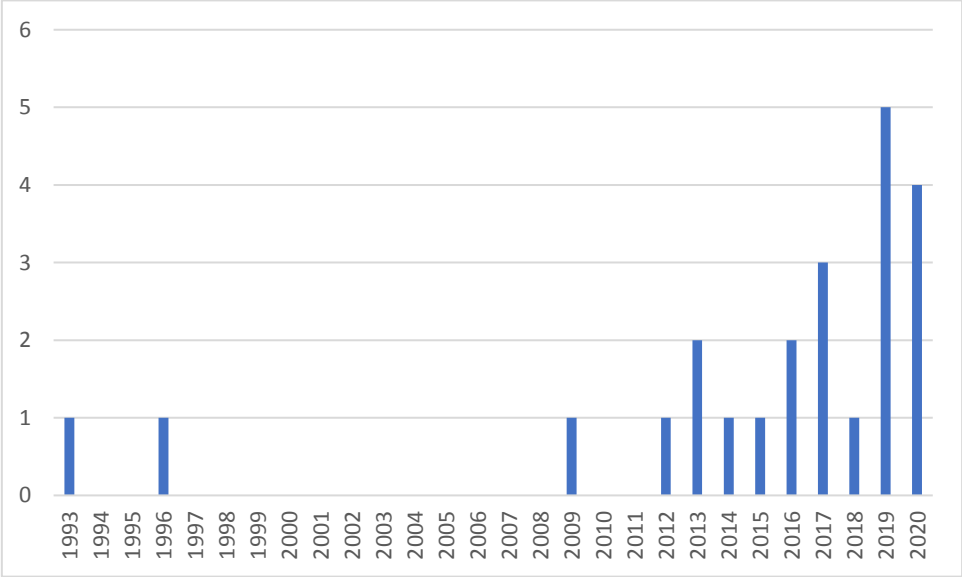

Figure 5 *Number of families identified with ATTRv in recent years (n=23) [13].*

5. Discussion

5.1. CA and AS

In our published retrospective study about the co-existence of AS and CA in 2019 [55], the prevalence of moderate to severe AS among consecutive, unselected CA patients was 9%. AL CA represented the majority of our patients. The explanation for this may be that our Department at Semmelweis University specializes in cardiology and hematology. Patients with ATTRwt CA have been diagnosed more frequently since 2016 [11]. Our patients, however, received their diagnosis between 2009 and 2019. This might be another reason why we have a relatively small percentage of ATTR CA.

Different authors found the prevalence of CA in AS to be 4.1 [52] to 16% [49] in the previous years. Although their methods and study design were different, these studies asserted that these had ATTR CA but not AL CA. According to two studies, those patients with both AS and CA have a worse prognosis than patients only having AS. This statement is true whether we examine patients after valve replacement [52] or if we pool them irrespective of valve replacement [50]. If we approach the other way around and look for AS among CA patients, we can find only a few studies. In these studies, however, the authors used ATTR CA patient registries. The prevalence of moderate to severe AS among ATTR CA patients was 16% by Sperry et al. [54]. In another retrospective study, Java et al. examined patients with different causes of amyloidosis [61]. The authors reviewed the outcome of aortic valve replacement in these patients. The database of the Mayo Clinic (Rochester, MN, USA) was screened for patients who had aortic valve replacement and were also diagnosed with any type of amyloidosis. Sixteen patients met their criteria, and only six had cardiac manifestation due to amyloidosis. Because these patients were not analyzed separately, the value of the publication is limited for calculating the common prevalence of AS and CA. The co-existence of ATTR CA and AS seems to be more common than the co-existence of AL CA and AS, based on these results. One plausible explanation for this is the age of the patients. Both ATTRwt and AS is strongly age-related [11, 62]. Thus, the co-existence is also increasing with age. On the other hand, because plasma cell dyscrasia affects younger patients, the patients with AL amyloidosis will have a lower median age, which means that the co-incidence with AS decreases.

Although CA was once regarded as a rare disease [10, 42], studies with HFpEF patients show that CA is unexpectedly common in this population, with a prevalence of

17 to 19% [63, 64]. As well as mentioned in the introduction, 25% of patients over the year 85 had amyloidosis on autopsy. The already mentioned recent publications about CA and AS also suggest that CA is more common than we thought. This relatively new knowledge has diagnostic importance and also therapeutic implications.

In our relatively small population, it was impossible to find relevant differences in echocardiographic, laboratory, clinical, or prognostic results between CA patients and patients with CA and AS. We found that moderate to severe AS is relatively common in a CA population where 80% of patients had AL amyloidosis. This is one of the novelties in our retrospective analysis.

The other novelty of our study was about the usage of dobutamine stress echocardiography in patients with low-flow, low-gradient AS, HFpEF, and CA. The dobutamine stress test is not recommended in current guidelines for patients with low-flow, low-gradient AS if they have a preserved EF [65]. The European Society of Cardiology (ESC) guideline emphasizes the diagnostic importance of low-flow, low-gradient AS. The recommendation includes an integrated step-by-step algorithm for these patients. The first step, as always, is clinical suspicion, usually raised by the morphology of the aortic valve. If the AVA is below 1 cm^2, the mean pressure gradient is below 40 mmHg, and the calculated stroke volume index (< 35 ml/m^2) is low, suggesting a low-flow state, then dobutamine stress echocardiography is recommended. It can separate true-severe AS from pseudo-severe AS. AVA will increase together with the stroke volume, but the mean transvalvular gradient will not change significantly. In pseudo-severe AS, in contrast to true-severe AS, where the gradient will increase, but the AVA will stay the same. The stress test will not help to make the correct diagnosis, of course, in case of diminished left ventricular contractile reserve. In this case, we should take into account other parameters. The ESC guideline does not recommend the dobutamine stress test in low-flow, low-gradient AS patients if their LVEF is preserved only in those with reduced EF. Data prove the dobutamine stress test to be helpful and safe in patients with low-flow, low-gradient AS, reduced stroke volume, and normal LVEF. In a study by Caval et al. [66], Fifty-five patients had the stress test to differentiate between pseudo- and true-AS. The average LVEF was 63%, and the conclusion was that the method is safe and valuable in this population. One may argue from the pathophysiological point of view that only reduced LVEF can be improved by dobutamine, but it is just speculation. We also performed the dobutamine stress echo test on our three patients with severe low-flow, low-gradient AS. Their LVEF was 48, 51, and 58%. The test was diagnostic in all

three cases. The significant increase in AVA in two patients clearly showed that their symptoms are caused by the CA rather than pseudo-severe AS. One patient had true-severe AS, as shown in the data in Table 4. Our results supported the possible review of related guidelines. The new ESC guideline on the management of valvular heart disease was published in 2021, after our article [67]. It still only suggests the dobutamine stress test in the case of low-flow, low-gradient AS in patients with reduced LVEF. For patients with preserved LVEF, an integrated approach is recommended. Part of this is the cardiac CT and the assessment of aortic valve calcification. However, AS severity is not necessarily proportionate with the calcification of the aortic valve in some cases of CA, possibly because of different pathophysiology [68]. Compared to the previous guideline from 2017 [69], which does not mention amyloidosis at all, the 2021 version states that CA is frequently associated with AS in the elderly and should be considered carefully. The ESC also published a position statement on the diagnosis and treatment of CA [70], but it does not address this problem. It highlights the 12 mm LV wall thickness plus one red flag as the indication for further evaluation toward CA.

It is speculated that CA and AS may have a connected pathomechanism, as aortic valve stenosis may be induced by amyloid deposition in the valve. However, there is no supporting evidence for that. CA and true-serve AS co-exist in large numbers apparently, but this is instead because of statistical distribution: the prevalence of AS is high in elderly patients, who statistically have a higher chance to have ATTRwt. In the previously mentioned studies, 6-16% of the AS patients had ATTRwt after screening for the disease [49, 50, 52, 53]. AL CA was excluded in these patients, or they were not screened. However, the incidence of AS is not published in an extensive series of CA patients. In a study focusing on the echocardiography, ECG, and clinical features of 149 ATTRwt patients, not a single patient was diagnosed with AS [71]. This may call into question the data but also highlights that the co-existence of CA and AS should always be considered. This seems necessary to make the current diagnosis and not to miss it. In our opinion, the presence of pronounced LV hypertrophy and diastolic dysfunction at the same time is a possible reason to overlook CA in AS. These anomalies are common in both diseases. LV hypertrophy is often interpreted as the consequence of AS. From the other perspective, it is also easy to miss AS in CA because gradients can be surprisingly low in CA due to the low stroke volume [72]. If the AVA is carefully calculated and, when necessary, dobutamine stress echocardiography is done, the diagnosis becomes more straightforward. We saw the "apical sparing" phenomenon in all our patients with AS and

CA, so this phenomenon may provide further help to make the correct diagnosis by raising the suspicion of CA in SA patients. Additional red flags for CA should also be looked for, like relative low voltage on ECG, clinical signs of amyloidosis, etc.

Only one patient in our study had true-severe AS. He was 66 years old with NYHA stage III HF. He only survived the diagnosis by two months. AS, CA and MM obviously limited his life expectancy. It is impossible to determine how much AS or CA contributed to HF in such cases. The postoperative prognosis of AS patients was studied in the UK. They found that the mortality rate during the follow-up period was higher in patients with CA than in patients with calcified AS: 50% (3/6) versus 7.5% (8/106) [52]. This information suggests that both CA and AS contribute to HF and the unfavorable prognosis if they co-exist.

In another study, 171 consecutive ATTR CA patients were retrospectively studied at the Cleveland Clinic Foundation (Ohio, USA) [54]. Sperry et al. based the diagnosis on endomyocardial biopsy, CMR, echocardiography (apical sparing strain pattern) and/or PYP scintigraphy. They examined the prognosis of patients with moderate (10 patients), low-flow, low-gradient (11 patients), or high-grade (6 patients) AS compared to those without AS. Eleven patients with AS had aortic valve repair, which may have influenced the outcome. Both groups' mortality was high without a statistical difference at two years: 37 and 33%, respectively. The conclusion was that in the case of low-flow, low-gradient AS, routine screening for ATTR CA might be helpful. Diagnosing the disease would influence therapy. AL CA patients were not included in this study, however. It is not mentioned if any dobutamine stress test was done, so it is unknown how many of the patients had true- or pseudo-severe AS. The article emphasizes the role of strain analysis with echo and PYP scintigraphy but fails to mention the dobutamine stress test as a diagnostic method.

Based on our experience, we underline the importance of dobutamine stress echocardiography in patients with low-flow, low-gradient AS. This test identifies patients with true-severe AS who benefit from a successful AVR or TAVI. Our findings show that low-flow, low-gradient AS patients who have CA, even with preserved EF, may benefit from this diagnostic test, as it helps to identify patients with true-serve AS, who may benefit from invasive treatment.

The major limitation of the study is the small sample size. Also, our population is not representative of Hungarian CA patients. AL amyloidosis is overrepresented because

hematology is a major profile of our Institution. Still, to the best of our knowledge, this is the first screening for AS in consecutive CA patients.

5.2. Patients with ATTRv in Hungary

We presented the first data on the prevalence, epidemiology, and geno- and phenotypes of ATTRv patients in Hungary. Apart from the endemic regions in Bulgaria with the ATTRGlu89Gln, from the Eastern and Central European regions, only Austrian data were available from a recent publication [12]. To our best knowledge, all patients diagnosed with ATTRv were included in our analysis.

The major epidemiologic finding in our study is the identification of ATTRHis88Arg and ATTRIle107Val mutations as the most prevalent ones in Hungary. There is limited information about these mutations in the literature. In 2013, ATTRIle107Val was recognized as a mutation causing a mixed phenotype: cardiac manifestations and polyneuropathy were also present [29]. On the other hand, ATTRHis88Arg was only described as a pathologic mutation in ATTR guidelines in 2019 [73]. Authors reported the ATTRIle107Val mutation in many countries: for example, in Japan [74, 75], France [76-78], Brazil [79], and Germany [80]. However, the case number is very low. In a recent review, only eleven patients with ATTRIle107Val were identified in Western Europe [81]. Available data show that this mutation is associated with a mixed phenotype: although the heart was predominantly involved, patients also had neurologic features. Our findings in the Hungarian patients with ATTRIle107Val partially correlate with this. All eight of them had mixed phenotypes; however, in five cases, polyneuropathy was the leading symptom, as it is shown in Table 6.

The first cases of ATTRHis88Arg were recently found in Sweden in recent years. All seven identified individuals were related and originated from the same region [82]. The leading symptom in their cases was HF, but neurologic involvement was also identified. With this mutation, we found ten patients in Hungary. The first symptom was HF in four of them, another four had polyneuropathy first, and the last two had both symptoms at onset. They all had some level of HF at the diagnosis, nevertheless. During follow-up, seven patients died. In five cases, a cardiac cause was identified. This implies that cardiac involvement is decisive in the prognosis, as presented in the introduction. This mutation is also infrequent worldwide. It was found in one Asian family [83]. But surprisingly, in a recent Austrian study, Auer-Grumbach et al. identified six families with ATTRHis88Arg, making it the most common variant in Austria [12]. The common

historical background of the countries may explain this co-incidence. The Austro-Hungarian Empire existed from 1867 to 1918, and its citizens mingled. As a rare mutation, ATTRHis88Arg was most likely introduced by a common ancestor to the Empire. This may be one topic for future investigation: trying to prove the theory of this founding effect. Another one may be a pooled analysis of these patients. This would further expand our knowledge about this mutation's clinical characteristics and expected penetrance, as it is already known in other ATTRv mutations [84, 85].

Our other major finding comes from the phenotype-genotype analysis in the case of the above-mentioned common Hungarian mutations. The absolute number of cases seems to be low in the case of the two mutations. However, these numbers are high if we put them into the context of other publications. One interesting finding is that ATTRIle107Val patients were almost ten years older at diagnosis, and they also had a more advanced stage of the disease: higher NYHA stage, higher PND score. This finding suggests that ATTRIle107Val is the more aggressive form of the two common Hungarian mutations. This information may have future therapeutic consequences.

The calculated prevalence of ATTRv in Hungary was 2.35 per 1 million, lower than the 5.2 cases per million in Europe [12], excluding the endemic regions. The clinical awareness of ATTRv improved significantly in the last decade, manifesting in the increased number of new cases shown in Figure 5. However, the disease may still be severely underdiagnosed in Hungary, which may be the reason for the discrepancy in prevalence. The number of new cases will probably increase as cardiologists and neurologists will more actively look for the "red flags" of amyloidosis [73, 86] now that the disease has targeted therapy.

We illustrated the geographical distribution of the Hungarian ATTRv patients in Figure 4. The inhomogeneous distribution does not necessarily reflect endemic regions. Instead, it highlights that the University hospitals of Szeged and Budapest specialize in amyloidosis patient care, and most patients were identified in these regions. To improve the awareness of ATTRv, I contributed to several other publications and held lectures at national congresses on the topic. These are listed in the bibliography.

We did not evaluate mortality, prognosis, disease progression, and therapy for many reasons. Most patients were diagnosed in recent years and had a short follow-up period. Also, the patient population was inhomogeneous as far as treatment was concerned. Ten received tafamidis, one taking 61 mg (approved for ATTR cardiomyopathy), the others 20 mg (approved for stage I ATTRv polyneuropathy) daily.

One patient was in a clinical trial receiving either vutrisiran or a placebo. Among symptomatic patients, thirteen (54%) died during the follow-up period, ranging from 1 to 195 months. Median survival was not calculated due to the short follow-up period of the surviving patients.

There are some limitations to our study. Because of its retrospective nature, in some instances, data were missing. For this reason, the ATTRvAsp18Gly patients identified by the literature search were only included in the epidemiologic analysis. Regarding the lower hungarian prevalence of ATTRv compared to non-endemic European regions, there may be undiagnosed patients present in Hungary whom we failed to identify despite the increasing awareness of this disease.

6. Conclusions

In conclusion, we found the prevalence of moderate to severe AS among consecutive, unselected CA patients to be 9%. This means that moderate to severe AS is relatively common among our CA population, where 80% of patients had AL amyloidosis. Our findings support the observation that the co-existence of ATTR CA and AS seems to be more common than the co-existence of AL CA and AS.

Based on our findings, we also concluded that dobutamine stress echocardiography has a valuable role in the diagnostic evaluation of patients with CA and AS, even though most of these patients have preserved LV EF, and the ESC guideline does not recommend this test with normal LV EF. CA patients typically have diastolic dysfunction with low stroke volume and gradients. AS is usually low-flow, low-gradient in this population and easily missed. Dobutamine stress echocardiography is a safe and helpful test to differentiate between true-severe and pseudo-severe AS in this scenario, so it may improve patient care.

Our major epidemiological finding of the Hungarian ATTRv patients is that ATTRHis88Arg and ATTRIle107Val are the most prevalent mutations in Hungary. This may have future therapeutic consequences. Both mutations are rare worldwide, although ATTRHis88Arg is also common in Austria, which can be the consequence of the common historical background of our countries. It also raises the question of a potential founder effect.

Regarding the genotype-phenotype correlation, we concluded that in ATTRIle107Val patients, polyneuropathy seems to be more dominant, although all patients had some degree of cardiac symptoms. At the same time, ATTRHis88Arg patients have an equally mixed phenotype. Our findings suggest that ATTRIle107Val is the more aggressive form of the two common Hungarian mutations. This may also have future therapeutic consequences.

7. Summary

Systemic amyloidosis is a classic internal medicine disease with many clinical forms and manifestations. The diversity of the disease warrants multidisciplinary evaluation most of the time. Its forms leading to cardiac manifestations like AL and ATTR amyloidosis are increasingly recognized in recent years, and the now available new therapeutic possibilities highlight the importance of early diagnosis of CA.

Our retrospective study aimed to investigate the co-existence of AS and CA. We assessed the prevalence, severity, and type of AS in consecutive patients with CA. We also evaluated the potential role of dobutamine stress echocardiography in the diagnostic process of these patients. Moreover, we retrospectively identified all available Hungarian patients with ATTRv CA in the other study. We investigated the prevalence, regional distribution, and genotypes of Hungarian patients with ATTRv and also studied their phenotype-genotype correlation.

We have observed that moderate to severe AS is relatively common (9%) in a CA population where 80% of patients had AL amyloidosis. Our findings also show that low-flow, low-gradient AS patients who have CA, even with preserved EF, may benefit from dobutamine stress echocardiography, as it helps to identify patients with true-severe AS who may benefit from invasive treatment. The major epidemiologic finding in our other study is that we identified ATTRHis88Arg and ATTRIle107Val mutations as the most prevalent ones in Hungarian ATTRv patients. ATTRHis88Arg patients seem to have a more balanced mixed phenotype with cardiac and neurologic manifestations. Patients with ATTRIle107Val are diagnosed later with more advanced disease, and in their case, polyneuropathy seems to be more dominant, but still, all of them had some degree of cardiac manifestation.

These findings highlight the importance of diagnosing CA in patients with AS and may also influence the diagnostic work-up in patients with low-flow, low-gradient AS and CA, incorporating dobutamine stress echocardiography. Our results also identify the most common Hungarian mutations in ATTRv patients and describe some phenotype-genotype correlations in them, which may have therapeutic consequences in the future.

Our work opened some new perspectives on cardiac amyloidosis.

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9. Bibliography of the candidate's publications

9.1. Publications related to the dissertation

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