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Primary and familiar cardiac amyloidosis:
from epidemiology to the clinical role of cardiac biomarkers

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Table of Contents

PART 2. ORIGINAL CONTRIBUTIONS

Non-standard abbreviations and acronyms

ACS: acute coronary syndrome
AH: amyloidosis related hospitalizations
AL: light chains amyloidosis
ATTR: transthyretin amyloidosis
ATTR-CM: transthyretin-related cardiomyopathy
ATTRv: hereditary transthyretin amyloidosis
ATTRwt: wild-type transthyretin amyloidosis
BNP: B-type natriuretic peptide
CA: cardiac amyloidosis
CAD: coronary artery disease
CCTA: coronary computed tomography angiography
CI: confidence interval
cTn: cardiac troponin
CTS: carpal tunnel syndrome
eGFR: estimated glomerular filtration rate
EMB: endomyocardial biopsy
HR: hazard ratio
Hs-cTn: high-sensitivity cardiac troponin
ICA: invasive coronary angiography
HF: heart failure
ICD-9: International Classification of Diseases
LVEF: left ventricular ejection fraction
NP: natriuretic peptides
NT-proBNP: N-terminal pro B-type natriuretic peptide
sFLC: serum free light chain
PYP: Technetium Tc 99m pyrophosphate scintigraphy

List of thesis original contributions

1) Hospitalization-based incidence of systemic and cardiac amyloidosis in the Veneto Region, Italy

2) Predictive value of high-sensitivity cardiac troponin T in patients undergoing technetium-99 m pyrophosphate scintigraphy for suspected transthyretin amyloid cardiomyopathy

3) High sensitivity cardiac troponin I in patients with transthyretin cardiac amyloidosis

4) Chest pain in cardiac amyloidosis: occurrence, causes and prognostic significance

Abstracts

Hospitalization-based incidence of systemic and cardiac amyloidosis in the Veneto Region, Italy

Background and aim. Defining the epidemiology of systemic and cardiac amyloidosis (CA) is a contemporary challenge. The present study aimed to estimate incidence and time trends in amyloidosis-related hospitalizations (AH) in Veneto Region (5 million inhabitants, Northeastern Italy).

Methods. International Classification of Diseases (ICD-9) codes were used to identify AH in Veneto Region from 2010 to 2020. Hospitalization for CA was defined as records with ICD-9 code for systemic amyloidosis and ICD-9 code for heart failure, cardiomyopathy or arrhythmia. Hospital/outpatient encounters for carpal tunnel syndrome (CTS) surgeries also were extracted. Incidence was estimated using a buffer of 5 years.

Results. In the time range 2015-2020, the incidence rate of AH was 23.5 cases per 106 (95% confidence interval, CI, 21.8; 25.3), mainly affecting patients>65 years (76.2%) and males (63.5%), with a progressively increasing trend (percent annual increase 17 %, 95% CI 12; 22%). The 10 year prevalence in 2020 was 124.5 per 106 (95% CI 114.9; 134.8). In 2020, annual hospitalized prevalent cases of CA were about 70% of all cases (159/228), mainly patients > 65 years and males. Among patients with multiple CTS surgeries, a subsequent code for cardiac disease was found in 913 after a median of 3.9 years, more frequently in men than in women (463/6.526 7.1% versus 450/11.406 3.9%).

Conclusions. In Veneto, we recorded a significantly increasing trend in the incidence of amyloidosis-related hospitalizations, with concordant increasing prevalence estimates. Tailored screening of selected patients with previous multiple CTS surgeries may be reasonable.

Predictive value of high-sensitivity cardiac troponin T in patients undergoing technetium-99 m pyrophosphate scintigraphy for suspected transthyretin amyloid cardiomyopathy

Background and aim. Technetium Tc 99m pyrophosphate scintigraphy (PYP) is used for the diagnosis of transthyretin amyloid cardiomyopathy (ATTR-CM). Cardiac biomarkers, particularly high sensitivity cardiac troponin (hs-cTn), might be helpful in identifying patients at low or high risk of ATTR-CM, discriminating the need for PYP investigation. The aim of this study was to investigate the predictive value of hs-cTnT (and N-terminal B-type natriuretic peptide, NT-proBNP) in patients undergoing PYP imaging for suspected ATTR-CM in a large US cohort.

Methods. Retrospective study of all patients with PYP imaging performed at the Mayo Clinic (Rochester, Minnesota) from 2013 to September 2022 and with at least one hs-cTnT available within 6 months from PYP imaging. Participants: Of 2291 unique patients with PYP imaging during the study period, 1442 met the inclusion criteria. Main outcomes and measures were the diagnostic performance of hs-cTnT and NT-proBNP for rule-out and rule-in ATTR-CM, in terms of negative and positive predictive value (NPV/PPV), sensitivity and specificity.

Results: A final diagnosis of ATTR-CM was made in 436/1442 (30%), 405 (93%) men, mean age 77 ($\pm$9) years. Patients with ATTR-CM had higher hs-cTnT [48 (32-67) versus 25 (13-48) ng/L, p<0.0001] and NT-proBNP [2145 (1016-3801) versus 1119 (326-2011) ng/L, p<0.0001] values. The AUC of hs-cTnT for the diagnosis of ATTR-CM was 0.69 (95% CI: 0.66, 0.72). A hs-cTnT<6 ng/L (n=50, 3.5%) showed 100% NPV (93, 100) and 100% sensitivity (99, 100) for ruling out ATTR-CM. At increasing thresholds, the number of patients potentially ruled-out increased but with some false negative results. For rule in ATTR-CM, specificity increased at progressively increasing hs-cTnT values (95% for hs-cTnT=153 ng/L), but the PPV remained low (23% for hs-cTnT=153 ng/L). NT-proBNP values were available for 1378 patients; a value < 60 ng/L was present in 53 patients (3.8%) with 100% sensitivity and NPV. Combination of hs-cTnT<12 ng/L and NT-proBNP <60 ng/L identified 41 patients (3.0%) without false negatives.

Conclusions. In patients undergoing PYP imaging for suspected ATTR-CM, very low hs-cTnT can be of help in ruling out the diagnosis. At increasing thresholds, false negative results are present, and this should be taken into consideration in clinical practice. Similar results were found for NT-proBNP and for the combination of the two biomarkers. On the other hand, high hs-cTnT (as well as NT-proBNP) has limited predictive value in ruling-in disease.

High sensitivity cardiac troponin I in patients with transthyretin cardiac amyloidosis

Background and aim. Cardiac troponin (cTn), marker of myocardial injury, and natriuretic peptides [NP, particularly N-terminal fragment (NT-proBNP)], markers of myocardial stretch and stress, are powerful prognostic markers in cardiac amyloidosis (CA), both light chain and transthyretin (ATTR). Validated risk stratification models are based on cTnT or high-sensitivity cTnT (hs-cTnT) values. Nowadays, multiple hs-cTnI assays are available for clinical use, with different analytical characteristics. The aims of this study were twofold: first, to assess the prognostic performance of hs-cTnI measured at baseline in patients with ATTR-CA; second, to derive assay-specific thresholds for risk stratification of these patients.

Methods. Retrospective observational multicenter study of patients diagnosed with ATTR amyloidosis. We investigated the prognostic significance of hs-cTnI measured at baseline in different cohorts with different assays, including the Abbott Architect Stat High Sensitive Troponin I assay, the Beckman Coulter Access High Sensitivity Troponin I assay and the Siemens Centaur XPT High-Sensitivity TnI assay. Outcome was all-cause mortality.

Results. A total of 434 patients are included in the study. Of these, 123 were evaluated with the Abbott assay, 107 with the Beckman assay and 204 with the Siemens assay. In the Abbott cohort, derived optimal threshold for risk stratification for hs-cTnI was 80 ng/L (sensitivity 87%, specificity 68%), consistent also when considering only ATTRwt-CA. Hs-cTnI remained a significant and independent determinant of mortality even after adjustment for age and NYHA class, for elevated NP and eGFR < 45 ml/min/m2 and for left ventricular ejection fraction (LVEF, %) and E/e'. At Cox regression analysis with time dependent covariates, hs-cTnI > 80 ng/L remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of hs-cTnI > 80 ng/L of 10.0 (95% CI 3.46-29.1), p<0.001]. In the Beckman cohort, the derived optimal threshold for risk stratification for hs-cTnI resulted to be 80 ng/L (sensitivity 77%, specificity 79%), consistent also when considering only ATTRwt-CA (sensitivity 75%, specificity 78%). Hs-cTnI remained a

significant and independent determinant of mortality also after adjustment for age, for eGFR < 45 ml/min/m2 and for elevated NP. Hs-cTnI > 80 ng/L remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of hs-cTnI > 80 ng/L of 13.4 (95% CI 4.35-41.4), p<0.001]. In the combined cohort Abbott plus Beckman, the optimal hs-cTnI threshold for risk stratification confirmed to be 80 ng/L, consistent when excluding 25 (11%) ATTRv patients. A 2-variables staging system using hs-cTnI > 80 ng/L and NP (NT-proBNP > 3000 ng/L or BNP > 250 ng/L) was tested [age-adjusted HR was 5.82 (95% CI: 1.24-27.36) for one above and 21.25 (95% CI: 5.03 to 89.84) for two above, consistent in the subset of ATTRwt-CA patients]. A 3-variables staging system was also tested, based on hs-cTnI (> 80 ng/L), NP (> 3000 ng/L for NT-proBNP or 250 ng/L for BNP) and eGFR, with a population-derived threshold of eGFR < 50 ml/min/m2. With time-dependent AUC curves, the 3-variables staging system performed similarly to the 2-variables staging system at 12 and 48 months, while it was superior at 24 months (p=0.05) and at 36 months. In the Siemens cohort, derived optimal threshold for risk stratification for hs-cTnI resulted to be 56 ng/L (sensitivity 77%, specificity 58%). Hs-cTnI remained a significant and independent determinant of mortality even after adjustment for age and LVEF [HR of hs-cTnI > 56 ng/L of 3.14 (95% CI 1.23-8.05), p=0.017] and for age and elevated NP [HR of hs-cTnI > 56 ng/L of 2.70 (95% CI 1.09-6.71), p=0.033].

Conclusions. In patients with ATTR-CA, particularly ATTRwt-CA, hs-cTnI measured with three different assays in different cohorts has a strong and independent prognostic role for mortality. A threshold of hs-cTnI of 80 ng/L (for Abbott and Beckman assay) and of 56 ng/L (for Siemens Centaur assay) appears to be useful for risk stratification of ATTRwt-CA patients. One staging system based on hs-cTnI and NP and one based on hs-cTnI, NP and eGFR were tested and demonstrated good performance for risk stratification of patients with ATTR-CA, particular ATTRwt-CA. These biomarkers-based systems maintained their prognostic significance also when adjusted for disease-modifying therapy. Therefore, our findings suggest that staging models for ATTRwt-CA based on cTn can be applied also in Institutions utilizing hs-cTnI measured with these three assays.

Chest pain in cardiac amyloidosis: occurrence, causes and prognostic significance

Background and aim. Chest pain is experienced by patients with cardiac amyloidosis (CA), but a systematic investigation of its frequency, underlying etiologies and clinical significance is lacking.

Methods. Clinical, echocardiographic, laboratory characteristics, available coronary arteries imaging and endomyocardial biopsy (EMB) findings of 174 patients with CA (n=104 with transthyretin, ATTR; n=70 with light chains, AL) were analyzed.

Results. Chest pain was reported in 66 (38%) CA patients. Compared to those without, patients with chest pain had more frequently a history of coronary artery disease (CAD) (27%vs15%, p=0.048) and heart failure (HF) symptoms (62%vs43%,p=0.015), higher high sensitivity troponin I (hs-cTnI,101vs65 ng/L,p=0.032) and higher brain natriuretic peptide (597vs407 ng/L,p=0.024). Among CA patients with chest pain undergoing coronary arteries imaging (n=37), obstructive CAD was detected in 14 (38%), 13 of whom with ATTR-CA. Of these 37 patients, EMB was available in 10 and vascular/perivascular amyloid deposition was detected in 4/5(80%) of AL-CA patients and 1/5 ATTR-CA. Among patients with suspected acute coronary syndrome (n=22), obstructive CAD was detected in 9/17 (53%) ATTR-CA and 0/5 AL-CA; hs-cTnI levels were similar between those with and without obstructive CAD. During a follow-up of 17 (8-34) months, chest pain was a significant predictor of HF hospitalization (HR1.86,95% CI 1.02-3.39,p=0.042), even after adjustment for CA subtype and CAD.

Conclusion. Chest pain is a common symptom in patients with CA, reflects a more advanced cardiac impairment and predicts future HF hospitalization. The etiology of chest pain seems to differ, with obstructive CAD more frequent in ATTR-CA whilst amyloid vascular/perivascular involvement more common in AL-CA.

PART 1. BACKGROUND

Chapter 1. Introduction to systemic amyloidosis

1.1 A brief overview

Systemic amyloidosis is a protein misfolding disorder caused by extracellular deposition of amyloid leading to organ dysfunction. Amyloid is composed of highly organized proteinaceous fibrils, insoluble and resistant to proteolysis degradation[1]. To date, more than 30 different proteins have been identified as amyloidogenic and at least 17 of them can cause systemic disease, in which the amyloidogenic protein is produced in one site (for example bone marrow or liver) and is deposited at distant site(s) (like heart and kidneys)[1]. In the recent years, systemic amyloidosis has been the subject of a medical revolution in terms of diagnostic and therapeutic advancements.

The pathogenic mechanisms of systemic amyloidosis can be broadly categorized as: (1) excess protein production favoring amyloidogenicity; (2) mutated protein with a higher tendency for misfolding than the native protein; and (3) intrinsic tendency of normal (wild-type) protein to form amyloid[1].

There are several forms of systemic amyloidosis that are clinically relevant and can lead to multiorgan involvement. However, the two forms of systemic amyloidosis that most frequently affect the heart are light chain (AL) and transthyretin (ATTR) amyloidosis, either hereditary (ATTRv) or wild type (ATTRwt). These three diseases constitute different entities with peculiar clinical course, prognosis and specific therapies and will be addressed in detail in separate chapters[2].

Another form that can rarely involve the heart is AA amyloidosis, also known as secondary amyloidosis; this form is triggered by long-standing inflammatory activation and the precursor amyloidogenic protein is serum amyloid A (SAA), an acute phase reactant[1].

1.2 General pathophysiological mechanisms

The pathogenetic process underlying amyloidosis is the conversion of globular, soluble proteins into insoluble amyloid fibrils that deposit in vital organs and damage their functions[3]. This complex

process can be favored by several factors, such as mutations that destabilize the native protein structure and expose hydrophobic and protease-sensitive regions (such as in ATTRv), increased protein concentrations, owing to either greater protein synthesis or reduced clearance (such as in AL), or the intrinsic propensity of certain proteins to form amyloid fibrils that becomes apparent with ageing (such as in ATTRwt)[3]. Several proteins are involved in intracellular and extracellular proteostasis and these mechanisms are usually able to contrast protein aggregation. However, when proteostasis mechanisms fail due to diverse reasons including aging, aggregation and amyloid formation can occur. As studied in vitro in AL amyloidosis, the formation of amyloid fibrils begins from a solution of the monomeric native protein, which might misfold and assume a partially folded or misfolded conformation[3]. When the amount of partially folded proteins reaches a specific concentration, a critical fibrillar nucleus forms, which catalyzes protein aggregation and fibril development (Figure 1). This critical concentration can vary based on the stability of the amyloidogenic protein[3]. Interestingly, after nucleation, the velocity of protein aggregation and fibril formation dramatically increase with lower concentration of misfolded proteins needed to elongate the amyloid fibers. Moreover, amyloid fibrils promote the misfolding of the precursor protein and further oligomer formation[3].
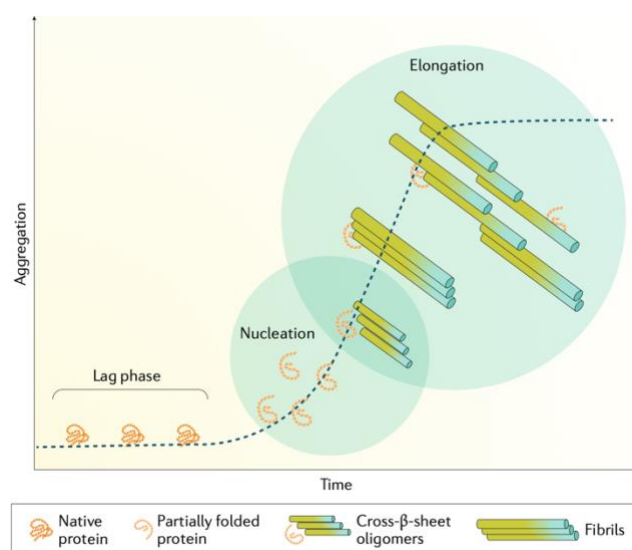
Figure 1. In vitro kinetics of amyloid fibrils formation. From Nat Rev Dis Primers. 2018 Oct 25;4(1):38[3].

Amyloid fibrils have a highly ordered cross-β-fibre structures and are characterized by antiparallel β-strands that are arranged perpendicular to the fibre, as demonstrated by X-ray diffraction[3]. Amyloid fibrils have a distinct diameter of 7.5–10.0 nm as determined using electron microscopy.

The diagnosis of systemic amyloidosis requires a clinical presentation suggestive of an amyloidosis syndrome with tissue confirmation of Congo red-positive extracellular deposits showing characteristics apple green birefringence under polarized light microscopy[1]. However, histology is similar in different forms of systemic amyloidosis and identification of the precursor protein is necessary to define the diagnosis. Amyloid typing can be performed with mass spectrometry, which, however, is an expensive method not widely available. Immunohistochemistry and immunoelectron microscopy, based on antigen-directed antibodies, can be alternative typing methods if performed in experienced laboratories[4]. In some cases, a further analysis with mass spectrometry might be still necessary to achieve a final diagnosis in doubtful cases.

1.3 Nomenclature

The structural proteins in or derived from amyloid fibrils are named after their precursors in abbreviated form preceded by the letter A for amyloid[5]. For example, immunoglobulin light chain amyloid protein is named AL and transthyretin amyloid protein ATTR. The protein can be specified further, e.g.ATTRv for variant, ATTRV30M for a specific mutation or ATTRwt for the wild-type form. For mutations, the Nomenclature Committee in 2022 recommended using the numbering based on the sequence of the mature protein, i.e. with-out leader sequence or propeptides[5]. To refer to specific diseases, the protein name followed by 'amyloidosis' should be used, for example ATTR amyloidosis or AL amyloidosis. Clinically useful specifications may be allowed, such as ATTR cardiomyopathy or AL neuropathy[5].

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Chapter 2. Systemic immunoglobulin light chain amyloidosis

This chapter is adapted from the manuscript: De Michieli L, Sinigiani G, De Gaspari M, Branca A, Rizzo S, Basso C, Trentin L, Iliceto S, Perazzolo Marra M, Cipriani A, Berno T. Light-chain cardiac amyloidosis for the non-expert: pearls and pitfalls. Intern Emerg Med. 2023 Jun 20.

2.1. Introduction

As mentioned earlier, systemic amyloidosis is an uncommon and progressive disease that, in the recent years, has been the subject of a medical revolution in terms of diagnostic and therapeutic advancements. This has been mainly related to the greater availability of non-invasive diagnostic strategies[1, 2] and novel effective therapies for the two most common forms that can affect the heart, such as the immunoglobulin light chains amyloidosis (AL) and transthyretin amyloidosis (ATTR)[3,4,5].

ATTR (both variant, ATTRv, and wild type, ATTRwt) and AL amyloidosis constitute substantially different conditions. ATTR is caused by misfolding of transthyretin, either due to destabilizing genetic variants in ATTRv or to complex and not completely elucidated mechanisms, including ageing, in ATTRwt[6]. The diagnosis of ATTR cardiac amyloidosis (CA) can be based either on histological demonstration of ATTR fibrils deposition or on a non-invasive approach in selected cases when AL amyloidosis has been excluded.

In AL amyloidosis, a plasma cell clone, or rarely a lymphoplasmacytic or marginal zone lymphoma, produces abnormal and toxic light chains that aggregate to form insoluble fibrils, with deposition in tissues and organ dysfunction[7,8]. All organs, except the brain, can be involved, with heart and kidneys most frequently affected; this multiorgan involvement accounts for the variable clinical presentation, frequently with nonspecific signs and symptoms[9]. For the diagnosis, monoclonal protein assessment should be performed including serum free light chains (FLCs) measurement and serum and urine protein electrophoresis with immunofixation[10]. Both lambda and kappa light chains can be involved and the difference between involved and uninvolved free light chain (dFLC) has prognostic significance[11].

Histological demonstration of amyloid deposits, with identification of AL type with mass spectrometry, immunohistochemistry or immunoelectron microscopy, is required to achieve a final

diagnosis of AL amyloidosis[1]. Cardiac involvement, the severity of which is defined by cardiac troponin (cTn) and natriuretic peptides values[12], is a major determinant of prognosis with median survival <1 year in AL patients with advanced cardiac disease[12,13]. The diagnosis of AL cardiac amyloidosis (AL-CA) can be determined based on laboratory, echocardiographic and/or cardiac magnetic resonance (CMR) criteria together with cardiac or extracardiac histological demonstration of AL amyloid deposits[1,3].

AL amyloidosis can develop in patients with multiple myeloma in 10%–15% of cases or in patients with monoclonal gammopathy of undetermined significance (MGUS) in 9% of cases[13]. It is therefore recommended to screen for pre-symptomatic amyloid organ involvement in these patients with measurements of brain natriuretic peptide (BNP) or N-terminal pro-brain natriuretic peptide (NT-proBNP), albuminuria and alkaline phosphatase[3]. In the suspicion of initial cardiac involvement, CMR is a useful tool for early diagnosis[14].

Early recognition of AL amyloidosis remains a critical issue. As many as one third of patients with amyloidosis may visit five or more physicians before diagnosis[15], and treatment efficacy is strictly related to patients' stage at diagnosis[3]. Failure of a timely recognition of AL amyloidosis is catastrophic for patients' outcome. Through the description of three clinical cases, some crucial diagnostic errors and pitfalls that can contribute to delay in diagnosis and reduced survival are pointed out.

2.2. Case Reports

1) A negative bone scintigraphy does not exclude cardiac amyloidosis.

Sixty-nine years old woman in good health till hospital admission for pulmonary edema and heart failure during a hypertension emergency. Left ventricular hypertrophy was noted at echocardiography, together with reduced global longitudinal strain with relative apical sparing[16], and CMR findings were suggestive for CA. She was discharged and underwent bone scintigraphy one month later, which was negative for cardiac uptake. No further diagnostic testing for amyloidosis was (erroneously) performed. A few months later, she was admitted for acute stroke; at blood tests there was evidence of IgG lambda monoclonal component, serum FLC lambda were elevated (408 mg/L, dFLC 386 mg/L) with lambda Bence Jones proteinuria. After bone marrow biopsy, a diagnosis of multiple myeloma was achieved. Moreover, she underwent fat pad biopsy that resulted positive for Congo red staining; subsequent typing with immunoelectron microscopy demonstrated lambda-type AL amyloid. Cardiac biomarkers were severely elevated (high sensitivity cTn T, hs-cTnT, 115 ng/L, NT-proBNP 12540 ng/L, revised Mayo Clinic stage IV [11]). Specific treatment was started, but the patient died after 4 months for refractory heart failure.

2) Fat pad biopsy does not have a 100% sensitivity for systemic amyloidosis, and life-saving therapies cannot be precluded just based on this finding.

Man of 49 years in good health till he developed lower limbs paresthesia and weight loss. He visited various physicians without a definitive diagnosis for 10 months. He was subsequently admitted for suspected acute coronary syndrome, which was ruled out with a coronary angiography. Echocardiography showed left ventricular hypertrophy and CMR revealed typical CA findings, such as abnormal gadolinium kinetics, myocardial late gadolinium enhancement "zebra" pattern and increased extracellular volume[17]. Fat pad biopsy was performed, and Congo Red staining was negative for amyloid deposits. So, amyloidosis diagnosis was (erroneously) excluded, and the patient was discharged. Three months later, he was admitted for heart failure. Serum FLC lambda were

elevated (sFLC lambda 780 mg/L, dFLC 764, k/λ ratio 0.02), so were the cardiac biomarkers (hs-cTnI 1336 ng/L, NT-proBNP 35000 ng/L). With this finding, fat pat biopsy was repeated, and it resulted positive for Congo red staining (subsequently typed as lambda-type AL amyloid); bone marrow biopsy was also performed with documentation of 9% plasma cells. Based on cardiac imaging, clinical presentation, and overall laboratory testing (with 24-hour urine protein >0.5 g/day), a diagnosis of AL amyloidosis with cardiac, renal, and neurological involvement was achieved and specific therapy was initiated, though ineffective in preventing disease progression and death of the patient a few weeks later.

3) Congo red staining is not enough to start aggressive and potentially hazardous chemotherapy. Seventy-one years old man, with previous history of bilateral carpal tunnel syndrome, biceps tendon rupture, IgA kappa MGUS and left ventricular hypertrophy on echocardiography for three years. He was hospitalized for heart failure; a CMR was suggestive for infiltrative cardiomyopathy and a subsequent bone scintigraphy showed mild cardiac uptake (Perugini grade 1[18]). Following laboratory investigations, fat pad biopsy was performed, and amyloid was detected on Congo red staining. Bone marrow aspirate showed 11% of plasma cells infiltration and AL-CA was (erroneously) diagnosed; the patient was sent to the closest Cardiac Amyloidosis center for specific chemotherapy. However, fat pad biopsy specimen was re-analyzed with immunogold electron microscopy and transthyretin was identified as the amyloid fibrils precursor (Figure 1). Specific treatment with transthyretin stabilizer was initiated accordingly.

SUSPECTED CARDIAC AMYLOIDOSIS: A FOCUS ON LIGHT-CHAIN AMYLOIDOSIS (AL)

AL amyloidosis signs & symptoms may be nonspecific	Always rule out AL amyloidosis	Be aware of tissue biopsy pitfalls
• New onset HF/LVH + • Malaise • Weight loss • Proteinuria • Skin bruising • Macroglossia • Peripheral neuropathy • Autonomic dysfunction • GI disturbances • GU disturbances	Serum and urine immunofixation & S-FLC kappa S-FLC lambda FLC kappa/lambda + Bone scintigraphy → Diagnostic algorithm	• **Sensitivity of Congo red staining for AL** For fat aspirate: 75% For bone marrow biopsy : 69% • **Typing of amyloid deposits is mandatory** AL amyloidosis ATTR amyloidosis

A TIMELY DIAGNOSIS IS KEY

Figure 1. Suspected cardiac amyloidosis: a focus on light-chain amyloidosis (AL). Abbreviations: AL, light chain amyloidosis, HF, heart failure, LVH left ventricular hypertrophy, GI, gastrointestinal, GU, genitourinary, s-FLC, serum free light chain, ATTR, transthyretin amyloidosis. *: Congo red staining; #: immunogold electron microscopy. AL amyloidosis fat pad biopsy as in Figure 1. Adapted from Intern Emerg Med. 2023 Jun 20.

2.3 Discussion

Recent international Consensus Documents[1,5] and Guidelines[19,20] have underlined the possibility of non-invasive diagnostic algorithms for ATTR-CA in selected patients. However, every physician dealing with suspected CA should keep in mind that the time-sensitive matter in the diagnostic algorithm is the exclusion of AL amyloidosis, since diagnostic delay of this condition can result in treatment inefficacy and early patients' death. Both the European and the American Consensus Statements[1,5] underscore the need for laboratory hematological investigations together with 99^{m}technetium-labelled bone scintigraphy, and tissue biopsy if necessary, to achieve a definitive diagnosis. Furthermore, in the American Heart Association Scientific Statement[5] and in the more recent American Heart Failure Guidelines[19], the diagnostic algorithm for suspected CA depicts as the first step the search for a monoclonal component. Only after, or together with, the exclusion of a

21

monoclonal component (with sFLC measurement and serum and urine protein electrophoresis with immunofixation), bone scintigraphy or CMR can be performed and interpreted to achieve the final diagnosis. This is to further stress that, while both laboratory investigations and bone scintigraphy might be necessary for a definitive diagnosis, there should not be any delay or indecision in ruling out AL-CA. Through the presentation of three clinical cases, important errors in the diagnostic process of CA are described, to raise awareness of the diagnostic pitfalls that can be encountered when dealing with these patients. The three clinical cases were not particularly challenging from a diagnostic standpoint, but some crucial steps in the diagnostic algorithm were misinterpreted or not completely executed, leading to diagnostic delay or misdiagnosis.

As underlined in the first tragic clinical case report, patients with AL-CA most frequently show no or mild (grade 1) uptake at bone scintigraphy[21]. In the presence of echocardiographic and/or CMR findings suggestive of CA, a bone scintigraphy with no or mild (grade 1) cardiac uptake should be carefully interpreted together with exhaustive hematological investigations, and it should prompt clinicians to suspect AL-CA. More rarely, also patients with ATTR-CA can present with mild or no cardiac uptake at bone scintigraphy for different reasons, including early disease stage or certain transthyretin mutations like Phe64Leu and Val30Met [22,23]. Importantly, on the other hand, it should be kept in mind that up to 20% of patients with AL-CA can show grade 2 or 3 radiotracer uptake[2,24,25], underlying the need for thorough hematological investigations in every patient with suspected CA. In any case, the execution of bone scintigraphy should not delay the laboratory investigations necessary to rule out the presence of a monoclonal component.

Histological demonstration of AL-type amyloid deposits is necessary in any case of suspected AL amyloidosis[1]. Importantly, it should be reminded that the sensitivity of Congo Red staining for AL amyloidosis differs significantly based on specimen source, e.g., 69% sensitivity for bone marrow biopsy, 75% for fat pad aspiration, 100% for heart biopsy[26]. Thus, negative results of peripheral biopsy should not rule out AL diagnosis, especially if pre-test probability is high, as pointed out in the second case report.

Moreover, typing of amyloid deposits with mass spectrometry, immunohistochemistry, or immunoelectron microscopy remains essential[1,5] (Figure 1). In the third clinical case, while the patient was referred for suspected AL-CA, amyloid fibrils typing with adequate techniques revealed ATTR deposits and the patient is now being treated for this condition together with a rigorous hematological follow-up. In case of cardiac uptake at bone scintigraphy and at least one abnormal monoclonal protein test, histological confirmation with amyloid typing is recommended, usually with endomyocardial biopsy[1]. In this specific clinical case, after evidence of ATTR deposits at fat pad immunogold electron microscopy, we decided to proceed with close hematological and cardiological follow-up and to avoid for now an invasive procedure such as endomyocardial biopsy, also considering patient's informed preference. It should be remembered, however, that cases of two concomitant types of CA, although rare, have been reported with evidence at heart biopsy of both ATTR and AL as the main amyloidogenic proteins in the sample[27,28]. Therefore, endomyocardial biopsy should be considered and performed in selected cases[1] to achieve a definitive diagnosis.

Even though all the investigations mentioned herein are necessary for a correct diagnosis, the first essential step to diagnose AL-CA is disease suspicion. Several red flags can be helpful in suspecting the disease[1], keeping in mind that such a systemic and multiorgan disease requires a general and comprehensive approach[29] (Figure 2). Some red flags can be detected both in ATTR-CA and in AL-CA, whilst some clinical characteristics can be taken into consideration during the differential diagnosis amongst different amyloidosis forms[1,5]. Patients with AL amyloidosis are usually younger than ATTRwt patients, even though this is not always the case for ATTRv[30]. Both AL- and ATTR-CA patients can present with heart failure with preserved ejection fraction, right-side heart failure, atrial arrhythmias, and "cured" systemic hypertension. Patients with AL amyloidosis usually manifest also debilitating systemic symptoms for which they might be referred to various physicians; symptoms might include weight loss, malaise, periorbital purpura and macroglossia together with renal and gastrointestinal involvement, peripheral neuropathy and/or autonomic dysfunction. Regarding electrocardiography[31], low QRS voltages are a frequent feature of CA,

although more common in AL-CA[30,32], probably due to a higher myocardial cytotoxicity of light chains. At echocardiography, LVH is usually more evident in ATTR-CA patients, occurring in AL-CA patients in more advanced clinical stages[30].
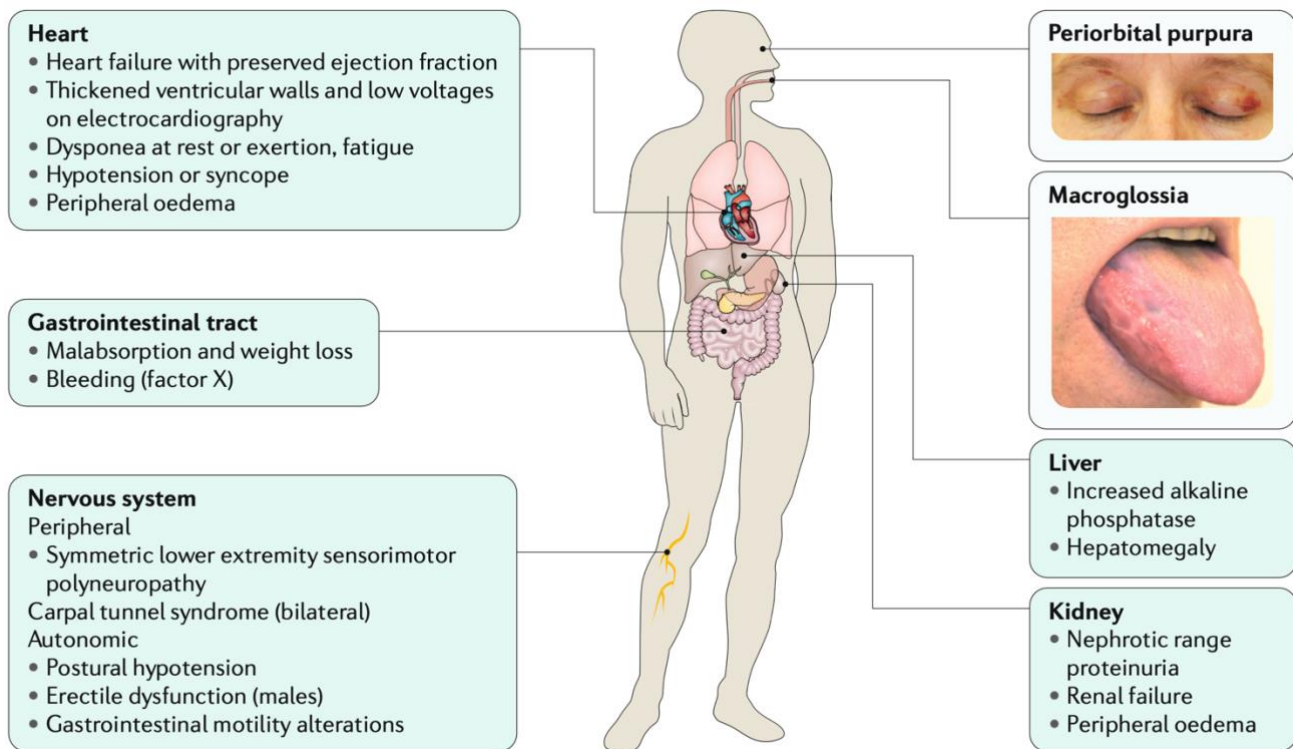
Figure 2. Organ involvement in systemic amyloidosis. From Nat Rev Dis Primers. 2018 Oct 25;4(1):38.[9]

Cardiac biomarkers can be useful in the diagnostic assessment of AL-CA. Besides its role in the screening of patients with MGUS and multiple myeloma, an NT-proBNP > 332 ng/L (in the absence of renal failure and atrial fibrillation) is indicative of cardiac involvement in established AL amyloidosis when mean LV wall thickness at echocardiography is > 12 mm[13]. Recent data suggest that a combination of very low hs-cTnT (< 14 ng/L) and NT-proBNP (<180 ng/L) can be useful in identifying patients at low risk for CA, whilst hs-cTnT> 86 ng/L could be relevant to spot those with high probability of the disease[33]. These aspects will be discussed further in the following chapters.

Prognostically, the severity of cardiac involvement[34], together with the depth and rapidity of hematological response to chemotherapy[35], are the main determinants of survival. Staging systems for AL-CA are available, based on cardiac biomarkers (cTn and natriuretic peptides) and sFLC values[11,36].

Treatment of AL amyloidosis aims to reduce the production of amyloidogenic light chains by suppressing the underlying plasma cell clone; treatment regimen should be risk-adapted and depends on the degree of organ involvement, the performance status, age, and bone marrow findings[37]. Based on their risk, patients can be candidate to autologous stem cell transplant as part of the upfront therapy (with only 20% of newly diagnosed patients eligible for this treatment), or combination chemotherapy without stem cell transplant. Several effective chemotherapy regimens are nowadays available, as reported in the most recent Guidelines[37] and whose complete description is beyond the scope of this thesis.

The role of the Cardiologists and the CA experts in the diagnostic and therapeutical process of AL-CA is certainly critical. However, patients with suspected CA might be evaluated first by other clinicians, with the need of a widespread awareness of amyloidosis red flags[1,5] and of the first, essential steps of the diagnostic algorithm that should be performed without delay and without uncertainties. Particularly, early diagnosis of AL amyloidosis among patients with the overmentioned systemic symptoms and/or with new onset heart failure/ unexplained left ventricular hypertrophy is a game changer in the management of these individuals. A systematic and holistic approach is crucial to correctly identify and address the multiorgan impairment typical of this disease. A prompt diagnosis when the disease is at an early stage and the patient is in good clinical conditions (low risk patients) can allow for a more aggressive and effective treatment, including autologous stem cell transplant[37]. A multidisciplinary counselling team including Hematologists and Pathologists will be then necessary for a correct interpretation of the investigations performed and to achieve the final diagnosis[10].

2.4. Conclusion

In conclusion, AL amyloidosis is a medical urgency that requires early diagnosis and specific treatment. Therefore, early suspicion of this condition and a correct diagnostic process is crucial for a quick referral to tertiary centers for guidelines-directed management and treatment. Some pitfalls in the diagnostic algorithm of this peculiar disease should be kept in mind and avoided to reduce diagnostic delay and improve patients' outcome.

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Chapter 3. Transthyretin amyloidosis

3.1 Introduction and pathophysiology

Transthyretin (TTR) amyloidosis, also called ATTR amyloidosis as per the official nomenclature, is an under-recognized, progressive disease characterized by deposition of misfolded TTR protein in various organs[1]. This condition results from age-related failure of homoeostatic mechanisms in wild-type ATTR (ATTRwt) amyloidosis (non-hereditary form) or destabilizing mutations in variant ATTR (ATTRv) amyloidosis (hereditary form)[2,3]. ATTRwt affects older individuals, frequently males[4], while manifestations of ATTRv are strictly related to the underlying mutation[5].

TTR, also known as prealbumin, is a 55-kDa protein normally composed of four monomers that circulates as a homo-tetramer and functions as a carrier protein for thyroxine and retinol binding protein.[1] TTR protein is mainly produced by the liver (95%) and secreted into the blood, with lesser amounts produced by the choroid plexus and retinal pigmented epithelial cells. The most widely explored pathophysiological mechanisms for TTR amyloid fibers formation involves dissociation of the TTR tetramer into dimers and monomers with subsequent misfolding and amyloid fibers formation. Transthyretin instability appears to be related to oxidative modifications, age-related failure of cellular homeostatic mechanisms, metal cations and genetic mutations; recently, a proteolytic pathway also potentially involved in amyloidogenesis has been reported[1]. The proteolytic cleavage of the native TTR protein would produce C-terminal fragments that can be amyloidogenic is certain conditions[6].

All these mechanisms (tetramer dissociation, oligomers formation and TTR proteolytic cleavage) lead to the formation of a nucleus with enough stability to grow by monomer addition, similarly to what described for AL amyloidosis. After this nucleation phase, the addition of preformed seeds can significantly shorten and complete this phase (seeding phenomenon). During the elongation phase, the addition of monomers to the nucleus results in formation of amyloid fibrils[1]. These fibrils can be composed of a mixture of both C terminal fragments and full-length TTR (type A fibrils) or by full-

length TTR only (type B fibrils)[1], and this will have important repercussion in the clinical application of nuclear imaging. Detailed description of the potential mechanisms of myocardial damage due to ATTR amyloidosis is reported in Chapter 5.
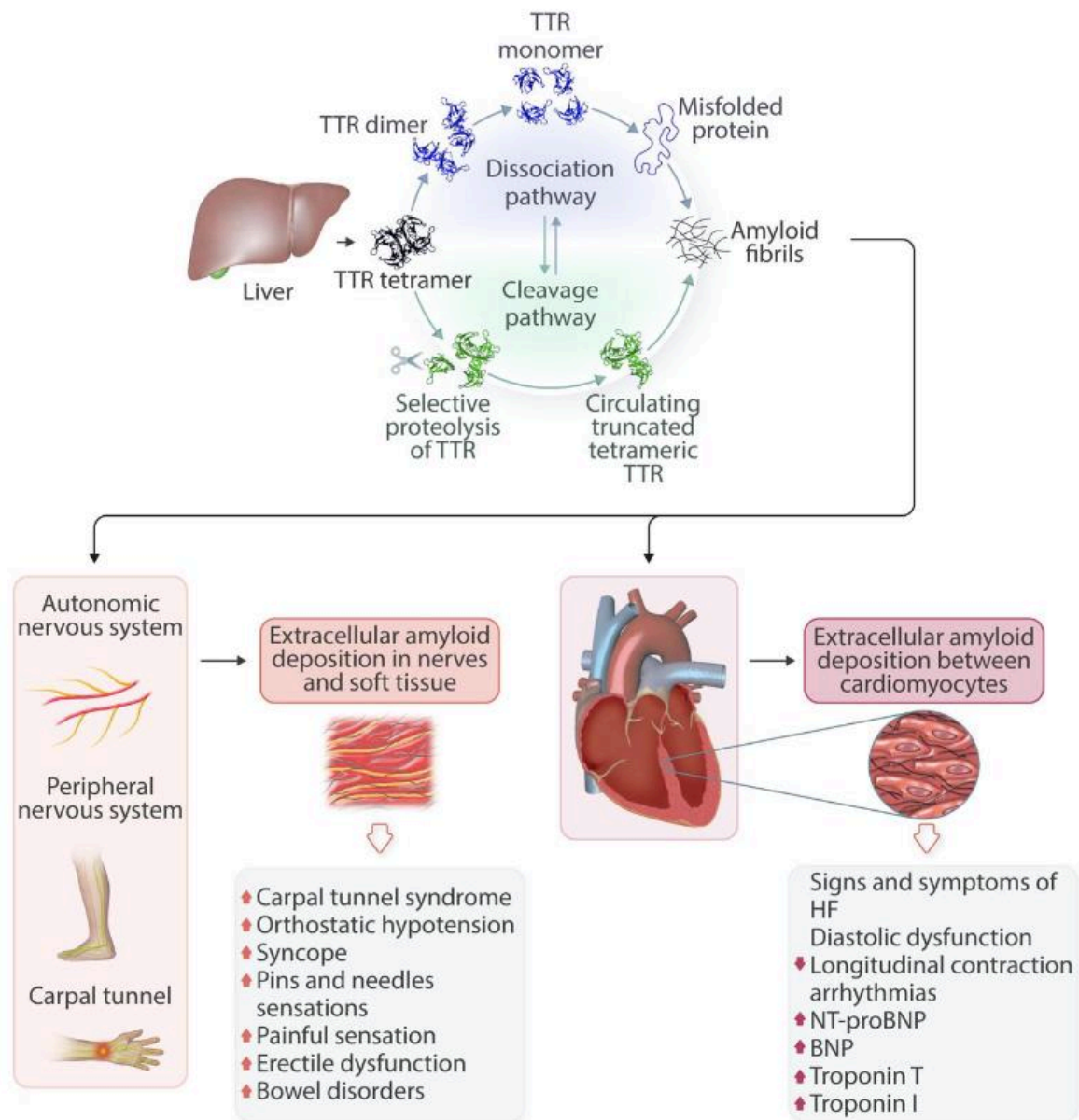
Figure 1. Pathophysiology of transthyretin amyloidosis and subsequent organ involvement. From Cardiovasc Res. 2023 Feb 3;118(18):3517-3535[1]. Abbreviations: NT-proBNP; N-terminal pro B-type natriuretic peptide; TTR, trahthyretin.

3.2. Clinical manifestations

3.2.1. Wild-type ATTR amyloidosis

ATTRwt mostly affects older individuals (75 years on average, usually > 60 years) and males[4,7], even though the diagnosis in women is not infrequent[8] and updated echocardiographic parameters (indexed by height) to define disease severity might be indicated in this setting[9]. Nevertheless, up to recent years, this condition was frequently mis- and under-diagnosed, also due to the need of invasive examinations to achieve a final diagnosis and to the lack of effective therapies.

ATTRwt affects the heart and soft tissues, leading to ATTR cardiomyopathy (ATTR-CM, or ATTR cardiac amyloidosis, ATTR-CA) and to osteo-articular manifestations such as carpal tunnel syndrome (CTS) and spinal stenosis (LSS)[4]. Atraumatic rupture of the brachial biceps tendon, finger disease and rotator cuff disease can also be present[10]. Although both peripheral and autonomic neuropathy can sometimes occur in ATTRwt, this is less severe than that in ATTRv[4].

From a cardiac standpoint, patients with ATTR-CA most commonly present with signs and symptoms of heart failure (HF) and increased left ventricular (LV) wall thickness with impaired diastolic filling and longitudinal systolic function[1]. Classically, ATTRwt-CA has been considered a restrictive cardiomyopathy with elevated left ventricular filling pressures and severely dilated atria; however, with the diagnostic improvements of the recent years, patients can be diagnosed earlier, and the structural and functional abnormalities can be less severe. Usually, patients present with HF with preserved ejection fraction (HFpEF), even though a non-negligible portion of patients has been identified also among patients with HF with reduced or mildy reduced ejection fraction[8].

Other manifestations include conduction abnormalities and bradyarrhythmias as well as supraventricular and, less frequently, ventricular tachyarrhythmias[11] (Figure 2). Atrial fibrillation (AF) is the most common tachyarrhythmia in patients with CA; specifically, it was reported in 9%, 11% and 38% of AL-, ATTRv- and ATTRwt-CA patients, respectively[11]. Less frequently, patients with CA can present with other types of SVAs including atrial flutter, atrial tachycardia, and atrioventricular nodal reentry tachycardias. The risk of intracardiac thrombus is increased in all

patients with CA and may occur even in sinus rhythm[12]. Unfortunately, stroke or systemic embolization is the presentation in some patients, usually because of unrecognized atrial fibrillation[4].
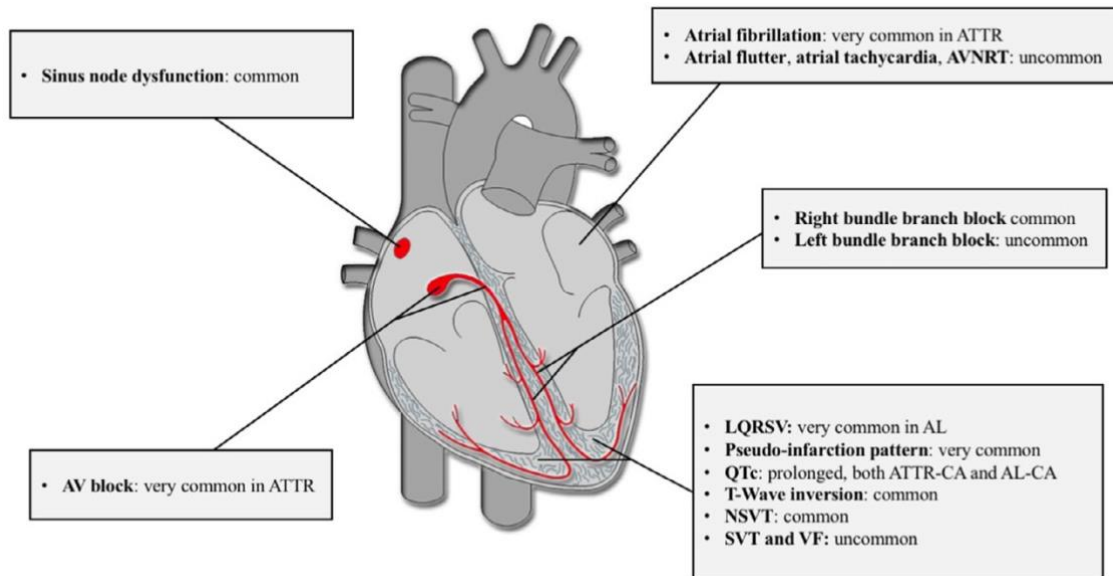
Figure 2. Electrocardiographic and rhythm abnormalities in cardiac amyloidosis. Abbreviations: ATTR-CA: transthyretin cardiac amyloidosis; AL-CA: immunoglobulin light chains cardiac amyloidosis; LQRSV: low QRS voltages; AV: atrio-ventricular; AVNRT: atrio-ventricular node reentry tachycardia; SVT: sustained ventricular tachycardia; VF: ventricular fibrillation; NSVT: non-sustained ventricular tachycardia. From Trends Cardiovasc Med. 2023 Feb 24;S1050-1738(23)00024-5.[11]

Bradyarrhythmias and conduction disturbances needing pacemaker implantation are common; in an Italian multicenter study, during a follow-up of 33 months, 11% patients with ATTRwt-CA required pacemaker implantation, particularly those with history of AF, long PR interval and wide QRS[13].

Though less common than SVAs, ventricular arrhythmias can be recorded in patients with CA, from non-sustained ventricular tachycardia (up to 74% of patients), to sustained ventricular tachycardia, electrical storm and ventricular fibrillation, possibly leading to sudden cardiac death[11]. Data on the benefit of implantable cardiac defibrillator for these patients are still controversial[14].

Low QRS voltages are present in around 35% of patients with ATTR-CA and are associated with a worse prognosis in terms of overall survival[15].

Regarding valvular heart disease, the association between aortic stenosis (AS) and ATTR-CA has been increasingly recognized; overall, around 8%[8] of patients with AS could be affected by ATTR-CA and the mechanisms linking the two conditions are a matter of debate[16]. Other valvular heart diseases are less frequent[16].

Regarding osteo-articular manifestations, CTS and CTS surgery are frequent in ATTR-CA, particularly ATTRwt-CA, and they might proceed the diagnosis of about 5-10 years[4]. A recent study demonstrated how screening for CA in patients with prior surgery for bilateral CTS finds approximately 5% with early- stage ATTR-CA and the clinical yield was higher (20%) in nonobese men ≥ 70 years[17]. LSS is associated principally with ATTRwt-CA[4]; the presence of amyloid deposition in the ligamentum flavum of older patients undergoing LSS surgery is frequent[18] but the clinical significance of this finding in terms of future development of cardiomyopathy is yet to be clarified.

3.2.2. Hereditary ATTR amyloidosis

While ATTRwt-CA is most frequently found in elderly men, ATTRv-CA presents at a younger age and has greater variability in gender predominance and clinical manifestations based on the specific TTR variant[1]. The prevalence of different genotypes is strictly related to the geographic area of interest, with valine to isoleucine at position 122 (Val122Ile) mutation more common in the United States, Caribbean and Africa (3-4% of Africo-Caribbean), threonine to alanine at position 60 (Thr60Ala) mutation in United Kingdom and Ireland and valine to methionine at position 30 (Val30Met) mutation in Portugal, Sweden and Japan[1]. In Italy, a variety of mutations have been described with Ile68Leu being the most frequent in those with an exclusively cardiac phenotype. Overall, among 186 Italian patients with ATTRv, phenotype was classified as exclusively cardiac (17%), exclusively neurologic (25%), and mixed cardiac/neurologic (58%) (Figure 3).
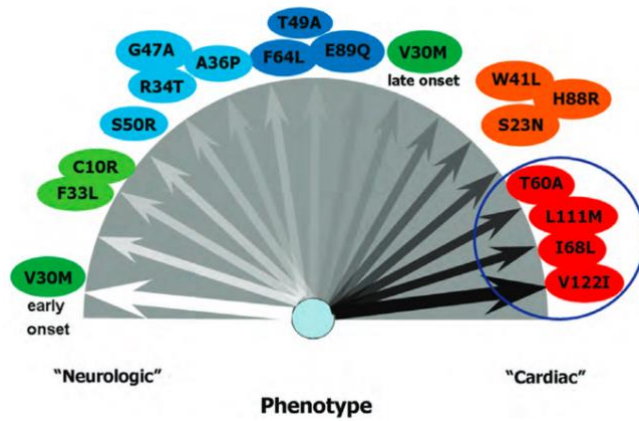
Figure 3. Possible spectrum of genotype –phenotype correlations in transthyretin-related amyloidosis. From European Heart Journal (2013) 34, 520–528[5].
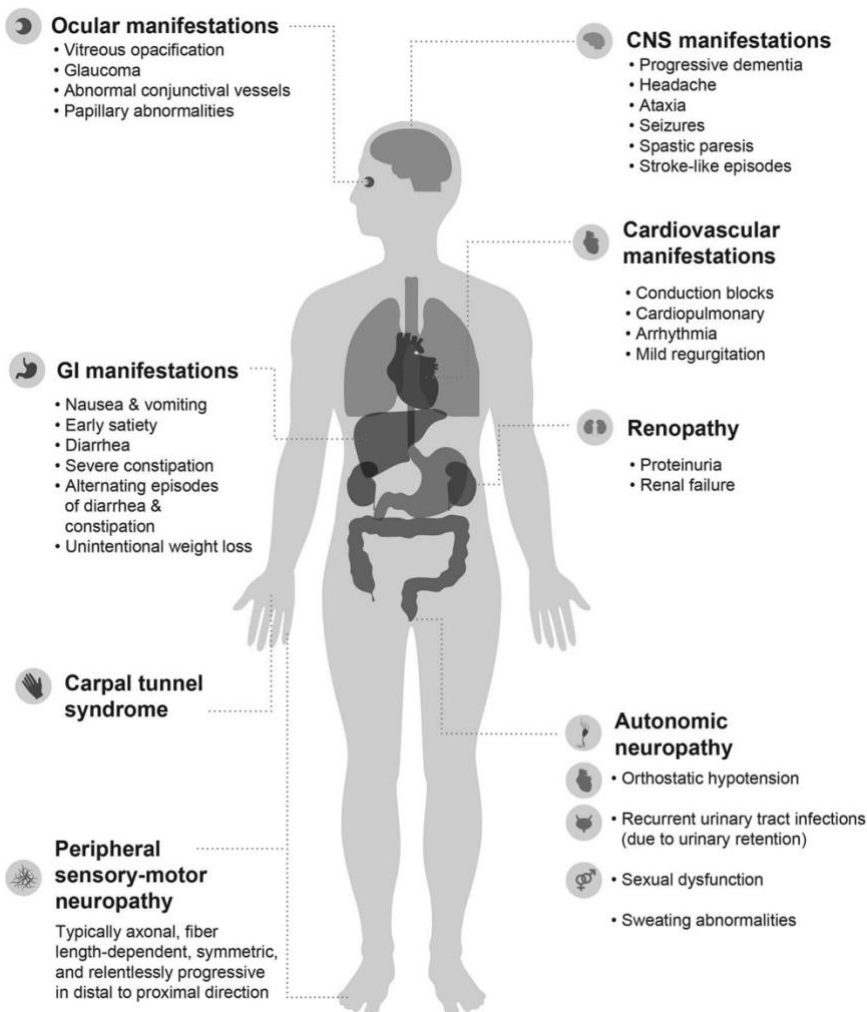
Figure 4. Clinical manifestation of ATTRv. From Expert Rev Clin Pharmacol. 2019;12(8):701-711[19].

Clinical manifestations of ATTRv are more diverse than ATTRwt and depend on the specific mutation. Besides cardiovascular manifestations, peripheral sensory-motor neuropathy is frequent, as well as autonomic neuropathy and gastrointestinal manifestations[19]. Detailed possible clinical manifestations of ATTRv are reported in Figure 4[19].

3.3. Diagnosis

Detailed description of all instrumental and imaging techniques available for the suspicion and diagnosis of ATTR-CA[1] is above the scope of this chapter.

After a clinical and imaging suspicion of CA, ATTR-CA can be diagnosed using both invasive and non-invasive diagnostic criteria. Invasive criteria include demonstration of amyloid fibrils within cardiac tissue or, alternatively, demonstration of amyloid deposits in an extracardiac biopsy accompanied either by characteristic features of cardiac amyloidosis on echocardiography or CMR.
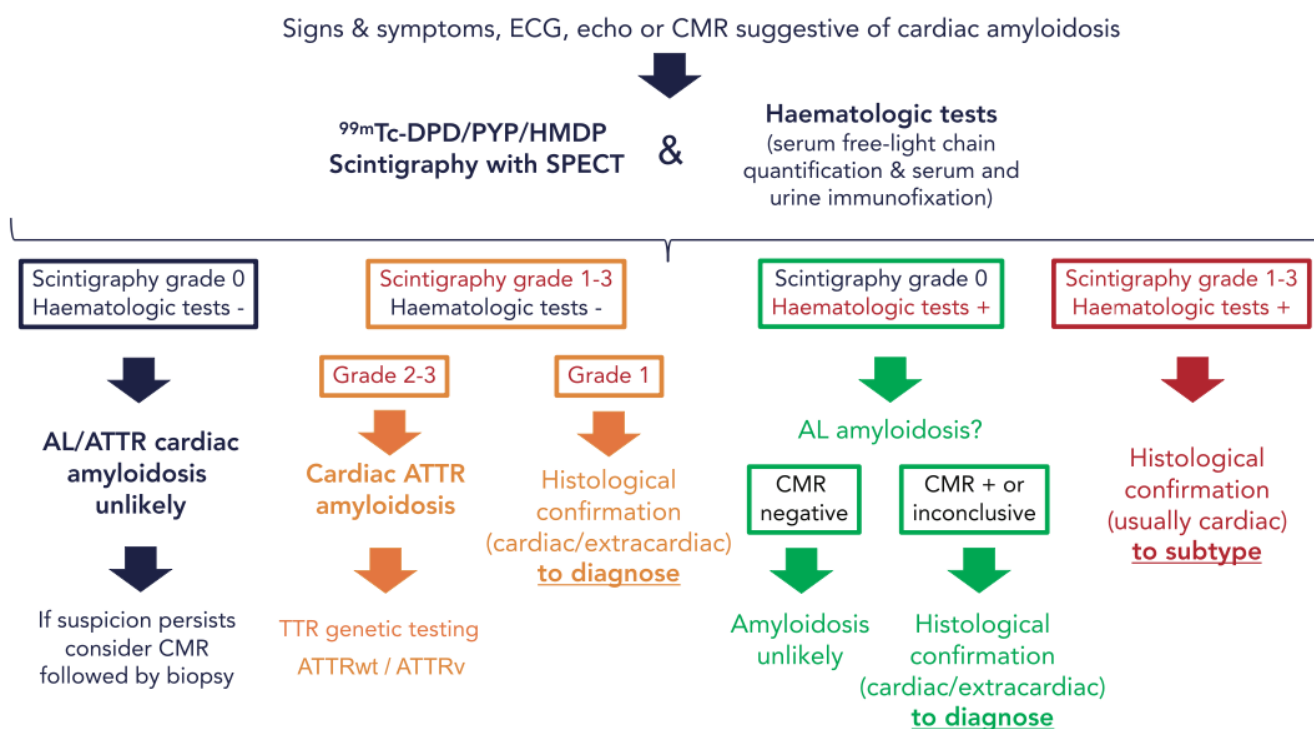
Figure 5. Diagnostic algorithm for cardiac amyloidosis. AL, light-chain amyloidosis;ATTR, transthyretin amyloidosis; ATTRv, hereditary transthyre- tin amyloidosis; ATTRwt, wild-type transthyretin amyloidosis; CMR, cardiac magnetic resonance; ECG, electrocardiogram; SPECT, single photon emission computed tomography; TTR, transthyretin. From European Heart Journal (2021) 42, 1554–1568 [20].

Non-invasive criteria can be applied after the exclusion of a plasma cell dyscrasia with serum and urine immunofixation and serum free light chain measurement (sFLC, see Chaper 2). If a monoclonal gammopathy is not present, planar and single-photon emission computed tomography (SPECT) scintigraphy with bone avid tracers such as 99mtechnetium-pyrophosphate (99mTc-PYP), 3,3-diphosphono-1,2- propanodicarboxylic acid (DPD) or hydroxymethylene diphosphonate (HMDP) can be used to achieve a diagnosis of ATTR-CA. In this case, a grade 2 or 3 myocardial radiotracer uptake according to Perugini score[21] is diagnostic for ATTR-CA (provided that AL has been excluded)[20,22].

False negative scans may occur in certain ATTRv genotypes[23]; false positives may be due to AL, recent myocardial infarction, long-term chloroquine use or blood-pool in the LV[24]. TTR genetic testing then is recommended in all ATTR patients regardless of age, also considered the high frequency of ATTRv also in older individuals[25]. In the presence of a monoclonal gammopathy, a final diagnosis of ATTR-CA can be achieved only with demonstration of amyloid infiltration and its typing on biopsy (usually cardiac) (Figure 5). A recent large multicentric collaboration guided by the National Amyloidosis Center (NAC)[26] reported that non-biopsy diagnostic criteria for ATTR-CA are highly specific [97% (95% CI 0.91-0.99)] and that the diagnostic performance can be further refined using new cut-offs for sFLC ratio in patients with chronic kidney disease (whose levels of sFLC, particularly kappa, are altered even in the absence of a monoclonal gammopathy[27]).

Detailed description of prognostic assessment of patients with ATTR-CA based on biomarkers is reported in Chapter 5.

3.4 Disease-modifying therapy

Modern therapeutic strategies aim at reducing the deposition of ATTR in the myocardium through stabilization of the circulating TTR tetramer or reduction in hepatic synthesis of TTR. Further therapeutic techniques such as gene editing[28] and removal of ATTR from the myocardium with antibody-mediated treatment are under investigation[29].

3.4.1. TTR stabilizers

Tafamidis is small molecule stabilizes the circulating TTR tetramer and prevents dissociation in monomers by binding the T4-binding sites[30]. Tafamidis is the first disease-modifying drug to be approved for the treatment of ATTRwt and ATTRv-CA, since, as of now, all other available medications are only licensed for the treatment of polyneuropathy caused by ATTR amyloidosis. In the phase 3 clinical trial, tafamidis was associated with lower all-cause mortality than placebo (78 of 264 [29.5%] vs. 76 of 177 [42.9%]; hazard ratio, 0.70; 95% confidence interval [CI], 0.51 to 0.96) and a lower rate of cardiovascular- related hospitalizations, with a relative risk ratio of 0.68 (0.48 per year vs. 0.70 per year; 95% CI, 0.56 to 0.81). At month 30, tafamidis was also associated with a lower rate of decline in distance for the 6-minute walk test and a lower rate of decline in KCCQ-OS score[31]. The mortality benefit was evident only after 18–20 months of therapy and mostly in patients in NYHA I-II classes, which underscore the need of adequate patients' selection to initiate treatment.

AG10, or acoramidis, is a potent, highly selective TTR stabilizer that was designed to mimic the structural influence of the protective super-stabilizing T119M mutation. Acoramidis has the capacity to form hydrogen bonds with the same serine residues at position 117 that stabilize the T119M variant[32]. Recently, the phase 3 clinical trial was presented at the European Society of Cardiology meeting in Amsterdam (August 2023) and the reported results showed that there was a positive treatment effect across all components of the primary endpoint analysis, including a numerical reduction in all-cause mortality, with an absolute risk reduction (ARR) of 6.4%, relative risk reduction (RRR) of 25%, and hazard ratio (HR) of 0.772 (95% confidence interval [CI] 0.542 to 1.102; p=0.15). The cumulative frequency of cardiovascular-related hospitalizations was reduced by about half in the treatment arm. Change from baseline in NT-proBNP was lower in the acoramidis arm than in the placebo arm at month 30 (ratio of adjusted geometric mean fold-change 0.529; 95% CI 0.463 to 0.604; p<0.0001) and the decline in change from baseline in 6MWD was also reduced. Treatment with acoramidis was generally well-tolerated.

3.5.2. Inhibitors of TTR gene expression

Inotersen is an antisense oligonucleotide (ASO) inhibiting the hepatic production of TTR (both wild-type and variant TTR). In the phase 3 clinical trial NEURO-TTR trial, ATTRv patients treated with inotersen showed a reduction in the decline of neurological manifestations and improved quality of life. ATTRv-CA was present in 68% of the study population but no significant change in echocardiographic parameters was reported but the study was not powered for this endpoint. Patients on inotersen require regular check of kidney function and platelet count due to a 3% rate of glomerulonephritis and severe thrombocytopenia in the trial.

Patisiran is a small interfering RNA (siRNA) encapsulated in lipid nano- particles that blocks the expression of TTR (both wild-type and variant TTR) in the hepatocytes by disrupting the TTR mRNA. In the phase 3 APOLLO clinical trial[33] on patients with ATTRv polyneuropathy, patisiran administered intravenously at the dose of 0.3mg/kg once every 3 weeks for 18 months significantly improved neurological status. At exploratory analysis of patients with echocardiography, patients in the patisiran arm demonstrated reduced mean LV wall thickness, relative wall thickness and serum NT-proBNP levels. In a cardiac magnetic resonance study, reductions in extracellular volume by provided evidence for ATTR cardiac amyloid regression in a proportion of patients receiving patisiran[34]. Primary results from the Apollo-B trial, a phase 3 study of patisiran in patients with ATTR-CA, showed at 12 months benefits in functional capacity, health status and quality of life in patients treated with patisiran, with consistency across clinically important subgroups[35].

As of now, based on these premises, tafamidis can be prescribed for the treatment of ATTRwt-CA and ATTRv with both neuropathy and cardiomyopathy; inotersen can be administered in patients with ATTRv and polyneuropathy while patisiran in ATTRv patients with neuropathy with or without cardiomyopathy (Figure 6).
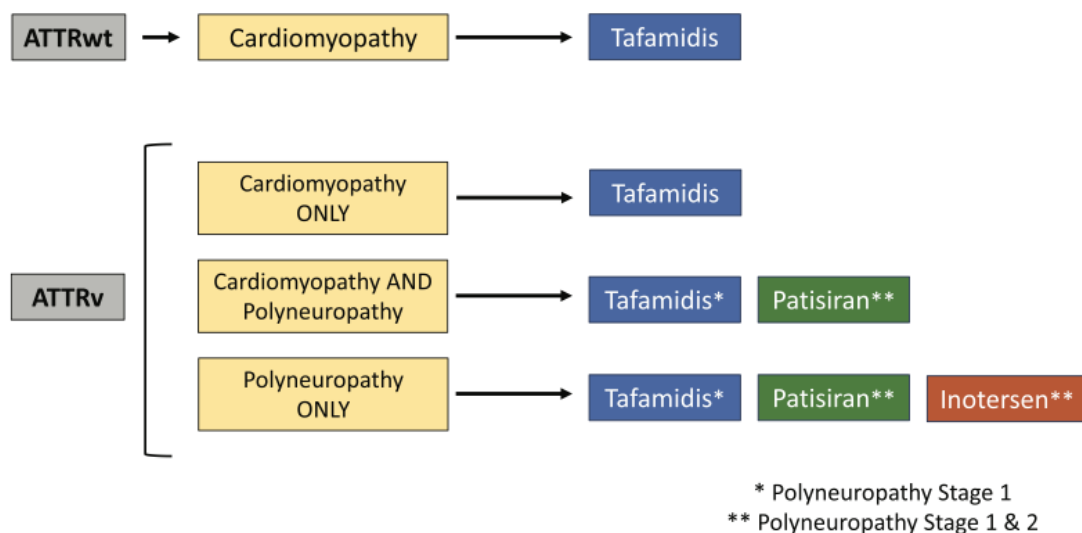
Figure 6. Proposed therapeutic alternatives in transthyretin amyloidosis patients. ATTRv, hereditary transthyretin amyloidosis; ATTRwt, wild-type transthyretin amyloidosis. From European Heart Journal (2021) 42, 1554–1568[20].

Second-generation drugs are conjugated to N-acetyl galactosamine and have a high affinity for the asialoglycoprotein receptor on hepatocytes, thus resulting in enhanced liver uptake compared to first-generation agents[1]. Two of these agents are vutrisiran[36] and eplontersen[37], that have showed promising results for ATTRv neuropathy and are now under investigation for ATTR-CA.

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Chapter 4. Epidemiology of systemic and cardiac amyloidosis

4.1 Introduction

To define the real prevalence and incidence of systemic and cardiac amyloidosis (CA) is a challenge of the present days. Up to some years ago, both light chain amyloidosis (AL) and transthyretin amyloidosis (ATTR) were considered as rare conditions. Moreover, before targeted treatment was available, the clinical implications of diagnostic delays of these conditions were limited. However, with the availability of a non-biopsy diagnostic algorithm for selected cases[1] and with the development of novel therapies for AL[2] and ATTR amyloidosis[3,4,5], greater physicians' awareness and increased diagnostic efforts have led to a rise in the number of patients diagnosed with this disease, particularly ATTRwt-CA. Moreover, advances in immunomodulating drugs, proteasome inhibitors, steroid therapy, and autologous stem cell transplantation have significantly improved survival in AL amyloidosis, especially in cases of early diagnosis[6]. Overall, timely diagnosis is paramount particularly for AL amyloidosis (which constitutes a medical emergency[7]) but also for ATTR. A comprehensive understanding of the epidemiology of these conditions is crucial to improve the management of affected patients.

4.2. Population-based studies

Autopsy data have found wild type ATTR (ATTRwt) amyloid deposits in the myocardium of over 20% of octogenarians with heart failure (HF)[8]. Several studies investigated the prevalence of CA in populations at risks, such as patients with HF with preserved ejection fraction (HFpEF), aortic stenosis (AS), carpal tunnel syndrome (CTS) and pacemaker (PM) implantation[9], but population-based studies are less extensive.

A study from Olmstead County performed in 1992, estimated 3200 new cases of AL amyloidosis per year in the United States[10]. A more recent study using commercial insurance data on AL amyloidosis patients found a prevalence rate of 40.5 per million in 2015 and an incidence rate of

14 cases per million in 2015[11]. Regarding ATTR, the THAOS (Transthyretin Amyloidosis Outcomes Survey) registry reported that in the United States ATTR was a disease of elderly men with a cardiac-predominant phenotype, with Val122Ile being the most common transthyretin mutation in those with ATTRv.

In 2019, Gilstrap et al reported the first contemporary estimate of the incidence and prevalence of CA among Medicare beneficiaries in the United States[12]. They included all Medicare beneficiaries aged ≥65 years enrolled in the Medicare Fee-for-Service program for at least 1 month between January 2000 and December 2014. Beneficiaries were counted in the cardiac amyloidosis prevalence cohort in each year that they had (1) ≥1 principal or secondary international classification of diseases (ICD)-9 code for systemic amyloidosis and (2) ≥1 principal or secondary ICD-9 code for HF or cardiomyopathy within 2 years after initial systemic amyloidosis claim. A beneficiary was counted in the incidence cohort only once during the year in which they first met criteria for the prevalence cohort. The prevalence cohort included 121122 CA patients, and the incidence cohort included 38254 newly diagnosed CA patients over the 12-year study period. The prevalence of CA increased from 18.0 (95% CI, 117.5–18.5) per 100 000 person-years to 55.2 (95% CI, 54.3–56.0) per 100 000 person-years (p<0.01) and the incidence of CA increased from 8 (95% CI, 7.5–8.2) patients to 16.6 (95% CI, 16.2–17.1) patients per 100 000 person-years (p<0.001). In their cohort, the highest prevalence was noted among black men (174 per 100000 person-years) and the highest incidence was also observed in this group (36 per 100 000 person-years). They concluded that the incidence and prevalence rates of CA among hospitalized patients are increasing, particularly in men, those ≥75 years old, and blacks. They argued that these trends might related to a combination of increased awareness and possibly a greater use non-invasive imaging in clinical practice.

Regarding Europe, Westin et al in 2021 aimed to describe the temporal trends in a Danish population of CA patients and to examine the changes in patient characteristics over the past 2 decades[6]. In 2015, Denmark counted for around 5.7 millions of inhabitants. All patients diagnosed with amyloidosis from 1998 to 2017 were examined for inclusion and CA was defined as any ICD-

10 diagnosis code for amyloidosis, combined with 1 possible cardiac manifestation of amyloidosis, whichever was registered first. They found that during each 5-year period of the study (Figure), the number of patients diagnosed with CA continuously increased; the incidence of CA among the entire Danish population ≥ 65 years of age increased from 0.88 to 3.56 per 100,000 person-years and from 1.21 to 5.19 per 100,000 person-years in men ≥ 65 years of age. Regarding patients with amyloidosis not meeting their criteria for cardiac involvement, the incidence of noncardiac amyloidosis rose from 1.61 to 2.36 per 100,000 person-years in those ≥ 65 years of age, from 1.52 to 2.93 in men ≥ 65 years of age and from 0.91 to 1.89 in women ≥ 65 years of age. Overall, they reported three trends in the temporal changes: first, CA is being increasingly diagnosed in Denmark; second, in patients with CA, the median diagnostic delay from overt heart disease to subsequent amyloidosis diagnosis was approximately 1 year; third, mortality decreased significantly over the study period. Even though over a time of 20 years the number of diagnosed CA patients per year quadrupled, the study reported how CA is still rare in Denmark. Nevertheless, the median age at the time of amyloidosis diagnosis (72 years in the time range 2013-2017) and the frequency of male patients (66% in the same time range) indicate that the diagnosis of ATTRwt is likely driving this increase.
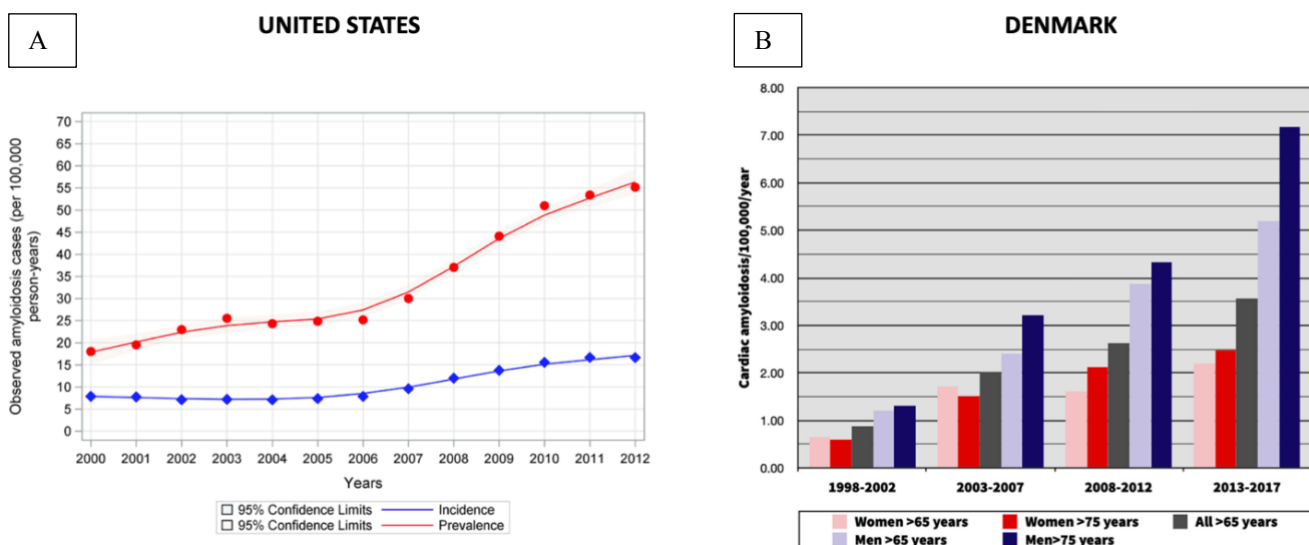
Figure 1. Incidence and prevalence trends of hospitalization for cardiac amyloidosis, 2000 to 2012, in the United States (Panel A) and changes in incidence of cardiac amyloidosis in Denmark (Panel B). Adapted from Circ Heart Fail. 2019;12:e005407[12] (Panel A) and J Am Coll Cardiol CardioOnc 2021;3:522–533[6] (Panel B).

Regarding data from Italy, in 2021 Zampieri et al.[13] examined the regional incidence of new AL diagnosis at Careggi University Hospital, in Italy. They reported an incidence of AL amyloidosis ranging from 5.3 to 13.7 cases per million person-years over a 16-years study period, with a mean value of approximately 9 cases per million person-years. Overall, they reported an epidemiological stability of the disease over time in the Tuscany region, despite the greater disease awareness towards amyloidosis and the improvement in effective therapies to treat AL amyloidosis; they concluded that AL appears to be truly rare, with stable rates over the years despite increasing awareness for amyloid disease in various disciplines. For ATTR amyloidosis, on the other hand, the situation is different. The same group reported that[14], among patients referred to their tertiary Center, ATTRwt showed an almost exponential increase in the number of new diagnoses in the last decade and that ATTR overall is the most common type of amyloidosis in their Center (Figure 2).

Distribution of amyloidosis diagnosis per year at Tuscan Amyloid Referral Centre 2000-2019

	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
wtATTR	0	0	0	0	0	0	0	0	0	1	0	5	11	17	16	21	35	41	61	66
vATTR	4	2	0	1	1	1	4	0	2	2	2	2	3	8	6	5	8	6	5	6
AL	8	8	11	12	7	12	17	17	7	14	18	12	17	12	14	15	20	16	27	17
AA	2	3	3	1	2	3	3	2	3	0	0	1	1	0	2	0	2	0	2	1
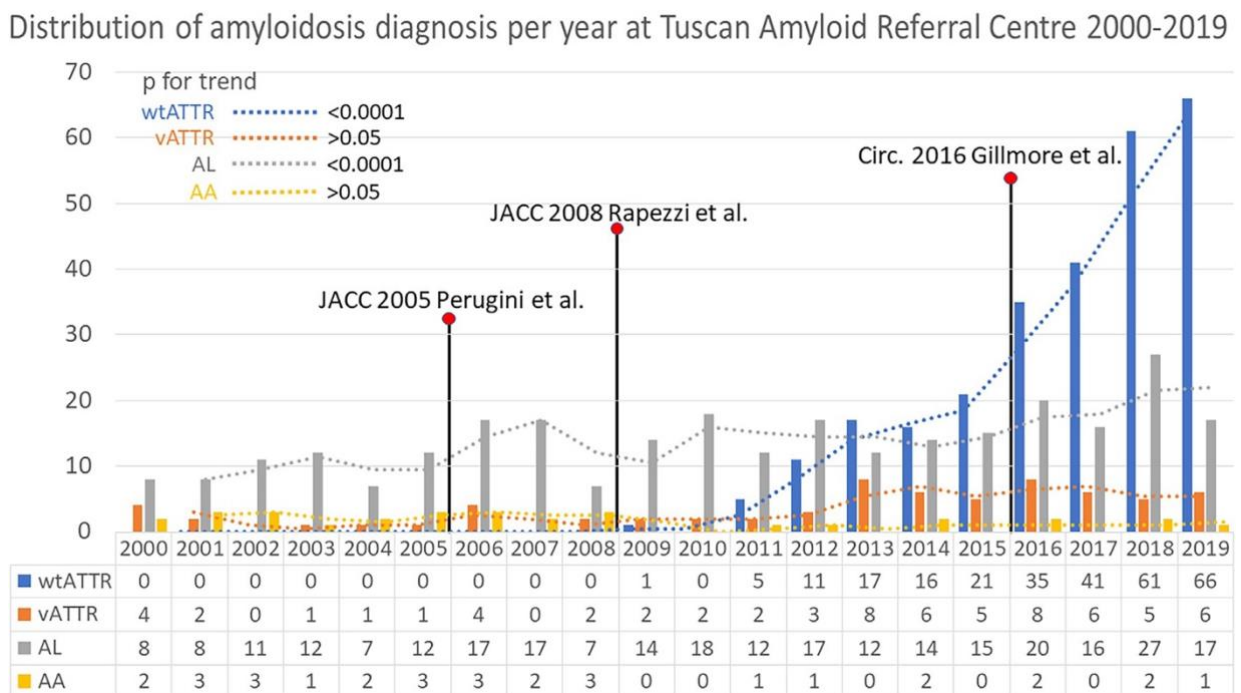
Figure 3. Diagnoses of Amyloidosis over a 20-year period at Careggi University Hospital, tertiary referral centre for amyloidosis. From Int J Cardiol. 2021;335:123-127[14]. Abbreviations: AL, light chain amyloidosis, ATTRwt, wild-type transthyretin amyloidosis. ATTRv, hereditary trnthyretin amyloidosis.

Recently, data from the population-based registry of Tuscany[15], which contains data on ATTR patients since 2006 also distinguishing ATTRwt from ATTRv, showed that on November 30th 2022, ATTRwt prevalence in this region is 90.3 per 1,000,000 persons and ATTRv prevalence is 9.5 per 1,000,000 persons, while the annual incidence ranges from 14.4 to 26.7 per 1,000,000 persons and from 0.8 to 2.7 per 1,000,000 persons, respectively. With this study, the Authors also confirmed the increasing trend in the reporting of ATTR in the community during the recent years.

4.3. Studies on patients at risk for cardiac amyloidosis

A different context is provided by the study of populations at risk to be affected by CA, such as patients with HFpEF, AS or CTS. A recent systematic review and metanalysis of screening studies, reported that searching for CA in specific settings can lead to the identification of a relatively high number of cases who may be eligible for treatment. In particular, ATTR-CA accounts for many cases of CA across the different settings, but also AL-CA was found to be not infrequent. In particular, 31 screening studies were ultimately selected and the prevalence values of CA in population at risks were: 1% among patients undergoing bone scintigraphy for non-cardiac reasons; from 2% to 33% in patients with HFpEF,; from 9% to 11% in patients with HF with reduced or mildly reduced ejection fraction; 2% among patients with conduction disturbances; from 3% to 10% in patients undergoing CTS surgery; from 5% to 9% in patients with hypertrophic cardiomyopathy phenotype; from 0% to 4% or from 8% to16% in patients with AS undergoing surgical or transcatheter valve replacement, respectively; from 4% to 29% in autopsy series of elderly individuals (Figure 4).

Based on these findings, it emerges how, in an era of important therapeutic advancement, appropriate screening in population at risks is crucial to identify patients that require specific, disease-modifying treatment.
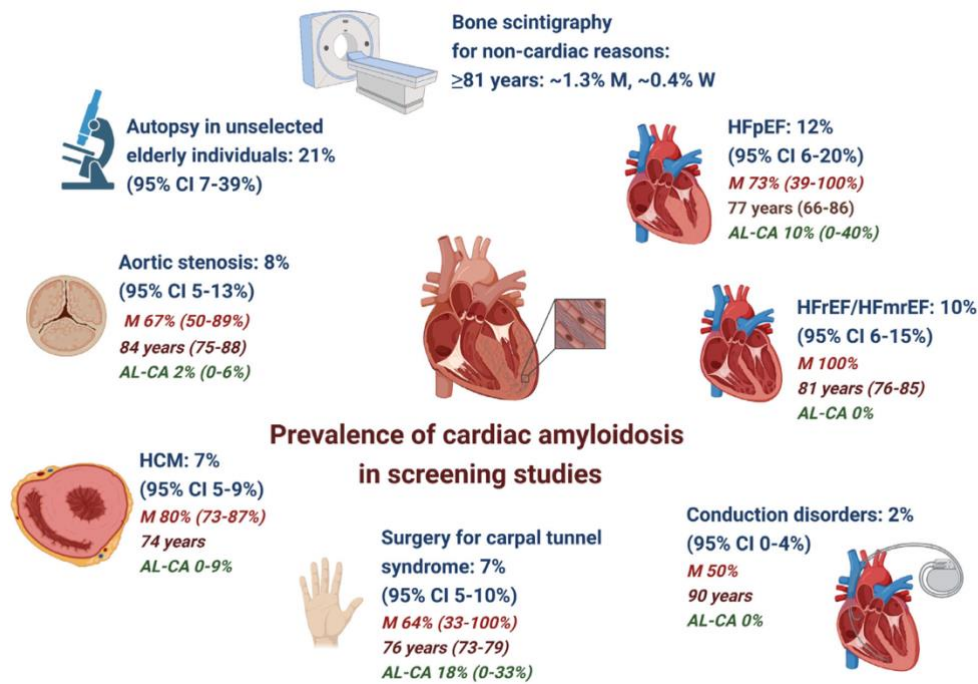
Figure 4. Prevalence of cardiac amyloidosis (CA) in different settings[9]. HCM, hypertrophic cardiomyopathy; HFmrEF, heart failure with mildly reduced ejection fraction; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; M, men; W, women. From Eur J Heart Fail. 2022;24(12):2342-2351[9].

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Chapter 5. Using Cardiac Troponin to Evaluate Patients with Light chain and/or Transthyretin Cardiac Amyloidosis

This Chapter is adapted from the manuscript: De Michieli L, Cipriani A, Iliceto S, Dispenzieri A and Jaffe AS. Using Cardiac Troponin to Evaluate Patients with Light chain and/or Transthyretin Cardiac Amyloidosis. Submitted to JACC: CardioOncology.

5.1. Introduction

Cardiac amyloidosis (CA) is an infiltrative disease due to the deposition of amyloid fibrils in the extracellular matrix, progressively leading to organ damage and dysfunction[1]. CA now is an increasingly recognized cause of heart failure (HF), particularly with preserved ejection fraction (HFpEF)[2]. The two main types of amyloidosis that can involve the heart, light chain (AL) and transthyretin (ATTR) amyloidosis, are pathophysiologically different. In AL amyloidosis, the amyloid precursor proteins are immunologic light chains produced by a plasmacellular clone, whereas in ATTR amyloidosis, either variant (ATTRv) or wild-type (ATTRwt)[3], the precursor protein is transthyretin, which is produced mostly by the liver to carry thyroxine and vitamin A (retinol). Although the disease management, course and prognosis of AL and ATTR-CA are different, at present effective treatment options exist for both [4,5]. The efficacy of these treatment options is dependent on disease stage at the time of diagnosis, mainly determined by the severity of cardiac involvement [3], which can be assessed using cardiac biomarkers and imaging techniques. Cardiac troponin (cTn) is the biomarker of choice for the detection of myocardial injury[6] and it is frequently found to be elevated in patients with CA, in particular when high-sensitivity assays (hs-cTn) are used. The extent of abnormalities in cTn is of crucial prognostic significance in these patients[3]. In this review, we will elucidate the significant aspects of cTn biology, measurement and interpretation when it is used adjunctively to assist in the management of patients with CA. The multiplicity of possible causes for myocardial injury in these patients will be addressed with particular focus on the relevant pathophysiological mechanisms and common clinical scenarios.

5.2 Cardiac troponin: the basics

Since the development of cardiac troponin (cTn) assays in the late 1980s and early 1990s, this marker has assumed a progressively more predominant role in cardiovascular care. It is currently the biomarker of choice for the detection of myocardial injury and for the diagnosis of myocardial infarction (MI) [6]. It is significantly more specific than prior markers and much more sensitive which allows the identification of many new processes that can damage cardiomyocytes.

The troponin complex consists of three different proteins, TnC, TnI and TnT, encoded by separate genes. They are regulatory proteins, essential for the proper excitation–contraction (actin-myosin interaction) mechanism in cardiomyocytes.[7] Due to their cardio-specific isoform expression, cTnI and cTnT can be utilized as highly specific markers of myocardial damage[8]. Recent data suggest that the re-expression of cTnT (gene *TNNT2*) in patients with active myopathies and myositis can contribute to cTnT elevations in some patients, including those induced by immune checkpoint inhibitor treatment[9,10]. At present, there is no evidence of upregulation/re-expression of cTnI in diseased skeletal muscle[10]. Most of the 3-unit cTn complex is covalently bound to the contractile apparatus. However, there is also a more loosely bound pool (an "early releasable pool"), which is thought to be released acutely after myocyte injury and is likely responsible for the initial signals after cardiomyocyte damage[11]. The persistence of increased values observed for 10-14 days after cardiomyocyte damage likely comes from degradation of the structural pool as troponin is cleared from the blood rapidly [11]. cTn elevations occur not only in acute ischemic disease with overt necrotic cardiac damage, but in many other situations, including for example strenuous exercise[12]. It is clear that some of the release can occur due to cardiomyocyte apoptosis but there is ongoing debate regarding the possibility of release in the absence of cell death, for example through blebs and exospheres [13]. Even if present, it is unclear how one would differentiate between cTn elaborated due to cardiomyocyte cell death and that released for other reasons. After a MI, cTnI circulates in the blood mainly as a binary complex with cTnC, but also as free cTnI and a ternary complex, cTnI-C-

T. In addition, various molecular modifications and protein fragmentations have been described[14]. cTnT mainly circulates in a free form; cTnT fragments have been also reported[15].

5.3 Cardiac troponin measurement and the definition of myocardial injury

Over time, assays for cTnT and cTnI have improved in terms of analytical performance. The original assays were insensitive but present-day hs-cTn assays provide increased sensitivity and greater precision at low concentrations, so that they are able to identify patients at higher risk of adverse events in multiple situations [16]. According to the International Federation of Clinical Chemistry Task Force on Clinical Applications of Cardiac Bio-Markers (IFCC TF-CB) position statement, for an assay to be defined as high sensitivity, 2 analytical criteria need to be met [17]: a) the 99th percentile upper reference limit (%URL) should be measured with an analytical imprecision of ≤10% (% CV); and b) the assay should be able to measure cTn above the limit of detection in ≥50% of both healthy male and female subjects. The 99th %URL value (roughly 3 standard deviations above the mean) as determined in a healthy population is universally endorsed as the reference cut-off to define myocardial injury [17]. The use of sex specific cutoffs is recommended [6,16]. The correlation between values measured with contemporary (non-high sensitivity) cTn assays and hs-cTn assays is good at higher levels, but poor at low concentrations, especially with hs-cTnT. [18]

While only one hs-cTnT assay is in use for clinical practice, many hs-cTnI assays have been developed; the same is true for older contemporary assays. Because of the different antibodies used in each assay and the unique mix of cTnI forms in each patient sample, there is no harmonization between the different hs-cTnI assays and absolute values cannot be directly compared [19]. For each assay, dedicated sex-specific 99th %URLs have been defined and should be applied in clinical practice[17].

Myocardial injury is defined as a cTn value above the sex specific 99th % URLs [6], regardless of cause, and it is frequently present in patients with primary cardiovascular disease as well as those whose primary problem can be non-cardiac conditions such as critical illness[20]. Its prognostic relevance has

been established in multiple clinical scenarios [6,20–24]. With the transition from conventional to hs-cTn assays, the frequency of the detection of myocardial injury has increased [25], leading to a more precise stratification of patients at higher risk of adverse events, but also escalating the challenge of identifying the reasons of cTn elevations in each patient. In some clinical situations, values below the 99th%URL have prognostic information despite the fact that they are within the normal range[26,27].

5.4 Myocardial injury in cardiac amyloidosis

Since the 2000s, it has been appreciated that there almost invariably are elevated cTn levels in patients with CA [28]. Such elevations are common and manifest important prognostic implications (see below). The exact frequency of myocardial injury is difficult to determine definitively and likely relates to differences in the populations studied, differences in the stages of the disease process and the fact that there are so many different hs-cTn assays available with unique cut-offs to define myocardial injury. Nevertheless, multiple pathophysiological mechanisms of myocardial injury in patients with CA have been proposed (Central Illustration). It is important for clinicians to recognize that the increases in hs-cTn values in any given patient may have a multiplicity of causes as hs-cTn assays seem to integrate the amount myocardial involvement.

5.4.1 *Direct cytotoxic effect of amyloid precursors*

Amyloid precursors can have direct toxic effects on cardiomyocytes. In AL amyloidosis, proteotoxicity of light chains (LC) has been extensively reported[29]. Interestingly, infusion of LC from patients with severe AL amyloidosis with cardiac involvement causes marked impairment of ventricular relaxation on isolated mouse heart models [30]. Moreover, human amyloid LC alter the cellular redox state in isolated cardiomyocytes [31]. This results in direct impairment of cardiomyocyte contractility and relaxation, independent of fibril deposition, associated with alterations in intracellular calcium handling [31]. Amyloidogenic LC have been reported to provoke oxidative stress, cellular dysfunction, and apoptosis in isolated adult cardiomyocytes through activation of p38

mitogen-activated protein kinase (MAPK)[32], which also mediates the transcription of brain natriuretic peptide (BNP) [29]. A study performed on human cardiac fibroblasts and C. elegans reported a correlation between the overall conformational properties of native folded proteins (including flexibility, kinetic instability and dynamic state) and the proteotoxicity of cardiotropic LC [33]. In vitro experiments suggest that, whereas the LC-derived amyloid fibrils exhibit an inhibition of the cell growth and division, soluble LC proteins allow cell growth but cause cellular dysfunction and apoptosis in cardiomyocytes, suggesting that the mechanisms of cytotoxicity differs between soluble proteins and amyloid fibrils [29,34]. Clinically, serum free LC (sFLC), particularly lambda, significantly correlate with increases in cTnI and N-terminal (NT)-pro hormone BNP (NT-proBNP) as well as echocardiographic parameters[35,36]. Hence, the important prognostic value of sFLC has been integrated into multiparametric staging systems [37,38].

In ATTR-CA, the literature is less extensive. However, markers of tissue damage characteristic of inflammation, apoptosis and the stimulation of reactive oxygen species have been found in tissues of human and transgenic mouse models carriers of mutant TTR variants, well before amyloid deposits are detected [39]. However, the direct toxicity of AL precursors is thought to be more marked than ATTR, possibly contributing to the different clinical profiles of the two conditions, such that AL amyloidosis has more rapid progression and worse prognosis if untreated than ATTR [40].

5.4.2 Interstitial amyloid fibrils infiltration

Amyloid infiltration in the heart results in disruption of tissue architecture and subsequent replacement fibrosis, with cardiomyocyte damage. The extracellular fibrils have a significant impact on the mechanics and physiology of the target tissue [41]. In AL-CA models, in-vitro analyses has shown that amyloid fibrils rapidly surround cultured cardiomyocytes and recruit soluble LC, triggering cytotoxicity[41]. Extracellular amyloid fibrils also appear to disrupt cardiac matrix homeostasis and alter extracellular matrix turnover which is critical for the maintenance of myocyte-myocyte force coupling and proper myocardial function[42]. An over-expression of matrix

metalloproteinases [43] also has been reported. Moreover, fibroblasts are able to internalize both aggregated transthyretin[44] and amyloidogenic LC[45]. These mechanisms contribute to the expansion of extracellular spaces and to the development of interstitial, reactive fibrosis in response to tissue damage which may contribute further to myocardial disruption and damage.

A study from Pucci et al.[46] showed that extracellular volume (ECV) evaluated at cardiac magnetic resonance (CMR) correlates with the combination of amyloid deposition and interstitial fibrosis at endomyocardial biopsy of the left ventricle (LV). This combination of amyloid and fibrosis also correlates with hs-cTnT, both in AL-CA (r=0.622) and in ATTR-CA (r=0.533), suggesting that infiltration, fibrosis and extracellular disruption together contribute to myocardial injury. In a cohort of ATTRwt-CA, hs-cTnT correlated with native T1 and ECV at CMR and just modestly, if at all with amyloid load (r=0.354) at endomyocardial biopsy of the right ventricle (RV)[47].

5.4.3 *Coronary microvascular dysfunction and amyloid/non-amyloid related coronary artery disease*

Coronary microvascular dysfunction (CMD) and amyloid/non-amyloid related coronary artery disease (CAD) also contributes to myocardial injury in patients with CA. Amyloid deposits can be found in the perivascular regions and in the media of intramyocardial coronary vessels[48], with vascular and perivascular involvement being more frequent in AL-CA[49]. CMD can be present, related to three possible mechanisms: structural (amyloid deposition in the vessels wall with thickening and stenosis), extravascular (extrinsic compression of the microvasculature), and functional (autonomic and endothelial dysfunction)[48,50]. Dorbala et al.[48] reported on 21 CA patients without obstructive epicardial CAD who underwent evaluation of coronary microvascular function with a rest and vasodilator stress N-13 ammonia positron emission tomography/computed tomography. Compared to 10 patients with LV hypertrophy, CA patients had lower resting myocardial blood flow (MBF), lower stress MBF, lower coronary flow reserve (CFR) and higher minimal coronary vascular resistance. Coronary microvascular dysfunction was associated with increased LV mass and

myocardial relaxation abnormalities. Similarly, with echocardiography, patients with CA had significantly lower CFR (together with lower rest and stress GLS and lower myocardial work efficiency) and changes in CFR and deformation capacity were strongly associated with exercise tolerance [51]. Also, in 20 patients with AL-CA, stress-induced wall motion abnormalities at echocardiography were frequent (55%) despite the absence of significant epicardial CAD[52]. Other CV and non-CV comorbidities (such as diabetes) can contribute to endothelial abnormalities and CMD, particularly in older and comorbid ATTRwt-CA patients. Therefore, CMD is frequent in CA and likely is an important cause of myocardial injury[53]. Amyloid vascular and perivascular infiltration [49,54] can also be cause of chest pain, acute myocardial injury and MI in the absence of atherosclerotic CAD[55,56,57]. In addition, classic atherosclerotic epicardial CAD also can be present (particularly in older ATTRwt-CA patients[2]) and may contribute to a lower ischemic thresholds. ACS management and the chest pain differential diagnosis in these patients can be challenging, due to the underlying chronic myocardial injury. Validated cTn-based algorithms for the rule-in/rule-out of MI of those presenting without ST-segment elevation[58] are difficult to be apply, not only in CA patients but in all who have baseline hs-cTn increases. In this situation, serial cTn measurements resulting in marked changes (>20%), combined with a careful history including prior hs-cTn values, a physical examination and ECG modifications, can be of help to better characterize the cause of myocardial injury [6].

5.4.4   Diastolic dysfunction and heart failure

Elevation of LV end diastolic pressure (LVEDP) can lead to apoptosis and cTnI release, as shown in animal models[59]. Also clinically, cTnT levels correlate with the LVEDP in patients with HF [53]. Due to the restrictive hemodynamics of CA, increased LV filling pressure with elevated myocardial wall tension (an obstacle to subendocardial myocardial perfusion) can play an important role in the genesis of myocardial injury. This can result in a vicious circle characterized by elevated LV filling pressures causing subendocardial ischemia, reduced coronary perfusion pressure and diastolic dysfunction,

which in turn can lead to more elevations in LVEDP[60]. Moreover, both LV and RV pseudohypertrophy can enhance these mechanisms. Group 2 pulmonary hypertension [61], frequently detected in patients with CA [62], can also contribute to cTn release through myocardial ischemia and cell death due to increased wall tension of the RV with pressure and/or volume overload [63].

5.4.5 Acute on chronic myocardial injury

Not only multiple mechanisms can contribute to the genesis and progression of myocardial injury in patients with CA, but these patients are also at risk for acute events related to CA or independent of CA as well. Some of these may be due to ischemia, although clear signs and symptoms of myocardial ischemia are often difficult to appreciate in this complex milieu. Acute exacerbations of HF or arrhythmias are frequently observed. Patients with CA indeed are particularly prone to developing atrial fibrillation/flutter and/or bradyarrhythmias[64,65]. Non-sustained ventricular arrhythmias are also frequent, particularly in those with a more advanced cardiac involvement[66]. Both HF exacerbations and tachy-/bradyarrhythmias are known causes of cTn elevations[6], particularly in individuals with an underlying myocardial disease[67]. Hypotension is also frequent in CA patients, secondary to the primary cardiac disease but also due to autonomic dysfunction and, similar to tachy- and bradyarrhythmias, prolonged hypotension is counted among the possible causes of myocardial injury related to oxygen supply/demand imbalance[6].
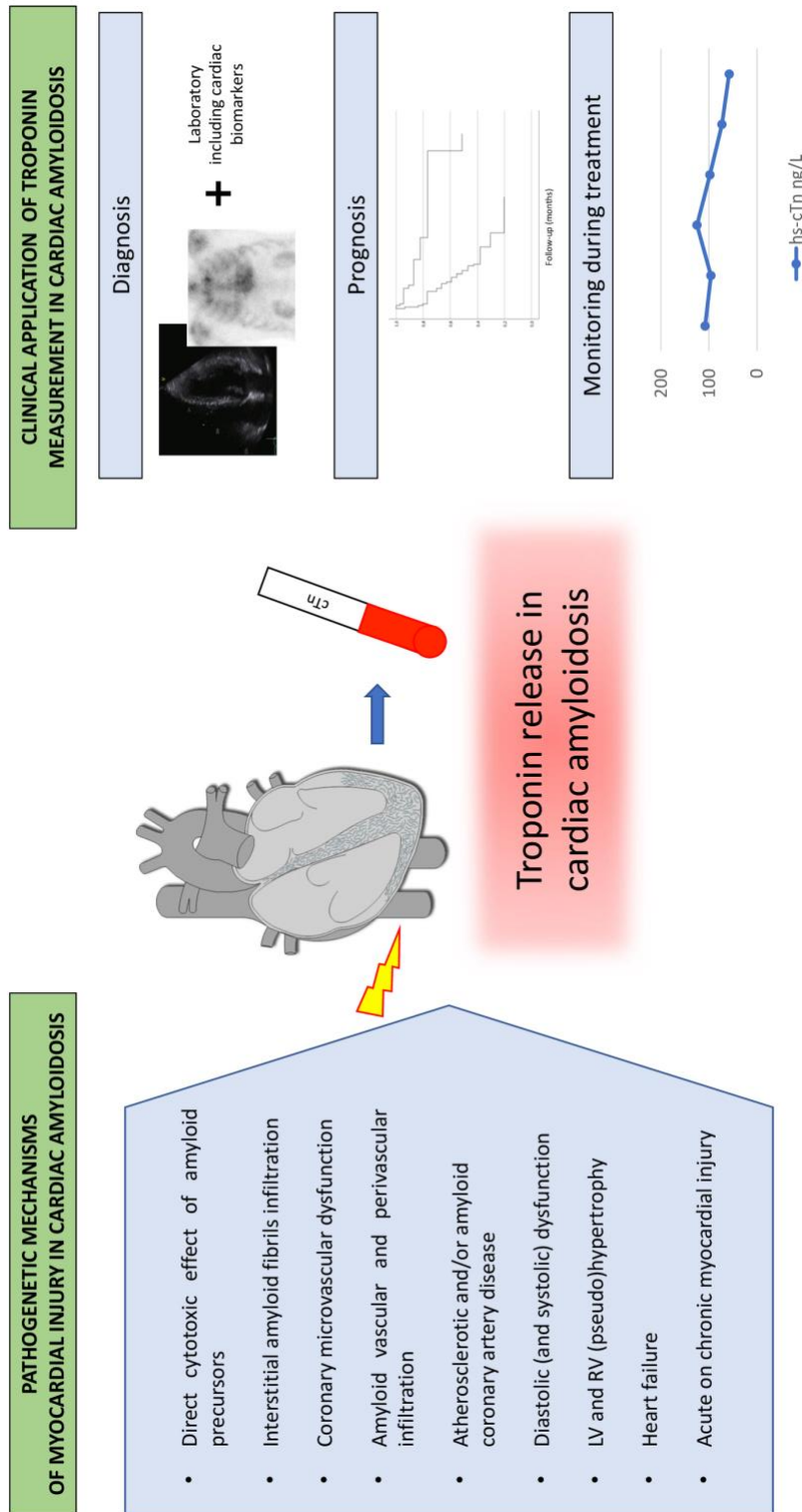
Central Illustration. Pathogenetic mechanisms of myocardial injury and clinical application of troponin measurement in patients with cardiac amyloidosis. Abbreviations: hs-cTn, high sensitivity cardiac troponin; LV, left ventricle; RV, right ventricle.

5.5 Clinical use of cardiac troponin in cardiac amyloidosis

The use of cardiac biomarkers like cTn and natriuretic peptides to aid in the evaluation of patients with CA was developed over many years. Today, they have a fundamental role in the prognostic assessment of patients with CA, alone and integrated in multiparametric staging systems. Moreover, they are helpful in monitoring the response to chemotherapy in AL amyloidosis. Emerging data suggest that they can also be of relevance when CA is suspected[68]. In the following paragraphs, the role of cTn in the different phases of CA management will be discussed (Table 1 for AL-CA and Table 2 for ATTR-CA).

5.5.1 Diagnosis

Compared to its role in the prognostic assessment of patients with CA, the diagnostic value of cTn has been less extensively investigated, in part because the metrics necessary to distinguish CA from other disease entities that can cause myocardial dysfunction has not been probed adequately. In addition, there are many assays for cTnI, both conventional and high sensitivity, and finding exact thresholds for diagnosis requires large studies for each.

In patients at risk of developing AL amyloidosis, including those with monoclonal gammopathy of uncertain significance, periodical screening for potential cardiac involvement can be performed with NT-proBNP and CV imaging parameters[69]. Some studies have started investigating the role of hs-cTnT in the diagnostic algorithm of CA[70,71,72]. Recently, Vergaro et al.[68] reported that hs-cTnT, alone and in combination with NT-proBNP, was useful when CA was suspected to identify patients in whom the diagnosis is unlikely and those in which it is much more likely. They suggested cutoffs <180 ng/L for NT-proBNP and <14 ng/L for hs-TnT as optimal rule-out thresholds. A hs-TnT ≥86 ng/L was a good rule-in threshold but the pre-test probability of CA was high; around 60% of patients had a confirmed CA in both derivation and validation cohorts. Only 9% of patients had both biomarkers below the cutoff values with 4 false negative. For the rule-in cutoff, around 20% of patients were correctly classified with 17 false positive in the validation cohort. Thus, for diagnosis,

other features must be present at least adjunctively. Further studies are needed to validate these findings in less selected populations.

5.5.2   Prognosis

a) AL amyloidosis

The prognostic role of cTn, and particularly cTnT, in CA has been extensively validated [73]. Dispenzieri et al [74] showed that survival of AL amyloidosis patients was significantly worse in those with raised cTnT/cTnI values compared to those with undetectable cTn. On multivariable analysis, cTnT was a better predictor than cTnI. With the evidence that NT-proBNP also had a strong prognostic value in AL amyloidosis[75], a staging system was developed integrating cTnT/cTnI with NT-proBNP.[76]   Patients were stratified in three stages (I, II and III) with median survivals of 26.4, 10.5, and 3.5 months, respectively. The proposed cutoffs were $\geq$ 332 ng/L for NT-proBNP, $\geq$ 0.035 µg/L for cTnT, and $\geq$ 0.1 µg/L for cTnI (using the Stratus CS assay). Subsequently, sFLC levels (particularly the difference between involved and uninvolved sFLC) were integrated in the model (Mayo 2012 model) with stratification of patients in four groups. In this model, cTnT was used at a threshold cutoff of $\geq$0.025 µg/L [77]. An European modification of the first Mayo Clinic stage was also developed, subclassifying stage III patients in IIIa and IIIb according to NT-proBNP levels using a threshold of 8500 ng/L[78]. The Boston University staging system, instead, was based on BNP (81 ng/L) and cTnI (0.1 ng/mL)[79]. Comparing the available staging systems suggests that the European 2015 model had better prediction for 1-year mortality but the Mayo 2012 model increased the ability to predict long-term survival[80].

When the hs-cTnT assay became available for clinical practice, a study from the Mayo Clinic[81] demonstrated that hs-cTnT numeric values could not merely be substituted for cTnT measurements in the original Mayo Clinic staging system. A threshold of 50 ng/L was derived with a quartic formula[73] and it is now used in the Mayo 2004 model. For the Mayo 2012 model, a threshold of hs-cTnT$\geq$ 40 ng/L was validated[82]. With hs-cTnT analytic performance, NT-proBNP might be no longer

necessary for prognostication[81], although still useful to follow up patients. Palladini et al [83] reported a prognostic cutoff of 77 ng/L for hs-cTnT to best predict mortality at presentation. Dispenzieri et al reported also on the clinical use of soluble suppression of tumorigenicity 2 in the prognosis of AL patients, together with cTnT, NT-proBNP and differential sFLC[84]. It is unclear how much these add to hs-cTnT alone.

Autologous stem cell transplant (ASCT) has been shown to be an effective therapy for patients with AL amyloidosis but only selected patients can undergo this procedure[5]. Cardiac biomarkers at baseline are useful for risk stratification of early death following ASCT. Specifically, cTnT > 0.06 μg/L (or hs-cTnT of 73/75 ng/L) and NT-proBNP > 5000 ng/L manifest optimal discrimination[85,73].

These approaches have invariably utilized cTnT. It is likely that over time, with large studies, the optimal threshold values for each of the hs-cTnI assays will be definable.

b) ATTR-CA

cTn, and particularly cTnT and hs-cTnT, are now recognized prognostic factors in ATTR-CA, especially in ATTRwt[86,87]. Grogan et al [88] developed a prognostic staging system for ATTRwt-CA based on a cTnT threshold of 0.05 ng/mL and an NT-proBNP threshold of 3000 pg/mL. The 4-year survival estimates were 57%, 42%, and 18% for stage I, stage II, and stage III respectively, with stage III patients having an increased risk of mortality after adjustment for age and sex compared with stage I patients. A widely used staging system, that avoids the issue of so many different cTn assays, was developed by Gillmore et al. for ATTRwt-CA and ATTRv-CA. It is based on NT-proBNP and estimated glomerular filtration rate (eGFR)[89]. In 175 ATTR-CA patients (133 ATTRwt-CA and 42 ATTRv-CA), this staging system provided better prognostic accuracy compared to one using NT-proBNP and contemporary cTnI [90]. Recently, a staging system combining hs-cTnT (> 50 ng/L), BNP (> 250 pg/mL), and eGFR (< 45 ml/mq) was published with good prediction of prognosis in 176 Japanese patients with ATTRwt-CA[91].

Diagnosis	Prognosis*	Response to therapy

Diagnostic score to define cardiac involvement in AL amyloidosis (70)
- **Hs-cTnT> 35 ng/L** *(1 point)*
- GLS≥ -17% *(1 point)*
- RELAPS ≥ 0.9 *(1 point)*
 	
CA very likely in patients with suspected CA (68)
- **Hs-cTnT ≥ 86 ng/L**
 	
CA unlikely in patients with suspected CA (68)
- **Hs-cTnT< 14 ng/L** *and*
- NT-proBNP < 180 ng/L
	Mayo Clinic 2004 (76)
	- **cTnT ≥ 0.035 µg/L**
	  or **cTnI (Stratus CS) ≥ 0.1 µg/L**
	  or **hs-cTnT ≥ 50 ng/L**
	- NT-proBNP ≥ 332 ng/L
	
	Mayo Clinic 2012 (77)
	- **cTnT >0.025 µg/L**
	  or **hs-cTnT ≥ 40 ng/L**
	- NT-proBNP ≥ 1800 ng/L
	- dFLC ≥ 180 mg/L
	
	European 2015 modification of Mayo 2004 (78)
	- **cTnT ≥ 0.035 µg/L**
	  or **cTnI (Stratus CS) ≥ 0.1 µg/L**
	  or **hs-cTnT ≥ 50 ng/L**
	- NT-proBNP ≥ 332 ng/L and > 8500 ng/L
	
	Boston University staging system (79)
	- **cTnI (assay not specified) > 0.1 ng/mL**
	- BNP > 81 ng/L
	
	Palladini et al (83)
	- **Hs-cTnT > 77 ng/L**
	  or **cTnI (Advia Centaur CP, Siemens) > 70 ng/L**
	
	ASCT candidates (73, 85)
	- **cTnT > 0.06 µg/L**
	  or **hs-cTnT > 75 ng/L**
	- NT-proBNP > 5000 ng/L
		Cardiac disease progression (92)
		- **cTn increase > 33%** *or*
		- NT-proBNP increase > 30% and > 300 ng/L *or*
		- LV ejection fraction reduction ≥ 10%.
		
		Cardiac disease response (92)
		- NT-proBNP reduction > 30% and > 300 ng/L (if baseline ≥ 650 ng/L) *or*
		- ≥ 2-class reduction in NYHA (if baseline 3-4)
		
		Graded cardiac response (94)
		- CarCR Nadir NT-proBNP ≤ 350 pg/mL or BNP ≤ 80 pg/mL
		- CarVGPR > 60% reduction in NT-proBNP/BNP from baseline level not meeting CarCR
		- CarPR 31%-60% reduction in NT-proBNP from baseline level not meeting CarCR
		- CarNR ≤ 30% reduction in NT-proBNP from baseline level
		
		Re-staging with Mayo 2004 and 2012 systems (95, 96)

Table 1. Clinical use of cardiac troponin (measured with contemporary and/or high sensitivity assays) in the management of patients with light chain amyloidosis.
For some staging system, alternative cutoffs for BNP (instead of NT-proBNP) are available. Criteria for response to therapy in AL amyloidosis are reported for completion, even if they do not include cTn/hs-cTn. Criteria for hematological response are not reported. * In prognostic staging systems, the stage for each patient is defined based on the number of variables above the specified thresholds.nAbbreviations: AL, light chain amyloidosis; ASCT, autologous stem cell transplant; BNP, brain natriuretic peptide; CA, cardiac amyloidosis; CarCR: complete cardiac response; CarNR: cardiac no response; CarPR: cardiac partial response; CarVGPR: cardiac very good partial response; cTnI, cardiac troponin I; cTnT, cardiac troponin T; GLS, global longitudinal strain; hs-cTnT, high sensitivity cardiac troponin T; LV: left ventricular; NT-proBNP, N-terminal pro-brain natriuretic peptide; RELAPS, relative apical sparing.

5.5.3 Response to treatment

a) AL amyloidosis

Cardiac response to chemotherapy has been defined as >30% decrease with a total decline of > 300 ng/L in NT-proBNP or ≥ 2-class decrease in New York Heart Association (NYHA) class (if baseline class 3 or 4). [92] Caution should be applied, however, in interpreting changes of NT-proBNP values <50% due to its high biological variability[93]. A recent paper reported that graded cardiac response (based on the extent of NT-proBNP reduction) allowed for a better assessment of cardiac improvement than the traditional binary response system[94]. On the other hand, cTn was proposed to define cardiac disease progression at 6 months, based on an increase ≥ 33%[92]. However, the assays used were contemporary cTnT and cTnI assays and the cTnI assay was not identified. Other criteria for disease progression were NT-proBNP increase >30% and > 300 ng/L or a reduction ≥10% in LV ejection fraction[92].

The current Mayo Clinic staging systems also are useful for re-staging during treatment at 3 and 6 months after chemotherapy initiation. A worsening stage at 3 and 6 months is associated with worse survival than maintenance at the same stage [95]. In patients with a disease relapse after a first-line therapy, both the Mayo 2004 and 2012 staging systems are useful for prognostic stratification with second-line therapy [96].

b) ATTR-CA

A multiparametric approach is recommended to evaluate disease progression in ATTR-CA; among the various criteria, an increase in cTn > 30% (which however is less than the reference change value,

i.e. the amount of change explained by conjoint analytical and biological variation) is considered indicative of disease progression[97]. Specific therapy for ATTR-CA has recently become available[3] but few studies have investigated the change of cardiac biomarkers over time to verify the response to treatment. Most trials and clinical studies report the trend of NT-proBNP, not cTn, over time[98,4]. Recently, a single center French study[99] showed that tafamidis therapy stabilizes NT-proBNP and hs-cTnT levels over time, especially in those with higher values at baseline. However, further studies on the role of hs-cTn for the monitoring of response to therapy in ATTR-CA are needed.

Diagnosis	Prognosis*	Response to therapy

CA very likely in patients with suspected CA (68)
- **Hs-cTnT ≥ 86 ng/L**
 	
CA unlikely in patients with suspected CA (68)
- **Hs-cTnT< 14 ng/L** *and*
- NT-proBNP <180 ng/L
	Grogan et al. 2016 (88) for ATTRwt-CA
	- **cTnT > 0.05 ng/mL**
	- NTproBNP > 3000 pg/mL
	
	Nakashima et al. for ATTRwt-CA (91)
	- **Hs-cTnT > 50 ng/L**
	- BNP> 250 ng/L
	- eGFR < 45 ml/mq
	
	NAC staging system for ATTRwt- and ATTRv-CA (89)
	- NTproBNP > 3000 ng/L
	- eGFR < 45 ml/min
		Disease progression in ATTR-CM (97):
		At least one marker in each domain:
		- clinical and functional domain
		- laboratory domain
		  i) **cTn increase > 30%** *or*
		  ii) NT-proBNP increase > 30% *or*
		  iii) Advance in NAC stage
		- ECG and imaging domain

Table 2. Clinical use of cardiac troponin (measured with contemporary and/or high sensitivity assays) in the management of patients with transthyretin cardiac amyloidosis. Abbreviations: ATTRv, variant transthyretin amyloidosis; ATTRwt, wild-type transthyretin amyloidosis; BNP, brain natriuretic peptide; CA, cardiac amyloidosis; cTnT, cardiac troponin T; hs-cTnT, high sensitivity cardiac troponin T; NT-proBNP, N-terminal pro-brain natriuretic peptide; eGFR, estimated glomerular filtration rate. National Amyloidosis Center (NAC) staging system has been reported for completeness, even if it does not include cTn/hs-cTn.
* In prognostic staging systems, the stage for each patient is defined based on the number of variables above the specified thresholds.

5.6 Conclusions

Cardiac troponin has dramatically changed the clinical practice in Cardiology in the last 30 years. It is now the biomarker of choice for the detection of myocardial injury and high sensitivity assays can identify even modest increases in a multiplicity of clinical conditions. Myocardial injury is particularly frequent in patients with CA due to multiple synergistic mechanisms that contribute to cardiomyocytes damage. Evaluation of cTn, alone and integrated in staging systems, is helpful in the management of patients with CA, which are now being increasingly recognized and treated. There

are still many potential areas of research in this field, including further addressing the diagnostic significance of cTn in suspected CA and its prognostic and monitoring role now that new effective therapies are available not only for AL, but also for ATTR-CA. Assay-specific thresholds (other than for cTnT/hs-cTnT) are also essential to allow for a wider dissemination of troponin-based diagnostic and prognostic scores.

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PART 2. ORIGINAL CONTRIBUTIONS

Chapter 6. Hospitalization-based incidence of systemic and cardiac amyloidosis in the Veneto Region, Italy

This Chapter is based on the manuscript: De Michieli L, Stoppa G, Sinigiani G, Previato L, Lorenzoni G, Salvalaggio A, Berno T, Perazzolo Marra M, Briani C, Iliceto S, Biggeri A, Catelan D, Cipriani A. Hospitalization-based incidence of systemic and cardiac amyloidosis in the Veneto Region, Italy. Submitted to Heart Failure Reviews.

6.1 Introduction

Systemic amyloidosis is a protein misfolding and deposition disorder leading to progressive organ dysfunction[1]. The two forms that most frequently affect the heart are light chain amyloidosis (AL) and transthyretin amyloidosis (ATTR), either variant (ATTRv) or wild type (ATTRwt)[1].

Unlike some years ago, cardiac amyloidosis (CA), particularly ATTR-related (ATTR-CA), is a widely recognized cause of heart failure (HF) and cardiovascular mortality. Recent diagnostic[2] and therapeutic advancements[3] have led to greater disease awareness and an increasing number of patients appropriately diagnosed and treated[4]. In particular, ATTR-CA is now often recognized in clinical practice, more typically in elderly men with HF and history of carpal tunnel syndrome (CTS)[5]. Among patients with HF and preserved ejection fraction, ATTR-CA frequency ranges between 6%[6] and 13%[7]; 9% of men undergoing bilateral CTS surgery have ATTRwt, raising to 21.2% in > 70 years and not obese[8]. The same phenomenon of increasing numbers of new diagnoses is not reported for AL amyloidosis[9,10], although new and effective chemotherapy regimens are available [11] with improved survival[12], particularly when a timely diagnosis is achieved[13].

Epidemiological data on incidence and prevalence of amyloidosis in specific regions of Italy are emerging[14]. Data from the Veneto region (Northeastern Italy), which counts almost 5 million inhabitants, are not available yet, and neither are data on amyloidosis-related hospitalizations (AH). Accordingly, we aimed to investigate AH and their temporal trend over the period 2010-2020 in this region. Moreover, we focused on the frequency of cardiac morbidities suspicious for CA among patients undergoing CTS surgery in Veneto over the same time range.

6.2 Methods

The Local Ethic Committee (136n/AO/21) of Padova University Hospital approved this retrospective observational study. International Classification of Diseases (ICD-9) codes reported in the hospital discharge summaries were used to identify AH in Veneto from 2010 to 2020 in patients aged ≥ 18 years. Moreover, among hospital summaries with a primary or secondary discharge code for systemic amyloidosis, we identified those presenting an ICD-9 code for cardiomyopathy, HF or arrhythmias. The following list of codes were utilized in this study for the identification of cases of systemic amyloidosis and cardiac amyloidosis:

1. primary or secondary diagnosis ICD-9 code for systemic amyloidosis: 277.3X, except 277.31.

2. For cardiac amyloidosis: a systemic amyloidosis code as above AND ≥ ICD-9 code for cardiomyopathy, heart failure or arrhythmias: 398.91, 402.01, 402.11, 402.91, 404.01, 404.11, 404.91, 404.03, 404.13, 404.93, 425.4, 425.7, 425.18, 425.8, 425.9, 428 (all

Hospitalization for CA was defined as the presence at discharge summary of (1) ≥1 principal or secondary ICD-9 code for systemic amyloidosis and (2) ≥1 principal or secondary ICD-9 code (contemporary or after the amyloidosis code) for HF, cardiomyopathy or arrhythmia[15].

To identify possible cases of CA, considering the association between CA and CTS[8], we also extrapolated hospital or outpatient discharge summaries with an ICD-9 code for CTS release surgery (single or multiple); among these, we identified those who also presented an ICD-9 code for cardiomyopathy, HF or arrhythmias. Patients presenting both types of codes were defined as suspected cases of CA. The following ICD-9 codes were utilized for this analysis:

1.   hospital or outpatient discharge summary with a ICD-9 code for carpal tunnel syndrome release surgery: 04.43,

2.   AND ≥ subsequent ICD-9 code for cardiomyopathy, heart failure or arrhythmias: 398.91, 402.01, 402.11, 402.91, 404.01, 404.11, 404.91, 404.03, 404.13, 404.93, 425.4, 425.7, 425.18, 425.8, 425.9, 428 (all).

Our primary outcomes were the incidence and prevalence of hospitalization for systemic and cardiac amyloidosis over the study period and the frequency of suspected cases of CA among patients undergoing CTS release surgery.

Statistical analysis

To estimate incidence, we considered the date of the first hospitalization, defined as the year of the hospital admission with a code of systemic amyloidosis without any other previous (last 5 years) amyloidosis-related hospital admission. Since the available hospitalization database refers to the period 2010-2020, we calculated incident cases from 2015 to 2020.

To estimate prevalence, we first considered all hospitalized patients in a given year. This is a poor estimate since it assumes that all the patients would have been hospitalized at least once a year during the disease course. Therefore, we also used the approach of Kumar et al.[16]. To estimate point prevalence at a given reference date, we multiplied the number of incident cases for the survival probability. We reported a five- and ten-year point prevalence and the one-year period prevalence. Period prevalence is the sum of old and new cases in a given time period. We used the survival rates from Kumar et al.[16], which are based on AL amyloidosis and are likely a slight underestimated of the survival for our casuistry, a mixture of all types of amyloidosis.

The following formulas were used to estimate prevalence:

Five-year prevalence at January 1st 2020:

=Inc15×Surv5yrs+Inc16×Surv4yrs+Inc17×Surv3yrs+Inc18×Surv2yrs+Inc19×Surv1yrs

Five-year prevalence at January 1st 2021:

=Inc16×Surv5yrs+Inc17×Surv4yrs+Inc18×Surv3yrs+Inc19×Surv2yrs+Inc20×Surv1yrs

Ten-year prevalence at January 1st 2021:

=Inc11×Surv10yrs+Inc12×Surv9yrs+Inc13×Surv8yrs+Inc14×Surv7yrs+Inc15×Surv6yrs+Inc16×Surv5yrs+Inc17×Surv4yrs+Inc18×Surv3yrs+Inc19×Surv2yrs+Inc20×Surv1yrs

Period prevalence for the year 2020:

- Five-year period prevalence

$$Inc15 \times Surv5yrs + Inc16 \times Surv4yrs + Inc17 \times Surv3yrs + Inc18 \times Surv2yrs + Inc19 \times Surv1yrs + Inc20$$

- Ten-year period prevalence:

$$Inc11 \times Surv9yrs + Inc12 \times Surv8yrs + Inc13 \times Surv7yrs + Inc14 \times Surv6yrs + Inc15 \times Surv5yrs + Inc16 \times Surv4yrs + Inc17 \times Surv3yrs + Inc18 \times Surv2yrs + Inc19 \times Surv1yrs + Inc20$$

We assumed that the number of non-resident cases hospitalized in the Veneto Region was equal to the number of Veneto residents hospitalized outside the region to estimate incidence and prevalence rates.

Since ICD-9 codes for systemic amyloidosis do not distinguish between AL and ATTR amyloidosis, considering the different demographic profile of the two conditions[17], we also performed separate analyses for patients aged more or less than 65 years, assuming that AL patients would be more frequently < 65 years, while ATTR > 65 years[4]. We summarized the time trend by Poisson regression analysis [18]. The data underlying this article cannot be shared publicly due to privacy reasons.

6.3 Results

6.3.1 Amyloidosis-related hospitalizations

A total of 688 new cases (with a median of 114.7 cases per year) hospitalized for systemic amyloidosis were observed in the time range 2015-2020 in the Veneto Region (about 4.9 million inhabitants in the same time period) with an incidence rate of 23.5 per million [95% confidence interval (CI) 21.8; 25.3]. We found a higher proportion of males (437/688, 63.5%) and elderly individuals (524/688, 76.2%). As reported in Table 1, by calendar year, we observed an increasing trend (percent annual increase of 17 %, 95% CI 12; 22%), particularly for men and patients > 65 years old (Figure 1, Figure 2).

Year	Number of cases	Person-years(*)	Rate (per 10^6)	Lower 95% CI	Upper 95% CI
2015	78	4911326	15.9	12.7	19.8
2016	84	4906283	17.1	13.8	21.2
2017	109	4894814	22.3	18.5	26.9
2018	114	4881862	23.4	19.4	28.1
2019	139	4874482	28.5	24.1	33.7
2020	164	4858788	33.8	29.0	39.3

(*) average population size

Table 1: Incident cases of amyloidosis-related hospitalization and annual rate for 1,000,000 inhabitants. Abbreviations: CI: confidence interval.
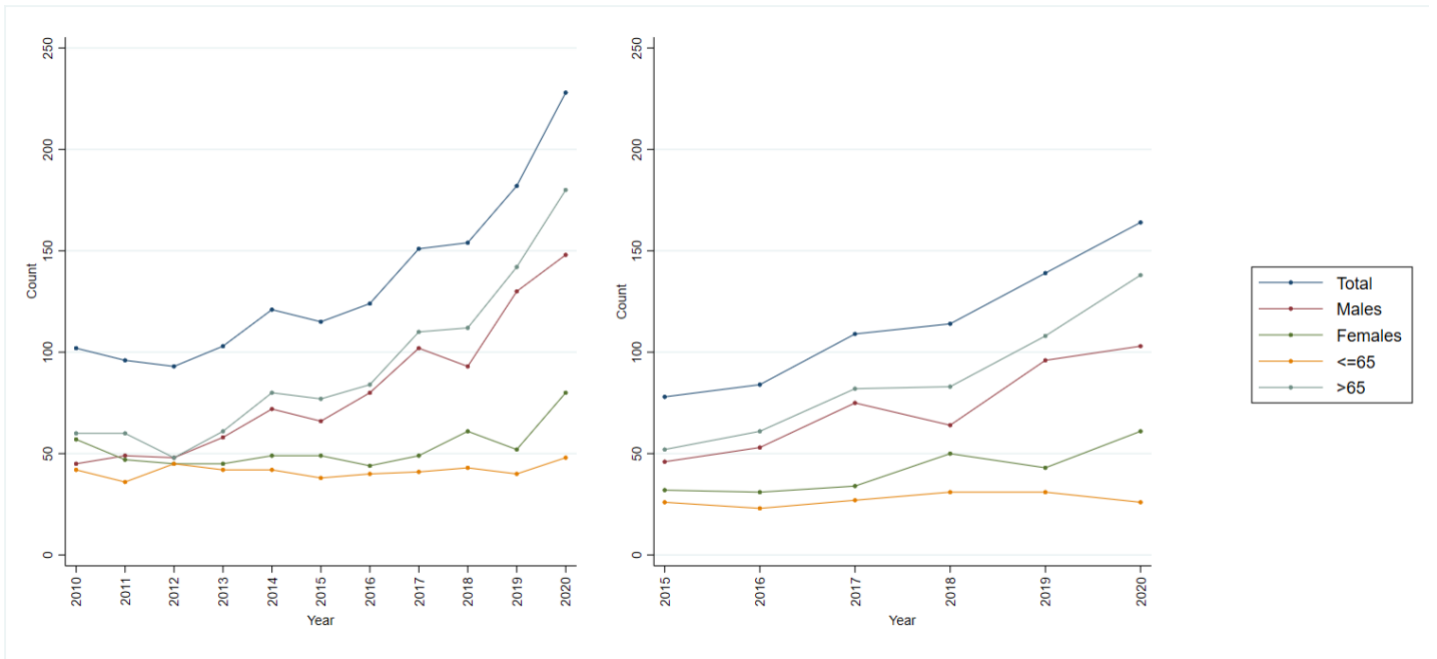
Figure 1. Panel a) annual hospitalized prevalent cases of systemic amyloidosis between 2010 and 2020. Panel b) annual hospitalized incident cases of systemic amyloidosis between 2015 and 2020.

The total number of discharge records with an amyloidosis code showed the same pattern, rising progressively from 148 in 2010 to 308 in 2020, with a steeper trend for men and >65 years old individuals.

Regarding prevalence estimates, from January 1, 2010, to December 31, 2020, 133.5 annual hospitalized prevalent cases per year were recorded in the Veneto Region. In 2010, 102 patients had at least one AH; in 2020, the number increased to 228 (Figure 1), corresponding to a patient-hospitalization rate of 21 per 10^6 in 2010 (95% CI 17.2; 25.4) and 47 per 10^6 in 2020 (95% CI 41.2;53.4) with a progressive increase of about 11.4 cases per year, around 11% increase per year since 2010. Similar computations show a higher annual percent increase for males (20%) and older adults (18%).

Using the estimated incident cases and the survival probabilities as in Kumar et al.[16], we estimated a 5-year prevalence on January 1, 2020, of 68.8 per 10^6 (95% CI 61.8; 76.6) and a 5-year prevalence on January 1, 2021, of 80.6 per 10^6 (95% CI 72.9; 88.9). The estimated 10-year prevalence on January 1, 2021, was 106.7 cases per 10^6 (95% CI 97.8; 116.3). The 5-year period prevalence in

2020 was estimated at 102.7 cases per 10^6 (95% CI 94.1; 112.2), while the 10-year period prevalence in 2020 was 124.5 per 10^6 (95% CI 114.9; 134.8).

6.3.2 Cardiac amyloidosis-related hospitalizations

In 2020, annual hospitalized prevalent cases of CA, compared to overall systemic amyloidosis hospitalizations, were about 70% (159 over 228 cases), mainly in individuals older than 65 years and males. On average, about 50 cases per year are recorded in Veneto – 556 patients in the time range 2010-2020 - with an increasing trend in the last years.

6.3.3 Carpal tunnel syndrome and suspected cases of cardiac amyloidosis

Considering the whole period, the number of patients with at least one record of CTS release surgery was stable (on average, about 8000 surgeries per year), with a slight decrease in 2020. Overall, CTS release surgery, both single and multiple, was performed more frequently in women (61,416 women/90,301 surgeries, 68%).

Among those with CTS, 3335 patients (3335/90301, 3.7%) presented a subsequent code of cardiomyopathy, HF or arrhythmia after a median of 4.2 years (min 0 days, max 11.1 years), and this was more frequent in men (1.361/28.885, 4.1%) than in women (1.974/61.416, 2.9%). Among patients with multiple CTS release surgeries, a subsequent code for cardiomyopathy, HF, or arrhythmia was present in 913 patients after a median of 3.9 years (min 4 days, max 10.8 years), and it was more frequent in men than women (463/6.526 7.1% versus 450/11.406 3.9%).

HOSPITALIZATION-BASED INCIDENCE OF SYSTEMIC AMYLOIDOSIS

Year	Number of cases	Person-years(*)	Rate (per 10^6)	Lower 95% CI	Upper 95% CI
2015	78	4911326	15.9	12.7	19.8
2016	84	4906283	17.1	13.8	21.2
2017	109	4894814	22.3	18.5	26.9
2018	114	4881862	23.4	19.4	28.1
2019	139	4874482	28.5	24.1	33.7
2020	164	4858788	33.8	29.0	39.3

ESTIMATED PREVALENCE OF SYSTEMIC AMYLOIDOSIS (BASED ON INCIDENCE AND SURVIVAL PROBABILITY)

	Estimated prevalence per 10^6 (95% CI)
10-year period prevalence in 2020	124.5 (114.9 – 134.8)

5 million inhabitants

Hospital discharge summaries from 2010 to 2020

Figure 2. Hospitalization-based incidence and estimated prevalence of systemic amyloidosis in the Veneto Region, Northeastern Italy. Veneto Region is a region of around 5 million inhabitants. International Classification of Diseases (ICD-9) codes were used to identify amyloidosis-related hospitalization (AH) in Veneto region from 2010 to 2020. On the right of the Figure, a progressively increasing incidence of AH is reported. Based on such incidence and survival probability, a 10-year period prevalence in 2020 of 124.5 per 10^6 was estimated. Abbreviations: CI, confidence interval.

6.4 Discussion

In this study, we report data on AH from 2010 to 2020 in the Veneto region, Italy. Moreover, the frequency of suspected CA among patients undergoing CTS surgery was analyzed.

We found several interesting findings: 1) hospitalizations for systemic amyloidosis are increasing over time, particularly in older (>65 years) patients and men; 2) there is also empirical evidence that hospitalizations for systemic amyloidosis and coexistent cardiomyopathy, HF or arrhythmias, consistent with CA, are increasing, again mainly in older patients and men; 3) although CTS release surgery was performed more often in women, men presented more frequently a following code for cardiomyopathy, HF or arrhythmia, notably if they underwent multiple CTS release surgeries. This association identifies patients potentially affected by CA and who might benefit from dedicated diagnostic assessment, therapy and follow-up.

Defining the real epidemiology of amyloidosis is a contemporary challenge. Recently, Italian data[10,14] showed that, over the past 20 years, the number of patients diagnosed with amyloidosis, particularly ATTR, has increased, especially after the publication of landmark studies[19,2] that facilitated a non-biopsy diagnosis and thus prompted access to new effective therapies[20,21,22]. Our data expand these findings providing information on AH in Veneto Region, a northeastern Italian region with about five million inhabitants. We analyzed hospitalization discharge records of all Veneto Region hospitals, avoiding the referral-center selection bias already described in previous literature. We reported a significantly increasing trend in the number of patients hospitalized for amyloidosis, also with alleged cardiac involvement, particularly in men and patients older than 65 years. Although we are not able to distinguish patients with AL and ATTR based only on ICD-9 codes, we applied a previously published[4] cutoff of 65 years to discriminate patients more likely to have AL from those

with ATTR. The results of our study are concordant with those reported on hospitalizations in the United States[15] and with data based on outpatient records and population registries[4,10,14], being driven by greater disease awareness and improved diagnostic efforts that allow the identification of a previously submerged portion of patients. Compared to previous Italian epidemiological studies[14,23] and international reports[16], we found consistent results. We did not include outpatients, which could explain the lower prevalence values if we based our calculation only on the number of hospitalized patients per year. Our data are consistent with recent studies analyzing the epidemiology of AL and ATTR in Tuscany, where data estimated an overall incidence of amyloidosis between 24.2 and 38.4 per million in the period 2006-2022[14,23], compared to 15.9 and 33.8 per million in the period 2015-2020 in Veneto.

Data from Tuscany reported a prevalence in 2022 of 90.3 per million for ATTRwt and 9.5 per million for ATTRv [14]. In the international literature, a 5 and 10 years point prevalence in 2018 of AL amyloidosis of 37.4 per million and 53.5 per million are reported, respectively [16]. Our result of an overall point prevalence (10 years) of 106 per million is consistent with previous literature, also considering the greater frequency of ATTRv in Tuscany (compared to the Veneto Region)[24] and the inclusion in our dataset only of hospitalized patients. Based on the estimates derived from the methodology from Kumar et al. [16], we should consider the rapid change in the number of cases in recent years and the period prevalence (10 years) of 124,5 per million as a reasonable estimate. Indeed, our data confirmed how, in a large population, CA is an emerging condition whose dimensions are increasing regarding patient care efforts and therapeutic management costs.

We also reported interesting data on patients with suspected CA among those undergoing CTS surgery. CTS is commonly associated with amyloidosis, particularly ATTR, such that 9% of men undergoing bilateral CTS surgery have ATTRwt, raising to 21.2% in those older than 70 years and not obese[8]. Our data showed that, even if the absolute number of procedures performed (both single and multiple surgeries) was higher in women, the percentage of patients with a subsequent code for cardiac disease was higher in men, up to 7% of men with multiple CTS surgeries. Although this is

clearly just an estimated number of patients potentially affected by CA (in particular ATTR-CA), our data may reinforce the opportunity to potentially identify CA in patients undergoing CTS surgery, particularly older men with multiple CTS surgeries. Tailored screening procedures, including electrocardiogram, echocardiogram, and cardiac biomarkers, may be reasonable in such population[8,25].

The present study has multiple limitations. It is not based on a disease-specific population-based registry but only on hospital records. While the severity of the disease will assume that hospitalization is an expected event in patient history, the date of first hospitalization could be delayed from the date of disease onset, especially in milder forms. However, since the relatively rapid course of the disease, we do not expect a major bias in identifying the incidence date. A misclassification of incident cases with the inclusion of prevalent cases is also unlikely to occur because we used a buffer of five years of hospital records. It is unlikely that an amyloidosis case would be treated out of the hospital for more than five years from the first hospitalization. Amyloidosis is listed as a rare disease, and the national health service entirely covers its costs. Even though we have hospital records from the Veneto Region, we did not obtain hospital records from Veneto residents admitted to hospitals outside the region. We should assume that this selection bias will be minor. In addition, the number of patients resident outside the Veneto region admitted for amyloidosis will compensate for the number of resident patients admitted to hospitals outside the region. Finally, we cannot distinguish AL and ATTR based only on ICD codes in the hospital admission records, but we measured the global burden of amyloidosis. We arbitrarily chose an age cutoff to distinguish AL amyloidosis from ATTR, and the results agreed with those in the literature, reassuring us about the accuracy and reliability of our data.

6.5. Conclusions

By analysing hospital discharge summaries in a 5-million inhabitants region of Italy, we demonstrated an increased incidence of AH, up to 33.8 cases/million in 2020, and a 10-year period prevalence in

2020 of 124.5 cases/million. Moreover, up to 7% of men undergoing multiple CTS release surgeries may represent cases of suspected CA, possibly deserving further testing. Our data confirm that systemic amyloidosis is an emerging condition whose epidemiological dimensions are increasing in terms of patient care. Further studies on tailored screening of selected populations, such as patients undergoing multiple CTS surgeries, are necessary.

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Chapter 7. Predictive value of high-sensitivity cardiac troponin T in patients undergoing technetium-99 m pyrophosphate scintigraphy for suspected transthyretin amyloid cardiomyopathy

This Chapter is adapted from the abstract presented at ESC 2023 in Amsterdam: L. De Michieli, OF. Abouezzeddine, MA. Abbasi, DR. Davies, CG. Scott, E. Muchtar, A. Dispenzieri, M. Grogan, M. Redfield, AS. Jaffe. Predictive Value of high-sensitivity cardiac troponin T in patients undergoing 99mTc pyrophosphate scintigraphy for suspected transthyretin amyloid cardiomyopathy.
Full manuscript is in preparation, to be reviewed by the co-Authors.

7.1 Introduction

Transthyretin (TTR) amyloid cardiomyopathy (ATTR-CM) is an infiltrative disease characterized by the progressive deposition of misfolded TTR into the extracellular matrix[1,2]. The condition is now recognized in 6[3] to 13%[4] of patients with heart failure with preserved ejection fraction (HFpEF) and increased left ventricular (LV) wall thickness.

Diagnostic scores[5,6] and "red flags"[7,8] have been proposed to identify patients who deserve further investigation. Once light chain amyloidosis is excluded, technetium Tc 99m pyrophosphate single-photon emission-computed tomography (PYP) can provide an accurate noninvasive diagnosis of ATTR-CM[7].

The role of cardiac biomarkers, and particularly high sensitivity cardiac troponin (hs-cTn), to identify those who require additional evaluations has not been investigated in a United States (US) cohort of patients with suspected ATTR-CA. The aim of this study was to investigate the predictive value of hs-cTnT in patients undergoing PYP imaging for suspected ATTR-CM. We particularly focused on whether there was a value that had sufficient negative predictive value (NPV) to deploy in clinic practice to rule-out the disease. We also probed the role of N-terminal pro B-type natriuretic peptide (NT-proBNP), both alone and combined with hs-cTnT, in the same setting.

7.2 Methods

7.2.1 Study design

The study was approved by the Mayo Clinic Institutional Review Board and funded by the Mayo Clinic Department of Cardiovascular Medicine. Only patients who provided written informed consent for use of their medical records for research were included.

All patients with PYP imaging performed at the Mayo Clinic (Rochester, Minnesota) from 2013 to September 2022 and with at least one hs-cTnT available within 6 months from PYP imaging were included. Some of these patients (n=607) were included in a previous study for the derivation and validation of a ATTR-CM score in patients with HFpEF[5]. Patients from a separate community HFpEF cohort (n = 261) are also included in this analysis. Their characteristics have been published[3] previously.

Hs-cTnT was measured with the Elecsys Troponin T Gen 5 STAT assay (Roche Diagnostics). Concentrations are reported as whole units in ng/L down to the limit of quantitation (LoQ) of <6 ng/L which per Food and Drug Administration is the lowest reportable value. Sex-specific 99th percentile upper reference limits (URLs) of 10 ng/L for women and 15 ng/L for men[9] are used to define myocardial injury[10]. A value of 14 ng/L for both sexes has been probed by others[11]. For PYP imaging, three hours planar imaging followed by single-photon emission computed tomography with computed tomography (SPECT/CT) imaging was performed for each patient. For a study to be considered as a 'positive' Tc 99m pyrophosphate SPECT/CT study that is highly suggestive of TTR-CA both visual interpretation of SPECT/CT image showing characteristic 'diffuse' tracer update greater than background blood pool and a heart-to-contralateral (H/CL) values ≥ 1.3 on the 3-hour planar image were required. In cases where the H/CL ratio was judged to be inaccurate, for example due to the patient's anatomy such as rib fractures, visual SPECT/CT interpretation was used alone. Each study was performed and co-interpreted by experienced nuclear cardiologists and radiologists/nuclear medicine physicians. Other laboratory and echocardiographic data (within 1 year of PYP) were extracted from electronic health records.

All patients undergoing PYP are assessed for serum free light chain abnormalities and the presence of a monoclonal protein in the serum and urine prior to imaging. A final diagnosis of ATTR-CM was

confirmed by one Cardiologist (LDM) and two Cardiology Fellows (MAA, DRD) using all available information. Dubious cases were evaluated by an expert Cardiologist (OAE) to achieve a final diagnosis. Our primary endpoint was not the frequency of a positive PYP scan but the final diagnosis of ATTR-CM.

7.2.2 Statistical analysis

Continuous variables are reported as median (with interquartile range, IQR), categorical variables as absolute numbers and percentages. Group comparison was performed with Kruskal-Wallis test for continuous variables and Chi-square test for categorical variables.

Logistic regression was used to define the area under the curve (AUC) with associated 95% confidence limits. Receiver operating characteristic (ROC) analysis was used to evaluate the performance of hs-TnT, NT-proBNP and their combination to rule out or identify ATTR-CM. The diagnostic performance of hs-cTnT and NT-proBNP for rule-out and rule-in ATTR-CM is described in terms of negative and positive predictive value (NPV/PPV), sensitivity and specificity with respective 95% confidence intervals (95% CI). The 95% confidence limits for these values were derived based on the exact binomial distribution. SAS version 9.4 was used for all analyses and two-sided p-values <0.05 were considered to be statistically significant.

7.3 Results

Of 2291 unique patients with PYP imaging, 52 were excluded due to lack of research authorization and 797 due to lack of hs-cTnT values within 6 months, leaving 1442 patients for analysis. Of those, 1378 had NT-proBNP values.

A final diagnosis of ATTR-CM was made in 436 (30%) patients. Most (93%) were men with a mean age of 77 (±9) years. For baseline characteristics, comorbidities, and echocardiographic parameters see Table 1. Patients with ATTR-CM had higher hs-cTnT [48 (32-67) versus 25 (13-48) ng/L, p<0.0001] and higher NT-proBNP [2145 (1016-3801) versus 1119 (326-2011) ng/L, p<0.0001]

values. Myocardial injury (a value above the sex specific 99th % value) was present in 415 (95%) patients with ATTR-CM versus 762 (76%) of patients without ATTR-CM, p<0.001.

7.3.1 Predictive value of hs-cTnT (Figure 1, Table 2 and 3)

The AUC of hs-cTnT for the diagnosis of ATTR-CM was 0.69 (95% CI: 0.66, 0.72). Among patients with a very low hs-cTnT value, i.e.<6 ng/L (n=50, 3.5% of the overall cohort), no patients had ATTR-CM, yielding an NPV of 100% (93, 100) and a sensitivity of 100% (99, 100) for ruling out ATTR-CM. A total of 277 (19%) patients had hs-cTnT <14 ng/L and, of those, 17/277 (6.1%, 1.2% of the overall cohort) had a final diagnosis of ATTR-CM for an NPV of 94% (90, 96) and a sensitivity of 96% (94, 97). When considering progressively increasing hs-cTnT values as potential thresholds to rule-in ATTR-CM (Figure 1, Table 3), we observed a progressive increase in specificity (95% for hs-cTnT=153 ng/L), but the PPV remained low (23% for hs-cTnT=153 ng/L).

7.3.2 Predictive value of NT-proBNP (Figure 1, Table 2 and 3)

NT-proBNP values were available for 1378 patients. The AUC of NT-proBNP was 0.63 (95% CI 0.60, 0.66) and increased to 0.68 (95% CI: 0.66, 0.71) when combined with hs-cTnT. For rule-out ATTR-CM, a value < 60 ng/L (n=53, 3.8%) showed a sensitivity and NPV of 100%. A value <180 ng/L (n=180, 13%) that has been advocated for by others[12] demonstrated a NPV of 93 % (88, 96) and sensitivity of 97% (95, 98) with 13/180 false negatives. For ruling-in ATTR-CM, we observed a progressive increase in specificity at high values (95% for NT-proBNP=12421 ng/L), but the PPV remained low (25% for NT-proBNP=12421 ng/L).

Variable	Total (n=1442)	No ATTR-CM (n=1006)	ATTR-CM (n= 436)	P value
Age, years	74 (67-81)	73 (65-80)	77 (72-83)	<0.0001
Male	1024 (71)	619 (62)	405 (93)	<0.0001
Caucasian[a]	1316 (93)	917 (92)	399 (93)	0.49
Black[a]	76 (5.3)	52 (5.2)	24 (5.6)	0.77
Asian[a]	13 (0.9)	10 (1.0)	3 (0.7)	0.58
Coronary artery disease	352 (24)	275 (27)	77 (18)	<0.0001
COPD[b]	257 (18)	209 (21)	48 (11)	<0.0001
Ischemic heart disease	102 (7.1)	84 (8.3)	18 (4.1)	0.0041
Chronic kidney disease	460 (32)	368 (37)	92 (21)	<0.0001
Hypertension	752 (52)	604 (60)	148 (34)	<0.0001
Diabetes	465 (32)	384 (38)	81 (19)	<0.0001
History of stroke	206 (14)	161 (16)	45 (10)	0.0046
Peripheral vascular disease	512 (36)	414 (41)	98 (23)	<0.0001
Atrial fibrillation	669 (46)	453 (45)	216 (50)	0.11
Carpal tunnel syndrome	159 (11)	98 (9.7)	61 (14)	0.02
Spinal stenosis	204 (14)	143 (14)	61 (14)	0.91
Systolic blood pressure, mmHg	129 (114-143)	131 (116-146)	122 (112-137)	<0.0001
Heart rate, bpm	70 (62-80)	71 (62-81)	69 (61-79)	0.03
Echocardiography				
Ejection fraction, %	58 (49-64)	60 (53-65)	52 (43-60)	<0.0001
Left ventricular end diastolic diameter, mm	49 (45-53)	50 (46-54)	47 (44-52)	<0.0001
Septal wall thickness, mm	13 (11-16)	12 (10-14)	16 (14-18)	<0.0001
Posterior wall thickness, mm	12 (10-14)	11 (10-13)	15 (12-16)	<0.0001
Relative wall thickness	0.49 (0.40-0,61)	0.45 (0.38-0.54)	0.60 (0.50-0.73)	<0.0001
Laboratory				
Creatinine, mg/dL	1.2 (1.0-1.6)	1.2 (1.0-1.6)	1.2 (1.1-1.5)	0.22
N-terminal pro-brain natriuretic peptide, ng/l[c]	1463 (462-3276)	1119 (326-2011)	2145 (1016-3801)	<0.0001
High-sensitivity cardiac troponin T, ng/l	33 (17-58)	25 (13-48)	48 (32-67)	<0.0001

Table 1. Baseline characteristics, comorbidities, echocardiographic and laboratory findings of the overall cohort and by final diagnosis (ATTR-CM versus no ATTR-CM). Abbreviations: ATTR-CM, transthyretin cardiomyopathy.

Data are median (IQR) or No. (%).
[a] Race was self-reported and missing in 19 patients.
[b] Chronic obstructive pulmonary disease.
[c] Values available for 1378 patients.
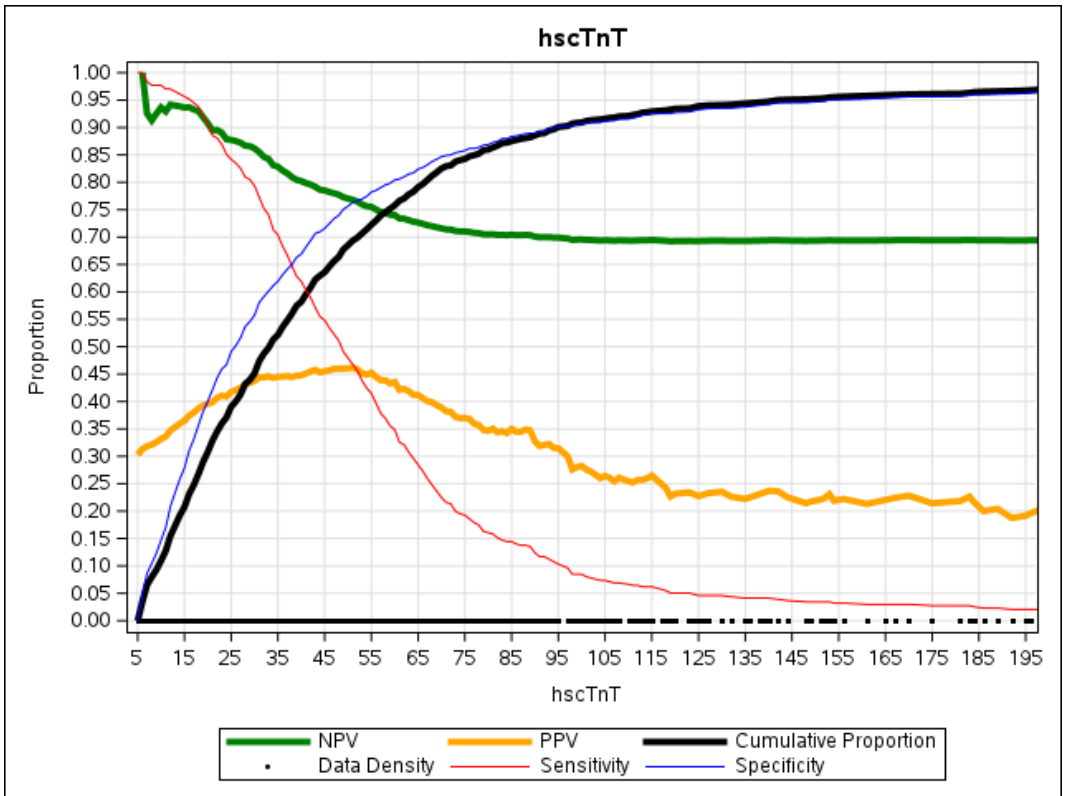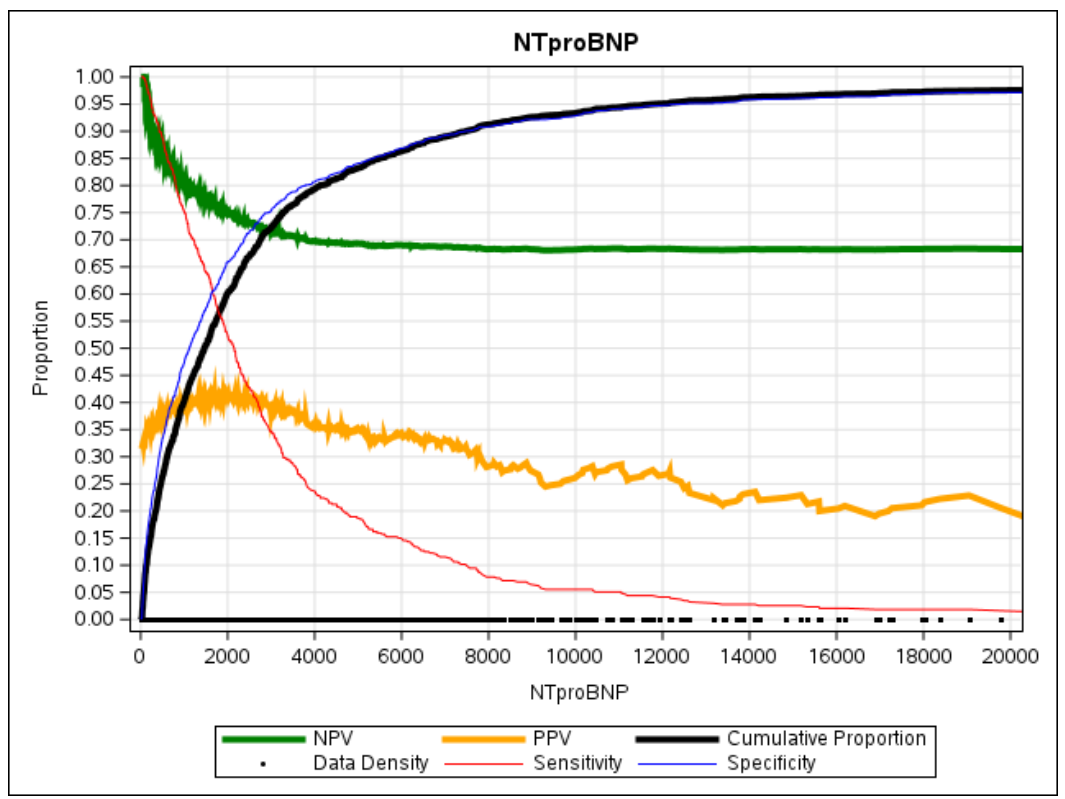
Figure 1. Diagnostic performance of hs-cTnT (Panel A) and of NT-proBNP (panel B) in patients undergoing PYP for suspected ATTR-CM at a large US referral center. Abbreviations: hs-cTnT, high sensitivity cardiac troponin T; NT-proBNP, N-terminal pro B-type natriuretic peptide, NPV, negative predictive value; PPV, positive predictive value.

Biomarker	Threshold	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95% CI)	PPV (95% CI)	TN n	FN n
Hs-cTnT	6 ng/l	100 (99, 100)	5 (4, 7)	100 (93, 100)	31 (29, 34)	50	0
	12 ng/l	97 (95, 98)	21 (18, 24)	94 (90, 97)	35 (32, 37)	210	13
	14 ng/L	96 (94, 97)	26 (23, 29)	94 (90, 96)	36 (33, 39)	260	17
	20 ng/l	90 (87, 93)	40 (37, 43)	91 (88, 93)	40 (37, 43)	403	41
	10 ng/l F 15 ng/l M	96 (92, 97)	22 (19, 24)	92 (88, 95)	35 (32, 37)	219	19
NT-proBNP	60 ng/l	100 (99, 100)	6 (4, 7)	100 (93, 100)	33 (30, 35)	53	0
	180 ng/l	97 (95, 98)	18 (15, 20)	93 (88, 96)	35 (32, 38)	167	13
Hs-cTnT/ NT-proBNP	6 ng/l 60 ngl	100 (99, 100)	2 (1, 3)	100 (79, 100)	32 (29, 34)	16	0
	6 ng/l 180 ng/l	100 (99, 100)	4 (3, 5)	100 (90, 100)	32 (30, 35)	36	0
	12 ng/l 60 ng/l	100 (99, 100)	4 (3, 6)	100 (91, 100)	32 (30, 35)	41	0
	12 ng/l 180 ng/l	99 (98, 100)	10 (8, 12)	96 (90, 99)	34 (31, 36)	97	4
	14 ng/L 180 ng/L	99 (97, 100)	11 (9, 14)	96 (90, 99)	34 (31, 36)	107	5

Table 2. Diagnostic performance of different hs-cTnT and NT-proBNP thresholds for ruling-out ATTR-CM in the overall 1442 patients undergoing PYP imaging.
Abbreviations: FN, false negative; hs-cTnT, high sensitivity cardiac troponin T; NT-proBNP, N-terminal pro B-type natriuretic peptide, NPV, negative predictive value; PPV, positive predictive value; CI, confidence interval; TN, true negative.

7.3.3 Combined hs-cTnT and NT-proBNP (Table 2 and 3)

The combination of hs-cTnT < 12 ng/L and NT-proBNP < 60 ng/L identified 41 patients (3.0% of

the overall cohort) without ATTR-CM, therefore with a 100% sensitivity and NPV for ruling-out

the diagnosis.

Biomarker	Threshold	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95% CI)	PPV (95% CI)	TP n (%)	FP n (%)
Hs-cTnT	153 ng/l	3 (2, 6)	95 (93, 96)	69 (67, 72)	23 (14. 35)	15	50
NT-proBNP	12421 ng/l	4 (2, 6)	95 (93, 96)	68 (66, 71)	25 (15, 38)	16	47

Table 3. Diagnostic performance of hs-cTnT and NT-proBNP thresholds for ruling-in ATTR-CM in the overall 1442 patients undergoing PYP imaging.
Abbreviations: FP, false positive; hs-cTnT, high sensitivity cardiac troponin T; NT-proBNP, N-terminal pro B-type natriuretic peptide, NPV, negative predictive value; PPV, positive predictive value; CI, confidence interval; TP, true positive.

7.4. Discussion

This is the first study to investigate the predictive value of cardiac biomarkers in a large US cohort undergoing PYP imaging for suspected ATTR-CM. We report several important findings. First, very low hs-cTnT values were present in few patients but when present, they demonstrated good performance to rule out ATTR-CM. Indeed, the highest sensitivity and NPV were at the lowest reportable value for the US (LoQ of the assay) of < 6 ng/L. Higher thresholds presented an increasing percentage of false negative results. Second, for ruling in ATTR-CM, a high hs-cTnT (153 ng/L) showed a specificity of 95%, but PPV remained low. Third, similar findings were found for NT-proBNP. Very low values showed good diagnostic performance for ruling out ATTR-CM; for ruling-in the disease, specificity was good at very high values (12421 ng/L) but PPV remained low. Fourth, the combination of hs-cTnT < 12 ng/L and NT-proBNP < 60 ng/L provided a sensitivity and NPV of 100% for ruling-out ATTR-CM, but it was present only in 3.0% of our cohort.

The ability to perform a non-invasive diagnosis of ATTR-CM in selected cases, together with new therapeutical advancements[7], has led to the need to identify patients at higher risk for ATTR-CM. For these reasons, scores and "red flags" have been proposed to identify patients with ATTR-CM among those with HFpEF, with aortic stenosis[6] or with carpal tunnel syndrome[8]. Conversely, there is also a need to identify those who do not need additional testing.

Recently, an European paper[12] reported that hs-cTnT and NT-proBNP hold diagnostic value in patients with suspected cardiac amyloidosis (CA). A final diagnosis of CA (either AL or ATTR) occurred in over 60% of cases. A low hs-cTnT defined as < 14 ng/L and NT-proBNP (<180 ng/L) were useful in identifying patients at low risk for CA, whilst hs-cTnT> 86 ng/L was helpful to identify those with high probability of disease. In the validation cohort, hs-cTnT of 14 ng/L had a 98% (96, 99) sensitivity and 91% (86, 96) NPV to exclude disease; similar results are reported for NT-proBNP at 180 ng/L. The combination of the two biomarkers below the thresholds was present in 78 patients of the validation cohort with 4 false negatives. For ruling in, hs-cTnT of 86 ng/L had a 95% (91,97) specificity and 89% (84, 94) PPV for CA. Results were consistent in the subgroup analysis of those referred for suspected ATTR-CM.

In this study of US patients with suspected ATTR-CM, we confirm that low hs-cTnT and NT-proBNP can identify patients at low risk of ATTR-CM. For hs-cTnT, the lowest reportable value in the US of < 6 ng/L (LoQ of the assay, present in 3.5% of the cohort) was able to avoid false negative results. This was similar for NT-proBNP; very low values < 60 ng/L (present in 3.8 % of the cohort) were able to avoid false negative results. At progressively higher thresholds, the number of patients ruled out increased but so did the number of false negative results. The number of false negatives remained low in the overall cohort but not negligible among those with cardiac biomarkers below the specific thresholds. The combination of hs-cTnT < 12 ng/L and NT-proBNP<60 ng/L probed by others in our data set provided sensitivity and NPV of 100% but was present in a minority (3%) of patients. Based on our results, it appears that very low thresholds can safely rule-out ATTR-CM. Higher thresholds, even below the 99th % upper reference limit of such tests, demonstrate a proportion of false negative results warranting closer clinical attention. Nevertheless, integrating cardiac biomarkers in the diagnostic algorithm of ATTR-CM is reasonable and might help in selecting patients needing further, more expensive imaging tests.

On the other hand, for ruling-in ATTR-CM, high values of hs-cTnT and NT-proBNP were probed and achieved a specificity of 95%, but PPV remained low. Therefore, we could not find specific,

clinically useful thresholds for hs-cTnT and NT-proBNP to rule in ATTR-CM. This may be due to the clinical characteristics of our cohort of all-comers for PYP imaging, with a rule in frequency for ATTR-CM of 30%. Similarly to our results, Vergaro et al[11] could not identify a reliable cut-off for NT-proBNP to rule in CA, due to the high percentage of patients with elevated NT-proBNP in the overall cohort. We encountered a similar issue for hs-cTnT, and we could not define a clinically applicable threshold. This probably reflects the clinical characteristics of contemporary cohort of patients with suspected ATTR-CM; such patients are frequently old and with a significant burden of comorbidities that are responsible for elevated hs-cTnT values even in the absence of ATTR-CM. Limitations exist. This is a retrospective study of patients in a referral-based population, which might introduce selection bias. The present study was performed in a large referral Center, and the same findings may not be confirmed in settings with lower disease prevalence. Moreover, only patients with available hs-cTnT values within 6 months were included, again with potential selection bias.

7.5 Conclusion

In patients undergoing PYP imaging for suspected ATTR-CM, very low hs-cTnT values can be of help in ruling out the diagnosis. At increasing thresholds, false negative results are present, and this should be taken into consideration in clinical practice. On the other hand, high hs-cTnT has limited predictive value in ruling-in disease. Similar results were found for NT-proBNP and for the combination of the two biomarkers.

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Chapter 8. High sensitivity cardiac troponin I in patients with transthyretin cardiac amyloidosis

8.1 Introduction

Systemic amyloidosis is a protein misfolding disorder caused by extracellular deposition of amyloid leading to organ dysfunction[1]. Depending on the precursor amyloidogenic protein, different organs can be involved. Cardiac amyloidosis (CA)[2] is nowadays increasingly recognized as cause of heart failure (HF)[3]. There are two main types of amyloidosis involving the heart: light chain (AL) amyloidosis and transthyretin (ATTR) amyloidosis; the later exists in two forms, variant/hereditary (ATTRv) and wild-type (ATTRwt)[1]. Although the prognosis of AL and ATTR amyloidosis is different, at present effective treatment options exist for both[4,5] and their efficacy is highly dependent on the disease stage at diagnosis.

Cardiac troponin (cTn), marker of myocardial injury, and natriuretic peptides [NP, particularly brain natriuretic peptide (BNP) and the N-terminal fragment (NT-proBNP)], markers of myocardial stretch and stress, are powerful prognostic markers for both AL and ATTR amyloidosis[6,7,8].

In AL amyloidosis, the heart and the kidneys are the most frequently affected organs[9] but the major determinant of outcome is the extent of cardiac involvement[10]. When combined in a staging system, cTn and NT-proBNP constitute a reliable and easily accessible tool for risk stratification[11,12,13,14].

Similarly, the clinical course and prognosis of ATTR-CA are related to the severity of cardiac involvement[8]. A staging system combining cTnT and NT-proBNP has been proposed for ATTRwt-CA, grading the disease severity from Stage I to Stage III[8] with a good correlation with survival. A different staging system based on NT-proBNP and glomerular filtration rate (GFR) has been validated for both ATTRwt-CA and ATTRv-CA[15], with the advantage of not being related to the type of troponin assay used at each Institution.

Both cTn T (cTnT) and I (cTnI) were initially assessed for their prognostic role in AL amyloidosis; cTnT had superior prognostic discrimination over cTnI in patients with AL amyloidosis using contemporary (not high sensitivity) assays[7]. Moreover, multiple cTnI assays were available, while

there was only one cTnT assay, which allowed for greater standardization and reproducibility using cTnT. Subsequently, with the development of a fifth generation cTnT assay, with analytical characteristics of a high-sensitivity (hs) assay, new thresholds were identified for the different staging systems[16]. Risk stratification models including hs-cTnT values performed better than those including contemporary cTnI values[11].

Nowadays, multiple high sensitivity cTnI (hs-cTnI) assays are available and validated for clinical use, with different analytical characteristics such that, for example, the sex specific 99th percentile upper reference limits (99th % URLs) are different and specific for each single assay. For this reason, for example, multiple assay-specific thresholds are available for the management of patients with suspected acute coronary syndrome[17]. Moreover, growing body of evidence is available showing the peculiarities and differences between hs-cTnI and hs-cTnT in clinical practice[18,19,20,21,22,23].

In this landmark, studies investigating and validating the prognostic role of hs-cTnI for risk stratification in patients with CA, particularly ATTR-CA, are still lacking. This is of crucial importance to allow for a more widespread and consolidated use of cTn-based CA staging systems, since many Institutions utilize hs-cTnI assays in clinical practice. The aims of this study were twofold: first, to assess the prognostic performance of hs-cTnI measured at diagnosis in patients with ATTR-CA; second, to derive assay-specific thresholds for risk stratification of these patients. Once validated, these thresholds could be used in clinical practice and integrated in the staging system for ATTR-CA.

8.2 Methods

This study was approved by the Institutional Review Board (AOP3013). This is a retrospective observational multicenter study of patients diagnosed with ATTR amyloidosis. Inclusion criterium was an established in vivo diagnosis of ATTR amyloidosis, as per the recent European Consensus Document[2] and Guidelines[24]. Exclusion criteria were the lack of availability of hs-cTnI values at

baseline or the availability of measurements only in acute clinical conditions, the lack of follow-up data and the enrollment in clinical trials.

8.2.1 Data Collection

For all patients, besides hs-cTnI values, we tabulated baseline clinical characteristics, comorbidities, if ATTR is wild-type or variant, and laboratory data at baseline. Moreover, we tabulated baseline electrocardiographic and echocardiographic parameters, treatment regimens (diuretic treatment and disease-modifying therapy, including gene-silencers[25,26] and tafamidis[5]) and outcome data (all cause death). Patients evaluated with the Siemens assay were all affected by ATTRwt-CA.

8.2.2 High-sensitivity cardiac troponin I

We investigated the prognostic significance of hs-cTnI measured at diagnosis in different cohorts with different assays, including the Abbott Architect Stat High Sensitive Troponin I assay (Cardiology Clinic, Department of Cardio-Thoraco-Vascular Sciences and Public Health, University of Padua, Padua, Italy; Cardiology, Department of Clinical and Molecular Medicine, Sapienza University of Rome, Sant'Andrea Hospital, Rome, Italy), the Beckman Coulter Access High Sensitivity Troponin I assay (Cardiology Unit, St. Orsola Hospital, IRCCS Azienda Ospedaliero— Universitaria di Bologna, Italy; Cardiologic Centre, Azienda Ospedaliero Universitaria of Ferrara, Italy; Cardiovascular Department, Azienda Sanitaria Universitaria Integrata, Trieste, Itay) and the Siemens Centaur XPT High- Sensitivity TnI assay (Amyloidosis Research and Treatment Center, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy). The first assay has a limit of detection (LoD) of 2 ng/L and 99th % URLs of 16 ng/L for women and 34 ng/L for men. The second assay has a LoD of 2.3 ng/L and recommended 99th %URLs of 11.6 ng/l for women and 19.8 ng/l for men. The third assay has a LoD of 1.6 ng/L and sex specific 99th % URLs of 40 ng/L for women and 58 ng/L for men. These cohorts have been analyzed separately; subsequently, based on assays characteristics and on available literature[27,28] showing high correlation

between hs-cTnI concentrations between the Beckman and Abbott assays, we also analyzed these two cohorts together. Baseline hs-cTnI value is defined as a value measured within 6 months before or after ATTR- CA diagnosis in stable clinical conditions, provided that no specific treatment is initiated between ATTR-CA diagnosis and hs-cTnI measurement. We did not collect hs-cTnI values measured during acute clinical scenarios, including acute HF or sepsis, but just those evaluated in stable clinical conditions. If hs-cTnI was only available during a hospitalization, we collected the value just before discharge.

8.2.3. Statistical analysis

Useful samples sizes were estimated based on the prevalence of adverse outcomes (n=281 for 15% mortality, n=215 for 20% mortality, n=176 for 25% mortality). Continuous variables are expressed as median with 25th and 75th percentiles (Q1-Q3) and were compared using the Wilcoxon rank sum test. Categoric variables are expressed as absolute numbers and percentages and were compared using the chi-square test or Fisher exact test as appropriate. Hs-cTnI was analyzed as a continuous variable and dichotomized according to assay-specific population-derived thresholds for risk stratification; when indicated, hs-cTnI values were log-transformed for the analysis. Receiver-operating characteristic (ROC) curves have been constructed to establish optimal hs-cTnI and eGFR cut-offs for risk stratification for mortality. The optimal cut-off value was defined as the point with the highest sum of sensitivity and specificity as calculated using the Youden method. For NP, high levels were considered as NT-proBNP >3000 ng/L or BNP >250 ng/L as reported in the Literature[8,15,29]. Cox proportional hazards regression models are used to test for association of different variables with mortality; one variable every 10 events was inserted in the models to avoid overfitting. Results are summarized with hazard ratio (HR) and 95% confidence interval (CI). Kaplan-Meier methods is used to plot survival by time, and the log- rank test was used to test for differences between groups. Cox model with time-dependent covariates was used to assess the impact of specific disease-modifying treatment (tafamidis or gene-silencers) on the prognostic role of hs-cTnI. Indeed, for many patients,

disease-modifying treatment was started during follow-up and not at diagnosis. Two staging system were developed for the Abbott and Beckman pooled cohort, one with 2 variables (hs-cTnI> 80 ng/L and elevated NP, based on the Mayo Clinic staging system[8]) and one with 3 variables, including also eGFR (with a population-derived threshold of < 50 ml/min/m2) based on previous observations[15]. Time-dependent area under the curve (AUC) of the corresponding ROC curves for the different staging systems were plotted and compared. The National Amyloidosis Center (NAC) staging system was calculated with previously published cut-offs[15] but utilizing NT-proBNP >3000 ng/L or BNP >250 ng/L to define elevated NP. Harrell's c-statistic was calculated to measure the discriminatory ability of each model. Further analyses with ROC curves cross validation to strengthen our findings are ongoing. For the analyses, R (v. 4.2.2) and IBM SPSS Statistics 28.0 package (New York, NY) were used.

8.3 Results

8.3.1 Study population

Study flow-chart is reported in Figure 1. Overall, a total of 434 patients with ATTR-CA were included, of which 123 evaluated with the Abbott assay, 107 with the Beckman assay and 204 with the Siemens assay. Baseline characteristics of the three cohorts are reported in Table 1.
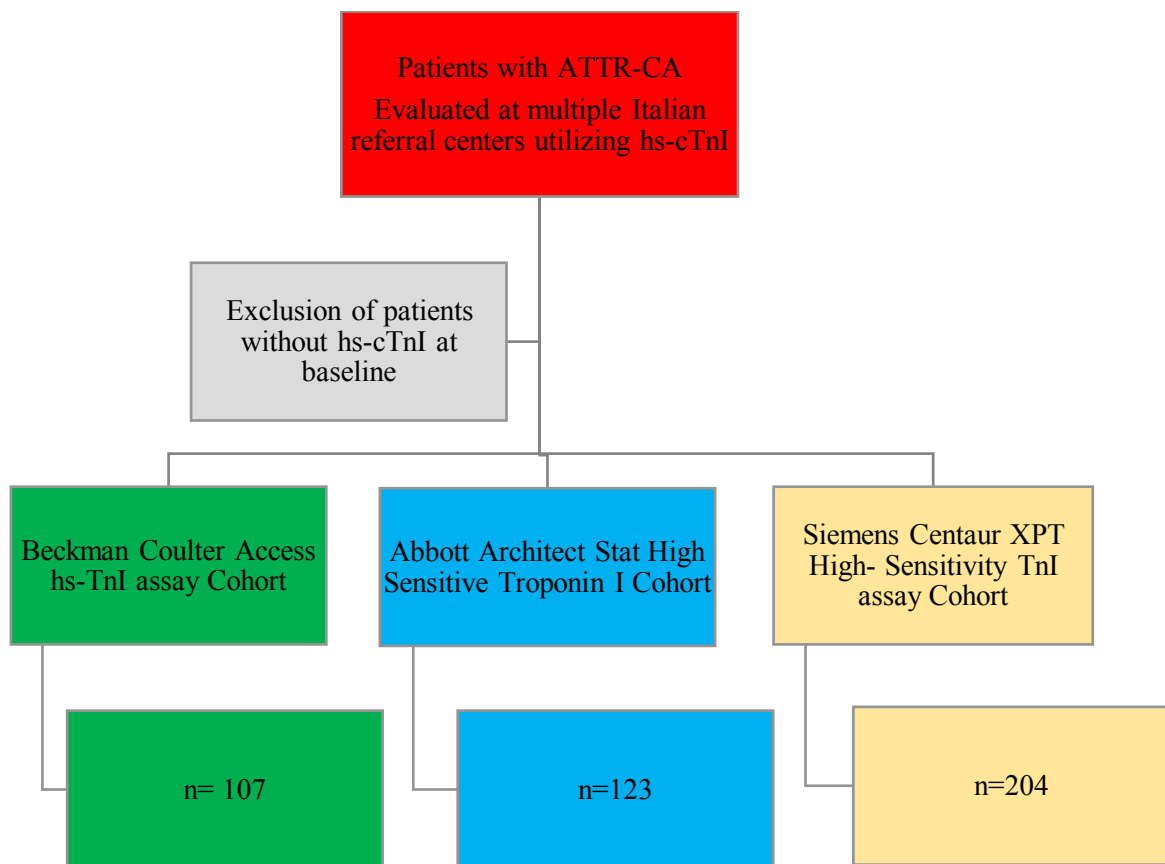

Figure 1. Study flow chart. Abbreviations: ATTR-CA, transthyretin cardiac amyloidosis; hs-cTnI, high sensitivity cardiac troponin I.

Variable	N	Abbott cohort n=123	N	Beckman cohort n=107	N	Siemens cohort n=204
Age	123	78 (72, 82)	107	77 (72-81)	204	78 (73-82)
Sex M	123	113 (92)	107	96 (90)	204	196 (96)
ATTRv	123	16 (13)	107	9 (8)	204	0 (0)
Hypertension	123	88 (72)	107	74 (69)	204	122 (61)
Diabetes	123	23 (19)	107	16 (15)	204	35 (17)
Significant CAD	123	36 (29)	107	20 (19)	204	32 (16)
AFib/flutter	123	77 (63)	107	55 (51)	204	106 (52)
PM/ICD	123	20 (16)	107	12 (11)	204	43 (21)
NYHA class	122		102		191	
I		21(17)		23 (23)		34 (18)
II		75(61)		62 (61)		130 (68)
III		27(22)		17 (17)		27 (14)
IV		0 (0)		0 (0)		0 (0)
Furosemide equivalent dose	119	25 (0-50)	101	25 (0-75)	196	25 (12.5-50)
Systolic blood pressure	123	120 (110-130)	103	130 (120-140)	191	134 (122-146)
Disease-modifying therapy	123	70 (57)	197	49 (46)	92	55 (60)
Months of disease-modifying therapy	123	16 (11-19)	197	10 (5-12)	92	13 (10-25)
Electrocardiogram at baseline						
Low QRS voltages	123	33 (27)	107	33 (31)	73	22 (30)
AFib at ECG	123	54 (44)	107	41 (38)	151	74 (49)
Echocardiogram at baseline						
IVS mm	123	17 (15-20)	107	18 (16-19)	179	17 (15-19)
PWT mm	123	16 (14-17)	107	16 (14-17)	150	15 (14-17)
LVEF	123	54 (45-59)	106	55 (47-61)	186	50 (42-55)
E/e'	111	15 (12-19)	103	19 (15-22)		---
sPAP mmHg	116	35 (28-45)	85	40 (32-50)	68	35 (27-42)
TAPSE	110	16 (14-20)	69	18 (16-20)	51	15 (13-17)
RV hypertrophy	122	81 (66)	100	57 (53)		---
Pericardial effusion (> 10 mm)	123	16 (13)	105	12 (11)	77	12 (16)
Laboratory values at baseline						
Hb baseline g/dL	115	13.9 (12.8-15.2)	89	13.7 (12.5-15)	196	13.9 (12.8-14.6)
eGFR baseline ml/min/m2	117	63 (52 – 80)	103	69 (48-81)	202	67 (51-80)
NT-proBNP baseline ng/L	95	1671 (842-4091)	57	1957 (891-4174)	195	3378 (1810-6369)
BNP baseline ng/L	91	405 (206-709)	45	278 (215-489)	31	391 (289-544)
Hs-cTnI baseline ng/L	123	74 (41-158)	107	57 (32-86)	204	55 (34-97)
NAC score	117		94		195	
Stage I		42 (36)		45 (48)		83 (43)
Stage II		61 (52)		38 (40)		94 (48)
Stage III		14 (12)		11 (12)		18 (9.2)

Table 1. Baseline characteristics of patients with ATTR-CA in the different hs-cTnI cohorts. Continuous variables are reported as median and interquartile range (Q1-Q3); categorical variables as number and

percentages. Abbreviations: AFib, atrial fibrillation; ATTRv, variant transthyretin amyloidosis; BNP, B-type natriuretic peptide; CAD, coronary artery disease; ECG, electrocardiogram; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; hs-cTnI, high sensitivity cardiac troponin I; IVD, interventricular septum; LVEF, left ventricular ejection fraction; M, males; NAC, National Amyloidosis Center; NYHA, New York Heart Association; NT-proBNP, N-terminal pro B-type natriuretic peptide; PM/ICD, pace-maker/implantable cardioverter defibrillator; PWT, posterior wall thickness; sPAP, systolic pulmonary artery pressure; TAPSE, tricuspid annular plane systolic excursion; RV, right ventricle.

8.3.2 Abbott Architect Stat High Sensitive Troponin I Cohort

In this cohort, over a median follow-up of 24 (17-44) months, a total of 30 (24%) patients died. At ROC curve analysis, the area under the curve (AUC) for hs-cTnI for mortality was 0.80 (95% CI 0.72-0.89) (Figure 2, panel A). The derived optimal threshold for risk stratification for hs-cTnI resulted to be 80 ng/L (sensitivity 87%, specificity 68%), consistent also when considering only ATTRwt-CA (sensitivity 88%, specificity 65%). Clinical characteristics of patients with hs-cTnI below/equal to 80 ng/L and above 80 ng/L are reported in Table 2. At Kaplan Meier analysis (Figure 2, panel B), patients with hs-cTnI > 80 ng/L had significantly worse survival than those with hs-cTnI ≤ 80 ng/L (Log rank p <0.001).
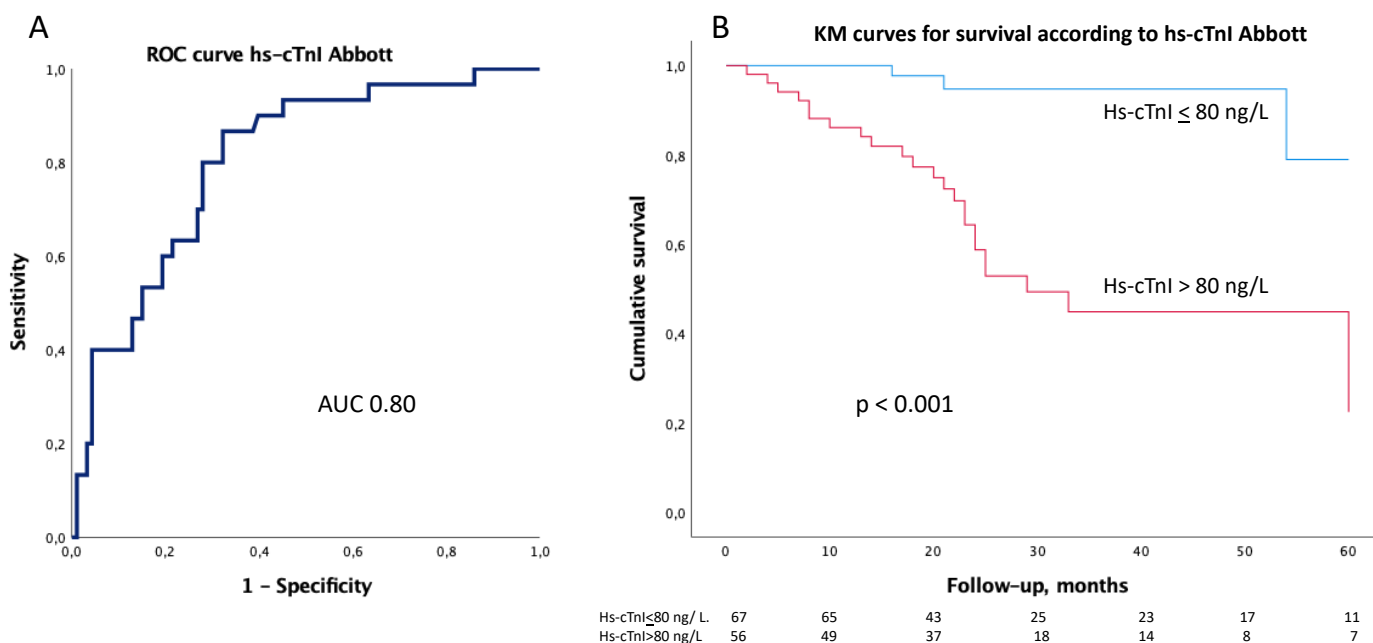

Figure 2. Receiver-operating characteristic (ROC) curve (Panel A) for hs-cTnI Abbott for mortality. Panel B: Kaplan-Meier (KM) curves for survival based on the threshold of hs-cTnI Abbott of 80 ng/L. Abbreviations: AUC, area under the curve; hs-cTnI, high sensitivity cardiac troponin I.

Variable	Abbott cohort			Beckman cohort			Siemens cohort		
	Hs-cTnI≤80 ng/L n=67	Hs-cTnI>80 ng/L n=56	P value	Hs-cTnI≤80 ng/L n=75	Hs-cTnI>80 ng/L n=32	P value	Hs-cTnI≤56 ng/L n=107	Hs-cTnI>56 ng/L n=97	P value
Age	76 (72-80)	81 (72-83)	**0.023**	76 (70-81)	80 (75-83)	**0.032**	77 (72-81)	79 (76-83)	**0.004**
Sex M	60 (90)	53 (95)	0.25	66 (88)	30 (94)	0.37	101 (94)	95 (98)	0.284
ATTRv	11 (16)	5 (8.9)	0.22	7 (9.3)	2 (6.3)	0.72	NA	NA	NA
Hypertension	41 (62)	47 (84)	**0.007**	47 (63)	27 (84)	**0.026**	64 (61)	58 (60)	0.938
Diabetes	13 (20)	10 (18)	0.80	12 (16)	4 (13)	0.77	20 (19)	15 (16)	0.543
Significant CAD	20 (30)	16 (29)	0.83	15 (20)	5 (16)	0.60	22 (21)	10 (10)	**0.044**
AFib/flutter	40 (61)	37 (66)	0.53	36 (48)	19 (59)	0.28	44 (41)	62 (64)	**0.001**
PM/ICD	6 (9)	14 (25)	**0.016**	9 (12)	4 (13)	1.00	18 (17)	25 (26)	0.118
NYHA class			**0.004**			0.64			0.536
I	16 (24)	5 (8.9)		18 (25)	5 (17)		20 (20)	14 (15)	
II	43 (64)	32 (57)		42 (58)	20 (67)		68 (68)	62 (68)	
III	8 (12)	19 (34)		12 (17)	5 (17)		12 (12)	15 (17)	
IV	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
Furosemide equivalent dose	9.75 (0-43.75)	50 (25-100)	**<0.001**	25 (0-75)	50 (25-100)	**0.017**	25 (0-50)	45 (25-75)	**<0.001**
Low QRS voltages	22 (33)	11 (20)	0.10	22 (29)	11 (34)	0.61	9 (27)	13 (18)	0.524
AFib at ECG	27 (41)	27 (48)	0.42	27 (36)	14 (44)	0.45	29 (39)	45 (62)	**0.003**
IVS mm	17 (15-19)	18 (16-20)	**0.009**	17 (16-19)	18 (15-20)	0.64	17 (15-19)	18 (16-20)	**0.006**
PWT mm	15 (13-17)	16 (15-18)	**0.003**	16 (14-17)	15 (14-17)	0.28	15 (13-16)	16 (14-18)	**<0.001**
LVEF %	55 (47-60)	50 (39-55)	**<0.001**	57 (50-61)	48 (44-61)	**0.020**	53 (44-58)	45 (40-53)	**0.001**
E/e'	14 (10-15)	18 (15-22)	**<0.001**	17 (15-21)	21 (17-25)	**0.015**	ND	ND	
sPAP mmHg	32 (27-40)	41 (34-48)	**<0.001**	38 (30-48)	40 (35-50)	0.13	31 (25-42)	36 (28-43)	0.170
TAPSE	18 (15-20)	15 (13-19)	**0.015**	19 (17-20)	17 (13-18)	**0.004**	17 (14-18)	14 (12-16)	**0.011**
RV hypertrophy	40 (60)	41 (75)	0.08	40 (58)	17 (55)	0.77	ND	ND	
Pericardial effusion (> 10 mm)	7 (10)	9 (16)	0.36	10 (14)	2 (6.3)	0.34	4 (11)	8 (20)	0.311
Hb baseline g/dL	13.9 (13.1-15.4)	14 (12.4-15-2)	0.40	13.8 (12.8-15.1)	12.8 (12.3-14.5)	0.10	13.9 (13.0-14.5)	13.7 (12.8-14.6)	0.778
eGFR baseline ml/min/m2	72 (60-86)	53 (42-65)	**<0.001**	74 (57-86)	54 (33-69)	**<0.001**	72 (62-85)	61 (47-72)	**<0.001**

Variable	Abbott cohort			Beckman cohort			Siemens cohort		
	Hs-cTnI≤80 ng/L n=67	Hs-cTnI>80 ng/L n=56	P value	Hs-cTnI≤80 ng/L n=75	Hs-cTnI>80 ng/L n=32	P value	Hs-cTnI ≤ 56 ng/L n=107	Hs-cTnI > 56 ng/L n=97	P value
NT-proBNP baseline ng/L	1079 (583-1966)	3326 (1285-5839)	**<0.001**	1702 (674-2330)	4753 (1949-7464)	**0.002**	2277 (940-3953)	4915 (3116-9346)	**<0.001**
BNP baseline ng/L	230 (111-415)	634 (401-816)	**<0.001**	254 (212-410)	428 (292-986)	**0.031**	332 (153-412)	423 (307-603)	0.093
Hs-cTnI baseline ng/L	42 (27-59)	167 (101-281)	NA	40 (24-60)	111 (90-180)	NA	35 (25-47)	101 (70-232)	NA
NAC score			**<0.001**			**<0.001**			**<0.001**
Stage I	37 (59)	5 (9)		38 (58)	7 (25)		62 (60)	21 (23)	
Stage II	25 (40)	36 (67)		27 (41)	11 (39)		37 (36)	57 (62)	
Stage III	1 (1.6)	13 (24)		1 (1.5)	10 (37)		4 (3.9)	14 (15)	

Table 2. Clinical characteristics of patients with ATTR-CA according to the assay-specific threshold in the three cohorts. Abbreviations as per Table 1.

Univariable analysis for mortality is shown in Table 3. Hs-cTnI remained a significant and independent determinant of mortality even after adjustment for age and NYHA class (Table 4, model 1), for elevated NP and eGFR < 45 ml/min/m2 (Table 4, model 2) and for left ventricular ejection fraction (LVEF, %) and E/e' (as estimate of left ventricular filling pressures) (Table 4, model 3). Other models with echocardiographic variables significant at univariable analysis were tested and only hs-cTnI>80 ng/L remained an independent determinant of mortality (data not shown). At Cox regression analysis with time dependent covariates, hs-cTnI > 80 ng/L remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of hs-cTnI > 80 ng/L of 10.0 (95% CI 3.46-29.1), p<0.001; HR of disease-modifying therapy 0.40 (95% CI 0.14-1.16), p=0.09].

	Univariate analysis		
	HR	95% CI	p
Age (years)	1.10	1.04-1.17	0.002
Sex, M	1.04	0.24-4-43	0.96
ATTRv	1.36	0.51-3.59	0.54
NYHA > 2	2.33	1.10-4.93	0.027
LV EF (%)	0.96	0.93-0.997	0.033
E/e'	1.11	1.05-1.17	<0.001
sPAP (mmHg)	1.04	1.01-1.08	0.007
TAPSE (mm)	0.86	0.76-0.97	0.015
eGFR (ml/min/m2)	0.95	0.93-0.97	<0.001
eGFR < 45 ml/min/m2	3.43	1.49-7.89	0.004
NT-proBNP Ln	1.77	1.14-2.75	0.012
Elevated NP	3.81	1.3-11.02	0014
Hs-cTnI Ln	1.96	1.35-2.85	<0.001
Hs-cTnI > 80 ng/L	7.44	2.57-21.52	<0.001

Table 3. Univariable analysis for mortality in patients with ATTR-CA evaluated with the Abbott assay. Abbreviations: CI, confidence interval; HR, hazard ratio; Ln, natural log; NP, natriuretic peptides; p, p value. Remaining as per Table 1.

	Multivariable analysis		
	HR	95% CI	p
MODEL 1			
Age (years)	1.10	1.04-1.17	0.002
NYHA > 2	2.33	1.10-4.93	0.027
Hs-cTnI > 80 ng/L	7.44	2.57-21.52	<0.001
MODEL 2			
eGFR < 45 ml/min/m2	1.74	0.73-4.12	0.21
Elevated NP	1.78	0.58-5.48	0.32
Hs-cTnI > 80 ng/L	5.00	1.56-15.96	0.007
MODEL 3			
LV EF (%)	0.997	0.96-1.04	0.88
E/e'	1.07	1.01-1.14	0.036
Hs-cTnI > 80 ng/L	6.43	1.78-23.20	0.004

Table 4. Multivariable analysis for mortality in patients with ATTR-CA evaluated with the Abbott assay. Abbreviations as per Table 1 and 3.

8.3.3 Beckman Coulter Access hs-TnI assay Cohort

In this cohort, over a median follow-up of 20 (12-29) months, a total of 17 (16%) patients died. At ROC curve analysis, the area under the curve (AUC) for hs-cTnI for mortality was 0.79 (95% CI 0.64-0.93) (Figure 3, panel A). The derived optimal threshold for risk stratification for hs-cTnI resulted to be 80 ng/L (sensitivity 77%, specificity 79%), consistent also when considering only ATTRwt-CA (sensitivity 75%, specificity 78%). Clinical characteristics of patients with hs-cTnI below/equal to 80 ng/L and above 80 ng/L are reported in Table 2. At Kaplan Meier analysis (Figure 3, panel B), patients with hs-cTnI > 80 ng/L had significantly worse survival than those with hs-cTnI ≤ 80 ng/L (log rank p <0.001). Univariable analysis for mortality is shown in Table 5. Hs-cTnI remained a significant and independent determinant of mortality also after adjustment for age (Table 6, model 1), for eGFR < 45 ml/min/m2 (Table 6, model 2) and for elevated NPs (Table 6, model 3). Other models with echocardiographic variables significant at univariable analysis were tested and only hs-cTnI>80 ng/L and sPAP remained independent determinants of mortality (data not shown). At Cox regression analysis with time dependent covariates, hs-cTnI > 80 ng/L remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of hs-cTnI > 80

ng/L of 13.4 (95% CI 4.35-41.4), p<0.001; HR of disease-modifying therapy 0.55 (95% CI 0.12-2.50), p=0.4].
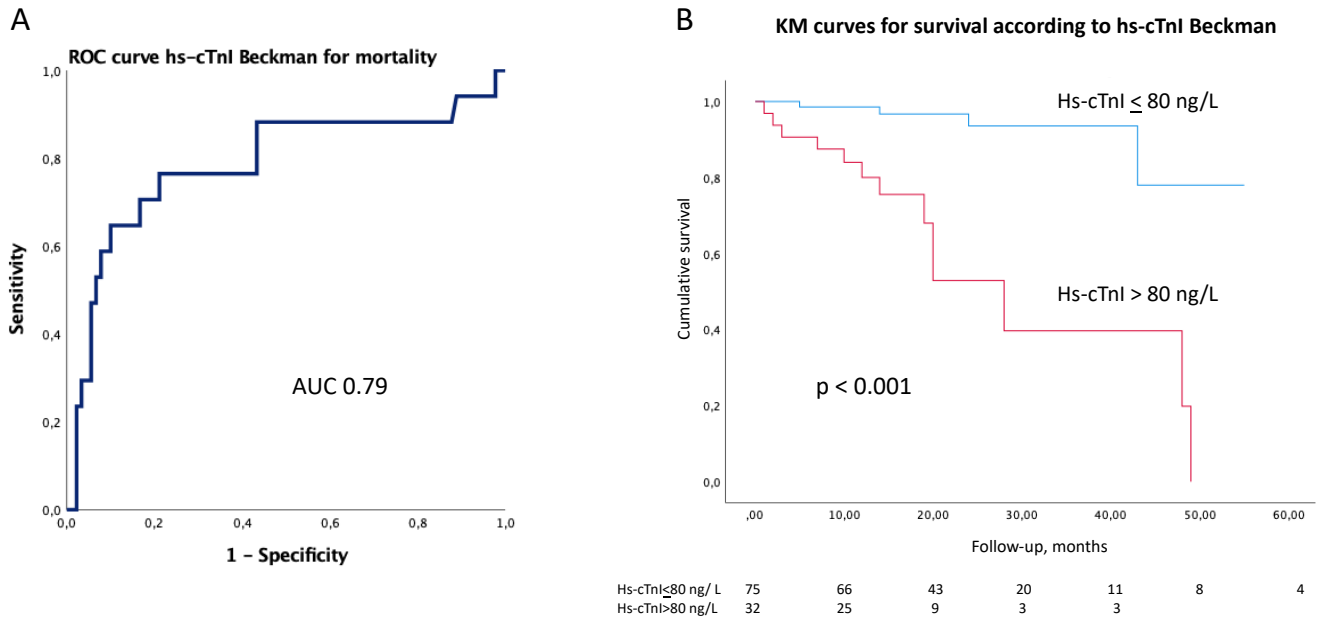

Figure 3. Receiver-operating characteristic (ROC) curve (Panel A) for hs-cTnI Beckman for mortality. Panel B: Kaplan-Meier (KM) curves for survival based on the threshold of hs-cTnI Beckman of 80 ng/L. Abbreviations as per Figure 2.

	Univariate analysis		
	HR	95% CI	p
Age (years)	1.12	1.03-1.21	0.008
Sex, M	1.53	0.34-6.83	0.58
ATTRv	0.45	0.06-3.43	0.44
NYHA > 2	1.49	0.48-4.58	0.49
LV EF (%)	0.95	0.91-0.99	0.018
E/e'	1.09	1.03-1.16	0.006
sPAP (mmHg)	1.05	1.01-1.10	0.029
TAPSE (mm)	0.87	0.77-0.98	0.019
eGFR (ml/min/m2)	0.96	0.93-0.98	<0.001
eGFR < 45 ml/min/m2	4.22	1.58-11.31	0.004
Elevated NP	7.21	1.97-26.37	0.003
Hs-cTnI Ln	2.08	1.36-3.19	<0.001
Hs-cTnI > 80 ng/L	12.17	3.9-37.88	<0.001

Table 5. Univariable analysis for mortality in patients with ATTR-CA evaluated with the Beckman assay. Abbreviations as per Table 1 and 3.

	Multivariable analysis		
	HR	95% CI	p
MODEL 1			
Age (years)	1.08	1.00-1.17	0.067
Hs-cTnI > 80 ng/L	9.8	3.09-31.1	<0.001
MODEL 2			
eGFR < 45 ml/min/m2	2.36	0.87-6.43	0.09
Hs-cTnI > 80 ng/L	9.8	3.06-31.37	<0.001
MODEL 3			
Elevated NP	2.06	0.52-8.16	0.31
Hs-cTnI > 80 ng/L	17.6	3.51-88.31	<0.001

Table 6. Multivariable analysis for mortality in patients with ATTR-CA evaluated with the Beckman assay. Abbreviations as per Table 1 and 3.

8.3.4 Combined Abbott and Beckman cohort

In the combined cohort of patients evaluated with the Abbott and Beckman assay (n=230), a total of 47 (20%) patients died during follow-up. At ROC curve analysis, the area under the curve (AUC) for hs-cTnI for mortality was 0.80 (95% CI 0.73-0.88) (Figure 4, panel A). The derived optimal threshold for risk stratification for hs-cTnI confirmed to be 80 ng/L (sensitivity 83%, specificity 73%). At Kaplan Meier analysis (Figure 4, panel B), patients with hs-cTnI > 80 ng/L had significantly worse survival than those with hs-cTnI ≤ 80 ng/L (lg rank p <0.001). Results were consistent when excluding 25 (11%) ATTRv patients. At Cox regression analysis with time dependent covariates, hs-cTnI > 80 ng/L remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of hs-cTnI > 80 ng/L of 11.3 (95% CI 5.24-24.3), p<0.001; HR of disease-modifying therapy 0.39 (95% CI 0.16-0.93), p=0.033].
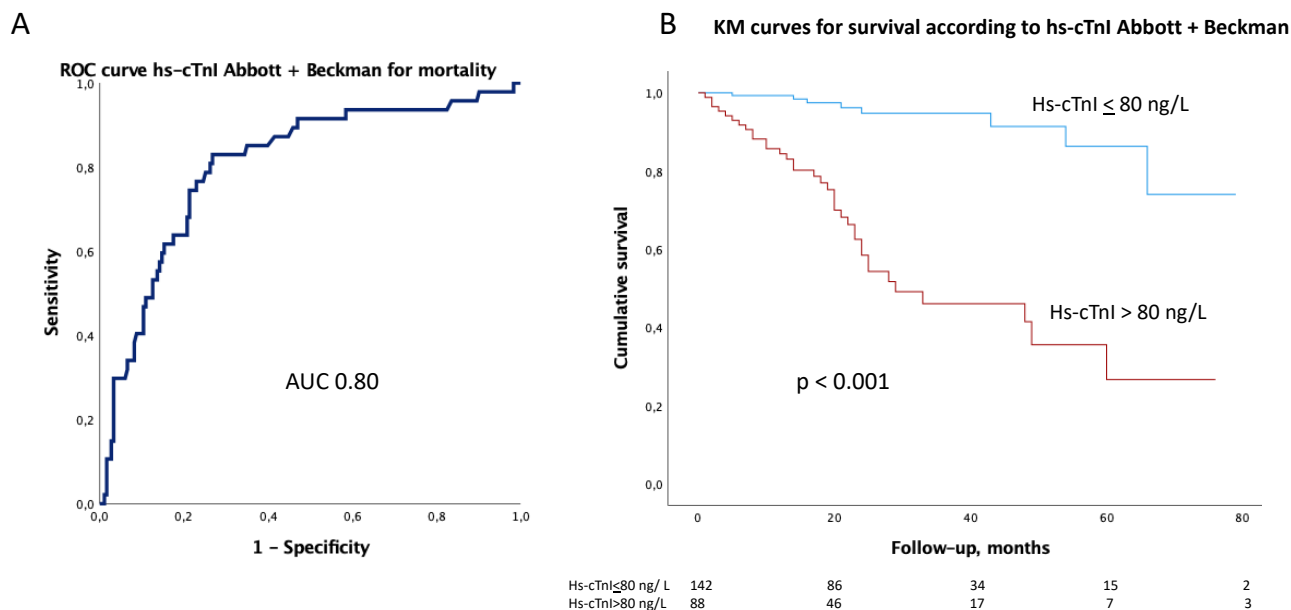
Figure 4. Receiver-operating characteristic (ROC) curve (Panel A) for hs-cTnI Abbott plus Beckman for mortality. Panel B: Kaplan-Meier (KM) curves for survival based on the threshold of hs-cTnI Abbott plus Beckman of 80 ng/L. Abreviations as per Figure 2.

Subsequently, a 2-variables staging system (based on the Mayo Clinic staging system[8]) was produced, using hs-cTnI (> 80 ng/L) and NP (NT-proBNP > 3000 ng/L or BNP > 250 ng/L); stage I was defined as both values being below the cutoff, stage II as one value being above, and stage III as both values being above. The age-adjusted HR was 5.82 (95% CI: 1.24-27.36) for one above and 21.25 (95% CI: 5.03 to 89.84) for two above for the overall cohort and results were consisted for the subset of ATTRwt-CA patients. In Figure 5 are reported the Kaplan-Meier curves for the overall cohort (Panel A) and for ATTRwt-CA (Panel B).

We also elaborated a 3-variables staging system based on hs-cTnI (> 80 ng/L), NP (> 3000 ng/L for NT-proBNP or 250 ng/L for BNP) and eGFR, with a population-derived threshold of eGFR < 50 ml/min/m2. Kaplan-Meier curves for the overall cohort (Panel A) and for ATTRwt-CA (Panel B) are reported in Figure 6. When comparing time-dependent AUC curves (Figure 7), the 3-variables staging system performed similarly to the 2-variables staging system at 12 and 48 months, while it was superior at 24 months (although borderline significant in the overall cohort) and at 36 months. Harrell's c-statistic for the different staging systems are reported in Table 7.

At Cox regression analysis with time dependent covariates, the 2-variables staging system remained a significant determinant of mortality also after adjustment for disease-modifying therapy [HR of the 2-variables staging system of 4.08 (95% CI 2.62-6-36), p<0.001; HR of disease-modifying therapy 0.43 (95% CI 0.18-1.02), p=0.056] and so did the 3-variables staging system [HR of the 3-variables staging system of 2.86 (95% CI 2.14-3.81), p<0.001; HR of disease-modifying therapy 0.49 (95% CI 0.20-1.17), p=0.11].
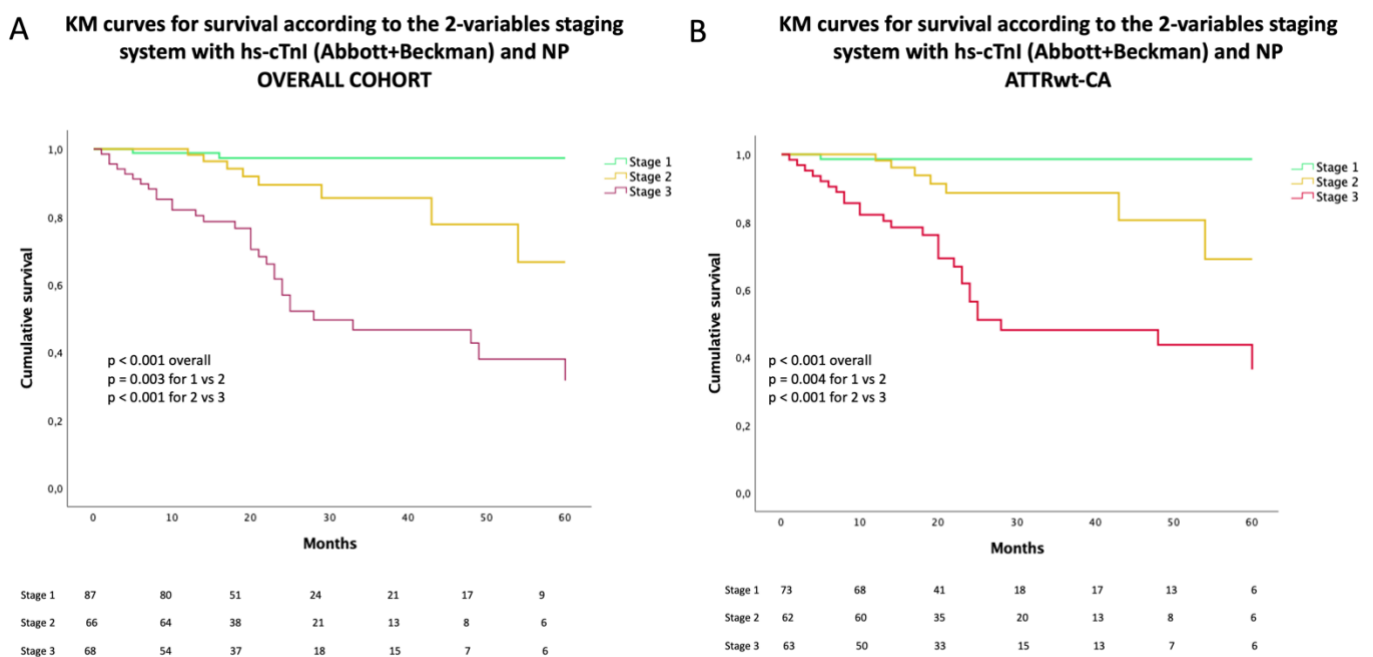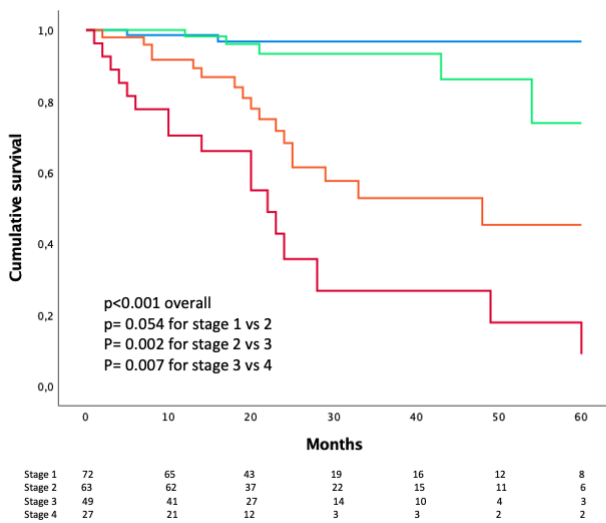

Figure 5. Kaplan-Meier (KM) curves for the overall cohort (Panel A) and for ATTRwt-CA (Panel B) according to the 2-variables staging system (based on Mayo Clinic staging system) with hs-cTnI (> 80 ng/L) and NP (NT-proBNP > 3000 ng/L or BNP > 250 ng/L). Abbreviations: ATTRwt-CA, wild-type transthyretin amyloidosis; hs-cTnI, high sensitivity cardac troponin I; NP, natriuretic peptides.
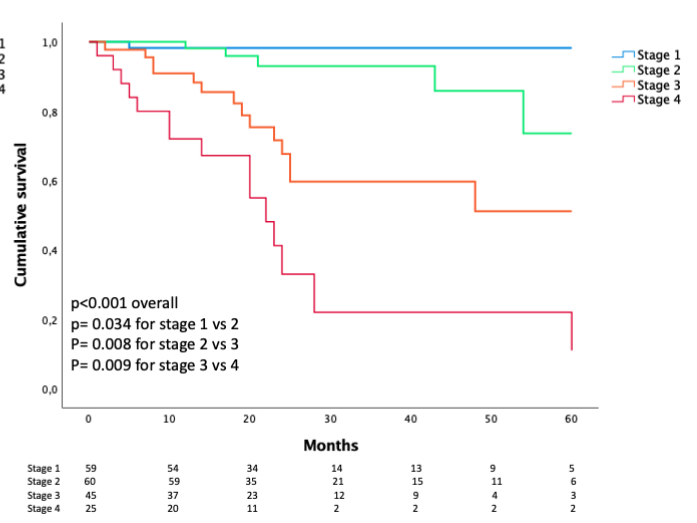
Figure 6. Kaplan-Meier (KM) curves for the overall cohort (Panel A) and for ATTRwt-CA (Panel B) according to the 3-variables staging system with hs-cTnI (> 80 ng/L), NP (NT-proBNP > 3000 ng/L or BNP > 250 ng/L) and eGFR (population-derived threshold < 50 ml/min/m2). Abbreviations: eGFR, estimated glomerular filtration rate. Remaining as per Figue 5.
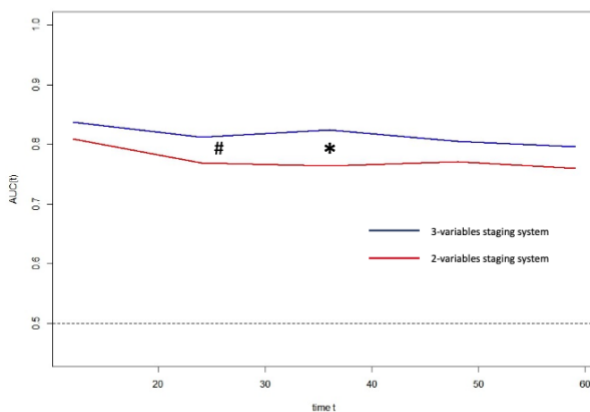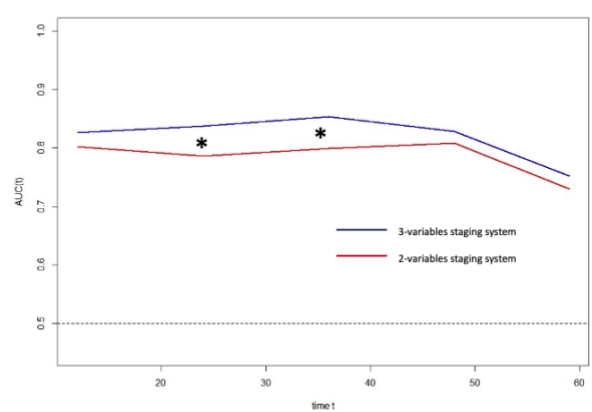
*P < 0.05 between AUCs at 36 months for the overall cohort and at 24 and 36 month in ATTRwt-CA; # p=0.05 at 24 months in the overall cohort.

Figure 7. Time-dependent area under the curve (AUC) of the 2-variables staging system (red) and the 3-variables staging system (blue) for the overall cohort (Panel A) and for ATTRwt-CA (Panel B). Abbreviations as per Figure 5 and 6.

	Global	12 months	24 months	36 months	48 months	60 months
Overall Cohort						
eGFR + NP (NAC system)	0.713	0.715	0.713	0.716	0.709	0.743
Hs-cTnI + NP (Mayo system)	0.771	0.786	0.772	0.780	0.773	0.778
Hs-cTnI + NP + eGFR	0.798	0.809	0.800	0.813	0.796	0.807
ATTRwt-CA						
eGFR + NP (NAC system)	0.709	0.689	0.718	0.727	0.715	0.738
Hs-cTnI + NP (Mayo system)	0.777	0.775	0.780	0.794	0.782	0.773
Hs-cTnI + NP + eGFR	0.804	0.791	0.813	0.824	0.802	0.800

Table 7. Harrell's C statistics for different the different staging systems (global and at different time points) in the overall Abbott + Beckman cohort and in patients with ATTRwt-CA.

8.3.5 Siemens Centaur XPT High- Sensitivity TnI assay Cohort

Overall median follow-up in this cohort was 20 (9-33) months. A total of 32 (15%) patients died during follow-up. At ROC curve analysis, the area under the curve (AUC) for hs-cTnI for mortality was 0.67 (95% CI 0.59-0.73) (Figure 8, panel A). The derived optimal threshold for risk stratification for hs-cTnI resulted to be 56 ng/L (sensitivity 77%, specificity 58%). Clinical characteristics of patients with hs-cTnI below/equal to 56 ng/L and above 56 ng/L are reported in Table 2. At Kaplan Meier analysis (Figure 8, panel B), patients with hs-cTnI > 56 ng/L had significantly worse survival than those with hs-cTnI ≤ 56 ng/L (Log rank p <0.001). Hs-cTnI remained a significant and independent determinant of mortality even after adjustment for age and LV EF [HR of hs-cTnI > 56 ng/L of 3.14 (95% CI 1.23-8.05), p=0.017] and for age and elevated NP [HR of hs-cTnI > 56 ng/L of 2.70 (95% CI 1.09-6.71), p=0.033]. Further analyses on the interaction of hs-cTnI and disease-modifying therapy, as well as the possible application of staging systems, are ongoing in this cohort.
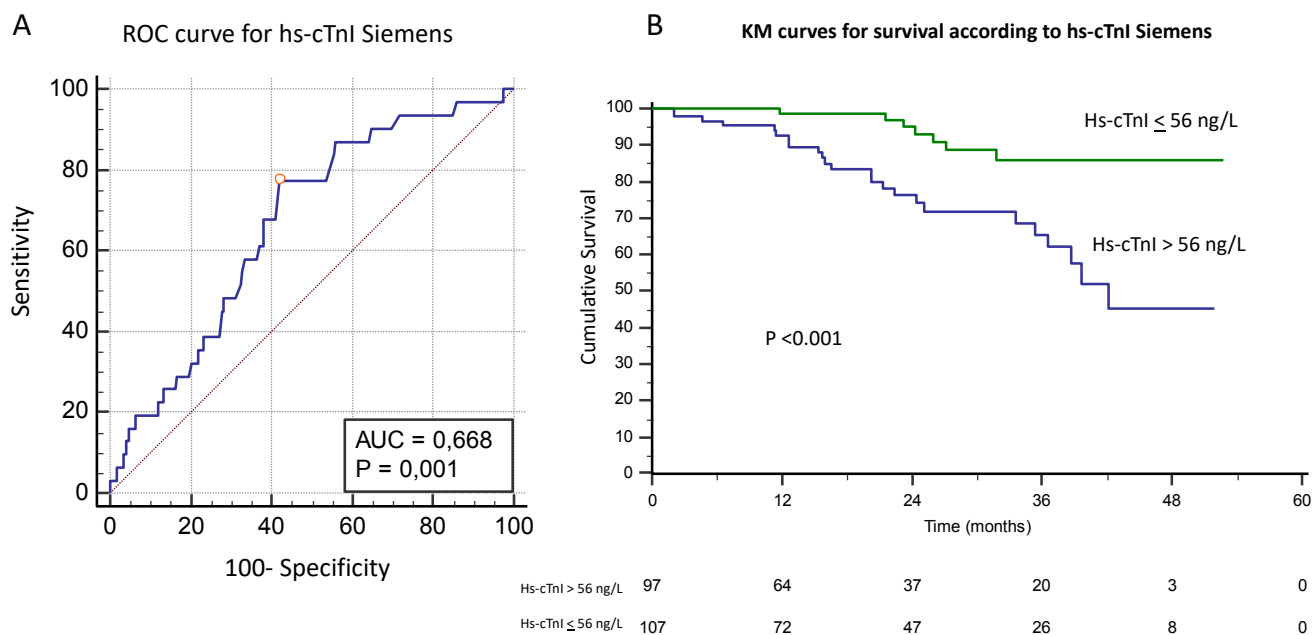
Figure 8. Receiver-operating characteristic (ROC) curve (Panel A) for hs-cTnI Siemens for mortality. Panel B: Kaplan-Meier (KM) curves for survival based on the threshold of hs-cTnI Siemens of 56 ng/L. Abbreviations: AUC, area under the curve, hs-cTnI, high sensitivity cardiac troponin I.

8.4 Discussion

In this multicenter observational study, we analyzed the prognostic role of hs-cTnI measured with three different assays in three different cohorts of patients affected by ATTR-CA. We report several important findings. First, hs-cTnI has a strong, independent prognostic role in patients with ATTR-CA, particularly ATTRwt-CA, consistent across the different assays and when adjusted for age, echocardiographic parameters and relevant laboratory markers including NPs and eGFR. For the Abbott and Beckman cohorts, we also verified its prognostic role when adjusted for disease-modifying therapy as a time-dependent covariate. Second, for the Abbott and Beckman hs-cTnI assay, a threshold of 80 ng/L appears to be useful in risk stratification of patients with ATTR-CA. Based on the number of patients included, we cannot infer on this threshold separately on patients with ATTRv, but we confirmed its prognostic role separately in those with ATTRwt-CA. Third, a 2-variables staging system (based on hs-cTnI Abbott/Beckman and NP) and a 3-variables staging system (based on hs-cTnI Abbott/Beckman, NP and eGFR) were developed and demonstrated good prognostic

performance for risk stratification of ATTR-CA patients, particularly ATTRwt-CA. Fourth, for the Siemens assay, a threshold of 56 ng/L appears to be useful in risk stratification of patients with ATTRwt-CA.

ATTR-CA is an increasingly recognized cause of cardiovascular morbidity and mortality, particularly in older individuals and males[30]. Recent evidence suggests that up to 12% of patients with HF with preserved ejection fraction might be affected by CA as well as up to 8% of those with aortic stenosis and 7% of those undergoing carpal tunnel release surgery[31]. Moreover, effective therapies are now available for patients with ATTR amyloidosis[2], including the TTR stabilizer tafamidis[5] and genetic silencers (such as inotersen[26] and patisiran[25]) for those with ATTRv. A correct risk stratification of patients with ATTR-CA in terms of adverse events is crucial to provide prognostic information when managing these patients in everyday clinical practice. A first ATTR staging system for patients with ATTRwt-CA was published by Grogan et al. in 2016[8] and it was based on two parameters, namely contemporary cTnT > 0.05 ng/mL and NT-proBNP > 3000 pg/mL. In 2018, a new staging system was published by Gillmore et al.[15], based on a eGFR < 45 ml/min/m2 and an NT-proBNP > 3000 pg/mL. This staging system was also validated in a cohort from France and its prognostic role was maintained in those with ATTRv. Moreover, this staging system was able to overcome the issue of the variety of cTn assays available in different Institutions and the transition from non-high sensitivity assays to more sensitive assays happening in the late 2010s. Recently, a staging system combining hs-cTnT (> 50 ng/L), BNP (> 250 pg/mL), and eGFR (< 45 ml/mq) was published with good prediction of prognosis in 176 Japanese patients with ATTRwt-CA[29]. It emerges from these data how the prognostic role of hs-cTnI and its significance in risk stratification have been poorly explored in ATTR amyloidosis. Indeed, many Institutions worldwide utilize different hs-cTnI assays for clinical practice. These different assays have peculiar analytical characteristics[27,32]; due to the unique mix of cTnI isoforms in each patient sample and the antibodies incorporated into each assay, what is measured by different cTnI assays in the same patient's blood will be different, limiting the between-assay comparability of cTnI concentrations[33]. Already in 2019, Muchtar[16] et al.

suggested that staging models based on cTn should not be discarded or made inaccessible purely based on lack of access to a desired assay and that it may be time to readdress the value of cTnI in this arena. Moreover, in recent years, a consistent body of evidence has become available analyzing the differences between hs-cTnI and hs-cTnT in several clinical situations. Among the latest, a study evaluating cardiomuscolar biomarkers in the diagnosis and prognostication of immune checkpoint inhibitor myocarditis[22] reported potential differences in the performance of cTnT and cTnI in relation to the assay used. It is also know, for example, that cTnT is elevated more frequently than cTnI in patients with renal failure[34], a condition that is common in those with ATTR-CA. Our present study is based on all these assumptions and observations.

We investigated the role of hs-cTnI measured with three different assays in three different cohorts of patients affected by ATTR-CA. We report that hs-cTnI has a strong prognostic role in patients with ATTR-CA, particularly ATTRwt-CA, regardless of the specific assay being used and after adjustment for variables that are known predictors of adverse events in ATTR-CA. Moreover, we identified two potential thresholds to be validated and applied in clinical practice for risk stratification of patients with ATTR-CA, particularly ATTRwt-CA, one for the Abbott and Beckman assays (80 ng/L) and one for the Siemens assay (56 ng/L). The hs-cTnI concentrations obtained from ethylenediaminetetraacetic acid plasma between the Beckman and Abbott assays are generally highly correlated, with possible differences in concentrations in a limited number of patients due to macrocomplexes[28]. Based on this literature and on the similar threshold found for the Abbott and Beckman assays, we also performed a pooled analysis of patients evaluated with these two assays. Although with limitations, this methodology has allowed us to perform subsequent analysis on a higher number of patients and events and to probe the performance of staging systems in this pooled cohort. Indeed, we developed a 2 variables staging system, based on hs-cTnI > 80 ng/L and elevated NP (NT-proBNP > 3000 ng/L or BNP > 250 ng/L) that demonstrated a good prognostic performance in risk-stratification of these patients and providing a useful alternative to the staging system based on cTnT[8] for the Institutions utilizing hs-cTnI. Moreover, we developed a 3-variables staging system

including also eGFR with a population-derived threshold of < 50 ml/min/mq. This staging system, at the expense of greater complexity by requiring three variables, appears to improve risk stratification compared to the 2-variables staging system particularly in ATTRwt-CA at 24 and 36 months after baseline evaluation. Both staging systems appeared to perform better than the NAC staging system although this might be due to the utilization of both NTproBNP and BNP which was not evaluated in the original study[15]. Further studies are needed in this regard.

For the Abbott and Beckman assays, we also performed a dedicated analysis to address the influence of disease-modifying therapy on the prognostic role of hs-cTnI and of the two staging systems and this was not performed in previous studies. We utilized time-dependent analysis because most patients were started on disease-modifying therapy during follow-up and not right away at diagnosis (because these medications were not available in Italy). Moreover, most patients in our cohorts, affected by ATTRwt-CA, are treated with tafamidis which, according to the dedicated trial[5], requires approximately 18 months for the effect on overall survival to emerge. Clearly, due to the retrospective design of the study, we cannot infer on the efficacy of disease-modifying therapy but we verified that the prognostic prognostic role of hs-cTnI and of the two staging systems was maintained when adjusting for such therapies.

For the Siemens assay, the first exploratory analyses performed demonstrated that a threshold of 56 ng/L was able to properly risk-stratify patients with ATTRwt-CA. The differences in thresholds found between the Abbott/Beckmann assays and the Siemens assays might be related to the populations included and to different analytical characteristics of these assays. The threshold of 56 ng/L for the Siemens assay is indeed just slightly higher than the 47 ng/L recommended by the manufacturer (40 ng/L for females and 58 ng/L for males) as 99th % URL. However, it should be considered that in a study performed using a universal sample bank on patients screened using a health questionnaire and surrogate biomarkers such as NT-proBNP, eGFR and Hemoglobin A1c, the overall and sex-specific 99th% URLs resulted much lower[32]. It might be hypothesized that patients included in the Siemens cohort were at earlier ATTR stage or in more stable clinical conditions at the

time of hs-cTnI measurement; however, baseline clinical characteristics, echocardiographic parameters and staging according to the NAC system were similar to the other two cohorts. Further analysis of these findings is ongoing.

Limitations exists. First, this is a retrospective study with the limitations related to study design. Second, in each cohort, patients that were evaluated with different cTn assays (as it might happen for those evaluated in external laboratories) were excluded from this study, therefore potentially introducing selection bias. Third, patients with ATTRv amyloidosis were not enough to perform a separate analysis but we did perform separate analysis for ATTRwt-CA and it confirmed our findings. Fourth, for some patients a NT-proBNP value was not available and, in these cases, the analysis was based on validated thresholds for BNP[29]. This might impare the comparison between the hs-cTnI staging systems and the NAC staging system. Fifth, a head-to-head comparison of hs-cTnT and hs-cTnI was not performed.

8.5 Conclusions

In patients with ATTR-CA, particularly ATTRwt-CA, hs-cTnI measured with three different assays in different cohorts has a strong and independent prognostic role for mortality, even after adjustment for variables known to influence survival in these patients. A threshold of hs-cTnI of 80 ng/L (for Abbott and Beckman assay) and of 56 ng/L (for Siemens Centaur assay) appears to be useful for risk stratification of ATTRwt-CA patients in clinical practice. One staging system based on hs-cTnI and NP and one based on hs-cTnI, NP and eGFR were tested and demonstrated good performance for risk stratification of patients with ATTR-CA, particular ATTRwt-CA. These biomarkers-based systems maintained their prognostic significance also when adjusted for disease-modifying therapy. Therefore, our findings suggest that staging models for ATTRwt-CA based on cTn can be applied also in Institutions utilizing hs-cTnI measured with these three assays.

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Chapter 9. Chest pain in cardiac amyloidosis: occurrence, causes and prognostic significance.

9.1 Introduction

Cardiac amyloidosis (CA) is a widely-recognized cause of heart failure (HF) and cardiovascular (CV) morbidity and mortality[1,2]. Transthyretin amyloidosis (ATTR), either wild-type (ATTRwt) or hereditary (variant, ATTRv), and light chains amyloidosis (AL) are the two subtypes that most frequently affect the heart; the severity of cardiac involvement is the main determinant of prognosis in these patients[3,4]. Fibrils deposition results in progressive organ disruption and dysfunction[5].

CA can manifest with a variety of clinical presentations, in particular HF (most often with preserved ejection fraction, HFpEF), tachy- or brady-arrhythmias, and multiple extra-cardiac findings, or *red flags,* that can help suspect the disease and achieve the final diagnosis[1]. However, it is a common experience in clinical practice to evaluate patients with CA suffering from typical or atypical chest pain or presenting with scenarios mimicking acute coronary syndromes (ACS)[6,7,8]. Atherosclerotic epicardial obstructive coronary artery disease (CAD) is a plausible etiology for chest pain in these patients, given their old age (particularly in ATTR-CA), male sex and inflammatory status[9,10,11,12]. However, myocardial injury and microvascular dysfunction may also occur as a result of amyloid fibrils infiltration in the myocardium and in the coronary vasculature and may play a significant role as a cause of myocardial ischemia[13,14,15]. Moreover, cardiac troponin (cTn) is typically persistently elevated in CA patients[3,4,16] and this further challenges the diagnostic process of those presenting with chest pain.

The aim of the present study is to assess the prevalence, characteristics, and prognostic role of chest pain in patients with CA, and to investigate the possible etiologies by evaluating the CAD investigations and endomyocardial biopsy (EMB) findings.

9.2 Methods

9.2.1 Study design and data collection

The study was conducted according to the Declaration of Helsinki and informed consent was waived due to the retrospective design of the study (protocol n. 80070). This is a retrospective, observational, single-center study including a cohort of 174 consecutive patients with either ATTR-CA or AL-CA, followed-up at the Cardiac Amyloidosis Outpatient Clinic of the University Hospital of Padova, from January 2019 until January 2022. Inclusion criteria were a definite diagnosis of CA established either with biopsy-proven deposition of amyloid in cardiac or extracardiac tissue or with non-invasive diagnostic algorithm for ATTR-CA in selected patients, according to the recent Experts Consensus Document[1]. Genetic testing was performed in all ATTR-CA patients to identify mutations in the transthyretin gene. Due to the small number of patients with ATTRv-CA in our cohort, we analyzed ATTR-CA patients as a unique group.

We collected data regarding demographic characteristics, comorbidities, electrocardiographic and echocardiographic parameters, and laboratory values including natriuretic peptides (brain natriuretic peptide, BNP) and high-sensitivity cardiac troponin I (hs-cTnI) values; all of the above data were collected at baseline, namely at the first evaluation in our Center. Moreover, we collected laboratory values, including hs-cTnI, in patients with acute presentation and subsequently undergoing coronary arteries imaging. At our Institution, hs-cTnI values were measured using the Abbott Architect Stat High Sensitive Troponin I assay (Abbott, Abbott Park, Illinois), which has a 99th percentile upper reference limit (99th % URL) of 16 ng/L for women and 34 ng/L for men. Finally, we tabulated the occurrence and date of the first HF hospitalization and all-cause death during follow-up.

9.2.2 Chest pain assessment

In the Cardiac Amyloidosis Outpatient Clinic of our Institution, patients are routinely questioned about their medical history and symptoms according to a fixed protocol, starting from general information (allergies, region of birth, family history, working activity), past medical history

(including carpal tunnel syndrome, spinal stenosis, and relevant red flags[1]) and cardiovascular symptoms (including dyspnea, syncope, palpitations and chest pain). When reported, relevant characteristics of chest pain are collected, including timing with diagnosis of CA, localization, and relationship with effort. For this study, typical chest pain was defined as central or left-sided oppressive chest pain with radiation to the arms or throat with sweating or clamminess[17]. Atypical chest pain was defined as right-sided chest pain or chest pain that radiated to the back or worsened on inspiration or palpation[17]. Accordingly, patients were classified as suffering from typical chest pain, atypical chest pain, or chest pain with elements of both typical and atypical pain.

9.2.3 Morphological and functional assessment of coronary artery disease
In patients with chest pain, data regarding ergometric test, stress CV imaging procedures (including stress echocardiography and single-photon emission computerized tomography, SPECT), coronary computed tomography angiography (CCTA) and invasive coronary angiography (ICA) were collected.

Indication to ICA was based on the latest guidelines for acute and chronic coronary syndromes[18,19] as per clinical practice. We tabulated the clinical indications for coronary angiography into five different categories which were mutually exclusive and hierarchical, meaning that patients with multiple indications for coronary angiography were counted only once in the category hierarchically higher. Possible coronary angiography indication to be considered were: 1) chest pain; 2) dyspnea and new onset/worsening HF symptoms; 3) electrocardiographic alterations, and arrhythmias (including rapid ventricular response rate atrial fibrillation or flutter [RVR AF], or ventricular arrhythmias); 4) new imaging findings (such as left ventricular [LV] kinetics abnormalities or systolic dysfunction); 5) other. The last one, denominated "other", included coronary angiography performed together with endomyocardial biopsy, for severe valvular heart disease such as aortic stenosis, pre-operative coronary angiography.

Obstructive CAD was defined as stenosis ≥ 50% of the left main coronary artery and/or ≥ 70% of the other epicardial vessels, including good-caliper secondary branches. Only data from index ICA (i.e. performed within 5 years before or after the diagnosis of CA) were collected, irrespective of the presence of previously treated CAD lesions. This was to analyse as accurately as possible the relationship between chest pain, CA and obstructive CAD, without the confounder of previously treated CAD lesions with no actual hemodynamic relevance.

9.2.4. Histo-pathology

Through the Cardiac Pathology Unit database, we identified all patients that underwent EMB (before the non-invasive diagnostic algorithm was available or as per the latest Consensus Document[1]) and had EMB samples available for further analysis. EMB samples were obtained at the junction of interventricular septum and right ventricular free wall. After processing for histological examination, EMB specimens underwent detailed histopathological analysis to assess the presence of cardiomyocyte vacuolization, necrosis, replacement-type fibrosis, edema, and inflammation. Amyloid load was expressed as the percentage of amyloid on the most severely involved sample. Amyloid vascular or peri-vascular involvement was also assessed; the presence of amyloid deposits in the intramyocardial vessels was evaluated in a qualitative mode in hematoxylin-eosin and Heidenhain's trichrome stained slides. Detailed methodology is reported in the supplemental material at the end.

9.2.5 Statistical analysis

Continuous variables are expressed as median with 25th and 75th percentiles (Q1-Q3) and were compared using the Wilcoxon rank sum test. Categoric variables were expressed as absolute numbers and percentages and were compared using the chi-square test or Fisher exact test when appropriate. The log-rank test and Kaplan-Meier survival analysis were used to explore survival and HF hospitalization in groups with and without chest pain. Moreover, competing risk between all-cause

mortality and HF hospitalization was explored using the Fine and Gray model[20]. Finally, a competing risk regression was performed to assess the impact of CA subtype and history of CAD on HF hospitalization accounting for the presence of chest pain, with death as a competing risk. Hazard ratios (HR) and 95% confidence intervals (CI) for HF hospitalization were calculated. For the analysis, we used the packages {survival}, {survminer}, {tidycmprsk}, and {condsurv} inside the R (v. 4.2.2) environment and IBM SPSS Statistics 27.0 package (New York, NY).

9.3 Results

9.3.1 Study population

The study population included 174 patients with CA, of whom 70 (40%) with AL-CA and 104 (60%) with ATTR-CA [including 12 (6.9%) patients with ATTRv-CA]. Median age at diagnosis was 75 (68-80) years and 140 (81%) were males. Study flow chart is presented in Figure 1.
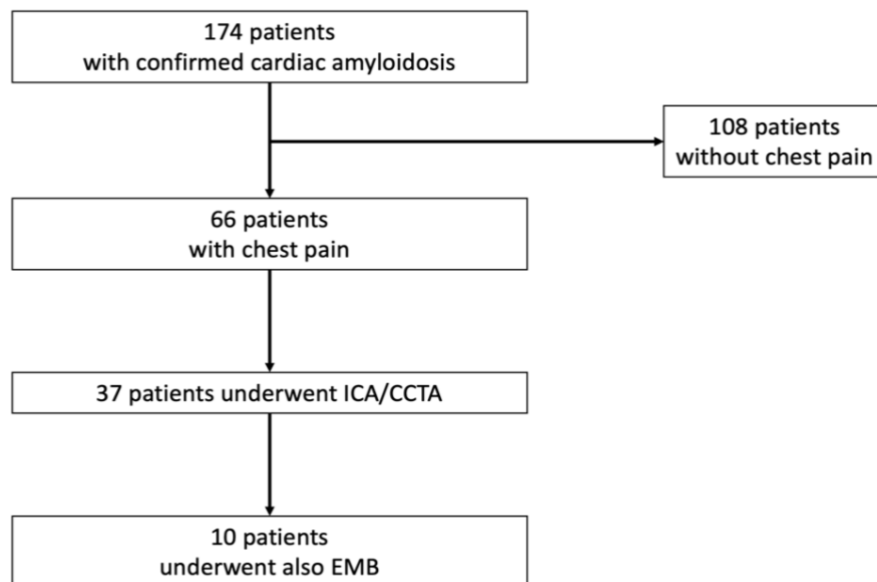

Figure 1. Study flow-chart. Abbreviations: CCTA, coronary computed tomography angiography; EMB, endomyocardial biopsy; ICA, invasive coronary angiography.

9.3.2 Prevalence and characteristics of chest pain in cardiac amyloidosis

Baseline characteristics of the overall population and by CA subtype according to the presence of chest pain are described in Table 1 and Supplemental Table 1. Among the 174 patients, 66 (38%) complained of chest pain, mostly at diagnosis or within one year from CA diagnosis [median time between CA diagnosis and reported episode of chest pain: 0 (0-6) months].

Of these patients, 22 (33%) complained of typical chest pain, 29 (44%) of chest pain with combined typical and atypical characteristics and 15 (23%) of atypical chest pain (Figure 2, Figure 3). Regarding the location of chest pain, in most cases (37/66, 56%) it was localized centrally in the retrosternal area, followed by lower sternal/epigastric area in 12/66 (18%) patients. In the remaining cases, it had more atypical locations such as left or right thorax or on the back. In 13/66 cases (20%), chest pain was effort related.

Patients with chest pain were younger than those without when analyzed overall (72 vs 76 years, p=0.048), but not when considered in subgroups (65 vs 73, p=0.23 for AL-CA, 77 vs 79, p=0.27 for ATTR-CA). Moreover, compared with those without, patients with chest pain had more frequently a past medical history of CAD (27% vs 15%, p=0.048) and presented more often with HF symptoms such as dyspnea and fatigue (62% vs 43%, p=0.015). Overall, no significant differences were found in terms of baseline electrocardiographic and echocardiographic characteristics (Table 1), besides in left atrial volume index (LAVi), which was greater in patients with chest pain (44 (35-53) ml/m2 vs 48 (42-58) ml/m2, p=0.045). Considering just AL-CA, patients with chest pain had higher E/e' ratio compared to those without chest pain (Supplemental Table 1). Regarding laboratory data, hemoglobin, creatinine and estimated glomerular filtration rate values did not differ among the two groups, while hs-cTnI [101 (45-219) vs 65 (41-129), p=0.032] and BNP values [597 (278-1675) vs 407 (203-653), p=0.024] were higher in patients with chest pain. At subgroup analysis, this difference in hs-cTnI and BNP values remained significant in AL-CA patients, but not in ATTR-CA (Supplemental Table 1). In 50 out of 66 cases (76%), the treating physicians modified patients' therapy by adding anti-ischemic medications such as beta-blockers or nitrates; these therapy

adjustments were more frequent in patients with typical chest pain, compared with those without

(91% vs 30%, p=0.042).

	N available	Overall n=174	No chest pain n=108	Chest pain n=66	P value
Age at diagnosis, years	174	75 (68-80)	76 (70-81)	72 (64-79)	0.048
Sex	174	M: 140 (81) F: 34 (19)	M: 88 (82) F: 20 (18)	M: 52 (79) F: 14 (21)	0.66
CA subtype	174	AL: 70 (40) ATTR: 104 (60)	AL: 40 (37) ATTR: 68 (63)	AL: 30 (46) ATTR: 36 (54)	0.27
Systemic hypertension	174	107 (62)	71 (66)	36 (55)	0.12
Diabetes mellitus	174	24 (14)	16 (15)	8 (12)	0.60
History of CAD	174	34 (20)	16 (15)	18 (27)	0.048
Atrial fibrillation/flutter	174	78 (45)	50 (47)	28 (42)	0.58
HF symptoms	174	87 (50)	46 (43)	41 (62)	0.015
Electrocardiography					
Low QRS voltages	174	62 (36)	39 (36)	23 (35)	0.83
Anterior pseudonecrosis	174	34 (20)	17 (16)	17 (26)	0.11
LBBB	174	9 (5)	5 (5)	4 (6)	0.69
RBBB	174	30 (17)	19 (18)	11 (17)	0.85
T-wave inversion	174	29 (17)	17 (16)	12 (18)	0.70
Echocardiography					
IVSd, mm	174	17 (15-19)	17 (15-19)	17 (15-19)	0.52
PWd, mm	174	15 (13-17)	15 (13-17)	15 (13-17)	0.45
LV EDV, ml/m^2	174	54 (43-64)	55 (44-63)	53 (43-71)	0.60
LV mass index, g/m^2	174	153 (128-185)	150 (126-177)	157 (130-189)	0.20
LVEF, %	174	54 (47-59)	55 (48-60)	52 (46-57)	0.05
GLS, %	113	-11 (-14 - -8)	-12 (-14 - -8)	-10 (-14 - -7)	0.33
E/e'	152	16 (12-21)	15 (11-21)	17 (14-20)	0.28
Restrictive filling pattern*	174	40 (23)	24 (23)	16 (25)	0.79
LA volume, ml/m^2	174	46 (37-54)	44 (35-53)	48 (42-58)	0.045
sPAP, mmHg,	144	34 (26-40)	35 (28-42)	32 (25-37)	0.06
TAPSE, mm	146	18 (15-21)	18 (14-21)	16 (15-20)	0.54
Pericardial effusion	166	48 (29)	25 (24)	23 (37)	0.09
≥moderate valve disease	174	32 (19)	17 (16)	15 (23)	0.25
Laboratory value					
Hb, g/dL	161	13 (12-15)	13 (12-15)	13 (12-15)	0.50
Creatinine, umol/L	160	102 (78-120)	101 (79-118)	107 (78-126)	0.51
eGFR, ml/min	160	60 (48-77)	60 (48-79)	57 (48-73)	0.50
dFLC, mg/L	131	53 (8-358)	48 (6-267)	97 (12-405)	0.23
Hs-cTnI, ng/L (Abbott assay)	139	81 (41-158)	65 (41-129)	101 (45-219)	0.032
Hs-cTnI, ng/L*	156	82 (41-158)	65 (37-124)	93 (44-219)	0.023
BNP, ng/L	102	463 (208-813)	407 (203-653)	597 (278-1675)	0.024

Table 1. Baseline characteristics of the overall population and by the presence/absence of chest pain.
Categorical variables are reported as n (%), continuous variables are reported as median (Q1-Q3). *Restrictive filling pattern was defined as a mitral inflow E/A greater than 2 and deceleration time<160 ms. sPAP was estimated based on tricuspid regurgitation velocity and estimated right atrial pressure, assumed by the size and distensibility of inferior vena cava. Abbreviations: AL, light chains amyloidosis; ATTR, transthyretin amyloidosis; BNP, brain natriuretic peptide; CA, cardiac amyloidosis; CAD, coronary artery disease; dFLC, differential free light chains; EDV, end diastolic volume; EF, ejection fraction; eGFR, estimated glomerular filtration rate; F, females; GLS, global longitudinal strain; Hb, hemoglobin; HF, heart failure; hs-cTnI, high sensitivity cardiac troponin I; IVS, interventricular septum; LA, left atrium; LBBB, left bundle branch block; LV, left ventricle; M, males; PW, posterior wall; RBBB, right bundle branch block; sPAP, systolic pulmonary.
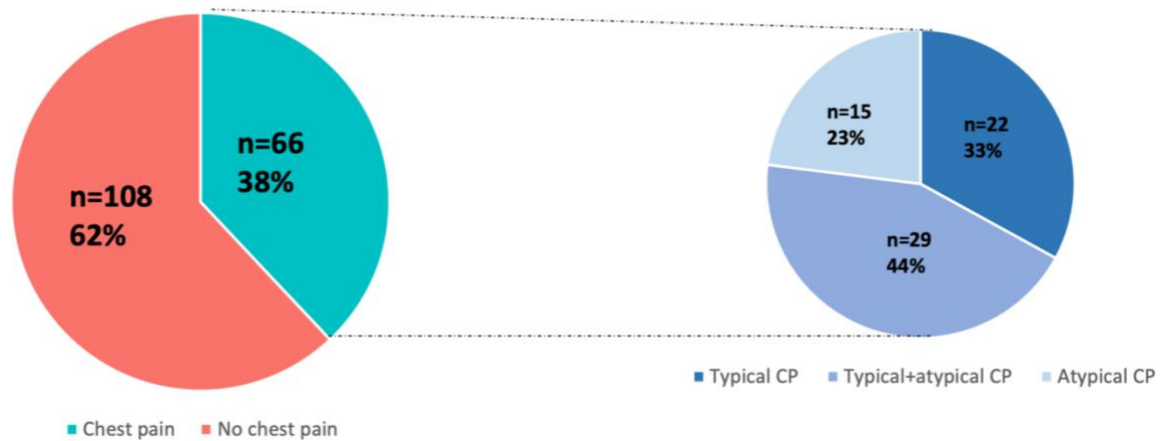
Figure 2. Frequency and characteristics of chest pain in cardiac amyloidosis. Pie charts with the distribution of cardiac amyloidosis (CA) patients without and with chest pain and, among the latest, the reported clinical characteristics of the pain.

9.3.3 Coronary artery disease investigations

Overall, chest pain triggered further investigations in 47 of 66 cases (71%); ergometric test was performed in 4 patients, stress echocardiogram in 4, SPECT in 2 and CCTA in 5 patients.

Regarding ICA, an index procedure was performed in 64/174 (37%) patients with CA, of whom 34 were patients with chest pain [median time difference between CA diagnosis and ICA 0 (0-6) months]. Overall clinical indications to ICA are described in the Supplemental Figure 1. Functional and morphological CAD investigations were performed more frequently in patients with typical compared with those with non-typical chest pain [20 (91%) vs 27 (61%), p=0.012]. Clinical characteristics of those who did not undergo further investigations are reported in Supplemental Material.

Overall, coronary arteries imaging (CCTA and/or ICA) was performed in 37 patients with CA and chest pain (Figure 1). Obstructive CAD was diagnosed at CCTA/ICA in 14/37 (38%) (Figure 3). Clinical characteristics of patients with CA and chest pain according to the presence/absence of obstructive CAD at index CCTA/ICA are reported in Table 2. In detail, patients with obstructive CAD were more frequently affected by ATTR-CA [13 (93) ATTR-CA vs 1 (7.1%) AL-CA, p=0.027]

and had more frequently classical CV risk factors such as systemic hypertension (100% vs 57%, p=0.006) and dyslipidemia (93% vs 44%, p=0.003). Typical chest pain did not occur differently among CA patients with obstructive CAD, compared with those without (57% vs 44%, p=0.42).

In patients with CA presenting with suspected ACS (n=22, 17 ATTR-CA and 5 AL-CA), obstructive CAD was detected in 9/17 (53%) of ATTR-CA patients, while it was absent in all 5 AL-CA patients. Out of the 9 ATTR-CA patients with obstructive CAD, coronary revascularization was performed in 6 (67%) while in 3 patients a medical therapy strategy was preferred. In Table 3 the results of urgent laboratory exams are reported. No significant differences in hemoglobin, creatinine and hs-cTnI values (first and second value available for each patient and first-to-peak absolute delta concentrations) were observed. First and second hs-cTnI values were obtained after a median of 3.5 hours (3-8). All but one patient with suspected ACS had at least one hs-cTnI value above the 99th % URL during the index encounter.

	Overall n=37	Nonobstructive CAD n=23	Obstructive CAD n=14	P value
Age at diagnosis, years	72 (68-78)	71 (66-78)	75 (71-80)	0.27
Sex	M: 32 (87) F: 5 (13)	M: 19 (83) F: 4 (17)	M: 13 (93) F: 1 (7)	0.63
CA subtype	AL: 11 (30) ATTR: 26 (70)	AL: 10 (44) ATTR: 13 (56)	AL: 1 (7.1) ATTR: 13 (93)	0.027
Typical chest pain	18 (47)	10 (44)	8 (57)	0.42
BMI, kg/m^2	28 (24-29)	27 (24-28)	28 (25-29)	0.38
Systemic hypertension	27 (73)	13 (57)	14 (100)	0.006
Diabetes mellitus	5 (14)	2 (8.7)	3 (21)	0.35
Dyslipidemia	23 (62)	10 (44)	13 (93)	0.003
Tobacco abuse	7 (19)	4 (17)	3 (21)	1.00
Family history of heart disease	16 (43)	9 (39)	7 (50)	0.52
CKD	14 (38)	6 (26)	8 (57)	0.059
Known CAD	8 (22)	3 (13)	5 (36)	0.10
Previous MI	4 (11)	1 (4.3)	3 (21)	0.14
AF/flutter	16 (43)	9 (39)	7 (50)	0.52
LV EF, %	48 (40-56)	48 (34-56)	50 (44-57)	0.53
ER admission	27 (73)	17 (74)	10 (71)	1.00
Revascularization performed	10 (27)	0	10 (71)	-

Table 2. Comorbidities and clinical characteristics of patients with cardiac amyloidosis and chest pain that underwent CCTA/ICA. Categoric variables are reported as n (%), continuous variables are reported as mean (±SD) or as median (Q1-Q3). Abbreviations: AF, atrial fibrillation; BMI, body mass index; CCTA, coronary computed tomography angiography; CKD, chronic kidney disease (estimated glomerular filtration rate <60 ml/mq/min); CP, chest pain; ER, emergency room; ICA, invasive coronary angiography; MI, myocardial infarction. Remaining as per Table 1.

	N available	Overall N=22	Nonobstructive CAD N=13	Obstructive CAD N=9	P value
Hb, g/dl	20	14.3 (12.5-15-5)	14.2 (12.5-15-5)	14.4 (12.3-15.6)	1.00
Creatinine, umol/L	19	87 (74-107)	81 (68-92)	95 (78-111)	0.13
First hs-cTnI, ng/L	19	96 (48-234)	102 (80-656)	42 (25-147)	0.05
Second hs-cTnI, ng/L	18	104 (45-289)	113 (75-582)	43 (21-156)	0.09
Peak hs-cTnI, ng/L	19	156 (49-656)	161 (94-656)	103 (44-618)	0.35
Absolute delta first hs-cTnI - peak hs-cTnI, ng/L*	19	10 (0-81)	5 (0-81)	10 (4-454)	0.66

Table 3. Laboratory values in patients with acute presentation undergoing CCTA/ICA Continuous variables are reported as median (Q1-Q3). First and second hs-cTnI values were obtained after a median of 3.5 hours (3-8). Abbreviations as per Table 1. *Absolute delta refers to the median difference in hs-cTnI values between the first and the peak value calculated for each patient.

9.3.4. Histological findings

Among CA patients with chest pain undergoing CCTA/ICA, EMB data were available in 10 (n=5 AL-CA and n=5 ATTR-CA), of which histological and CCTA/ICA findings are reported in Table 4. Median amyloid burden was 15% (3-45), myocardial vacuolization was found in 6 patients (60%) and replacement-type fibrosis in 4 (40%); amyloid vascular and perivascular involvement was described in 5 patients (50%). No patients had signs of myocardial necrosis.

Among the 5 AL-CA patients with chest pain and unobstructive CAD, 4 (80%) had evidence of amyloid vascular and perivascular involvement (Figure 4), 4 (80%) of myocytes vacuolization, 3 (60%) of replacement-type fibrosis, 1 of myocardial edema and inflammation. Among CA patients without chest pain who underwent CCTA/ICA and EMB (n=3, 2 with AL-CA and 1 with ATTR-CA), no amyloid vascular and perivascular involvement was detected.

A further analysis of patients that underwent EMB regardless of ICA/CCTA data (n=36) showed that chest pain was more common in the presence of amyloid vascular/perivascular involvement (Supplemental Table 2).

N	Amyloid type	Significant CAD at index CCTA/ICA	History of previous CAD	Amyloid burden	Myocyte vacuolization	Myocytes necrosis	Fibrosis	Vascular involvement	Myocardial edema	Myocardial inflammation
1	ATTR	Yes	Yes	3%	No	No	Yes (30%)	No	No	No
2	ATTR	Yes	No	37%	Yes	No	No	No	No	No
3	ATTR	Yes	No	45%	Yes	No	No	Yes	No	No
4	ATTR	No	No	3%	No	No	No	No	No	No
5	ATTR	No	No	15%	No	No	No	No	No	No
6	AL kappa	No	Yes	85%	Yes	No	Yes (5%)	Yes	No	No
7	AL kappa	No	No	10%	No	No	No	Yes	No	No
8	AL kappa	No	No	3%	Yes	No	Yes (5%)	No	No	No
9	AL lambda	No	No	45%	Yes	No	Yes (5%)	Yes	Yes	Yes
10	AL lambda	No	No	15%	Yes	No	No	Yes	No	No

Table 4. Description of histological findings of the 10 cardiac amyloidosis patients with chest pain undergoing endomyocardial biopsy and CCTA/ICA. Abbreviations. AL: light chains amyloidosis; ATTR, transthyretin amyloidosis; CAD, coronary artery disease; CCTA, coronary computed tomography angiography; ICA, invasive coronary angiography
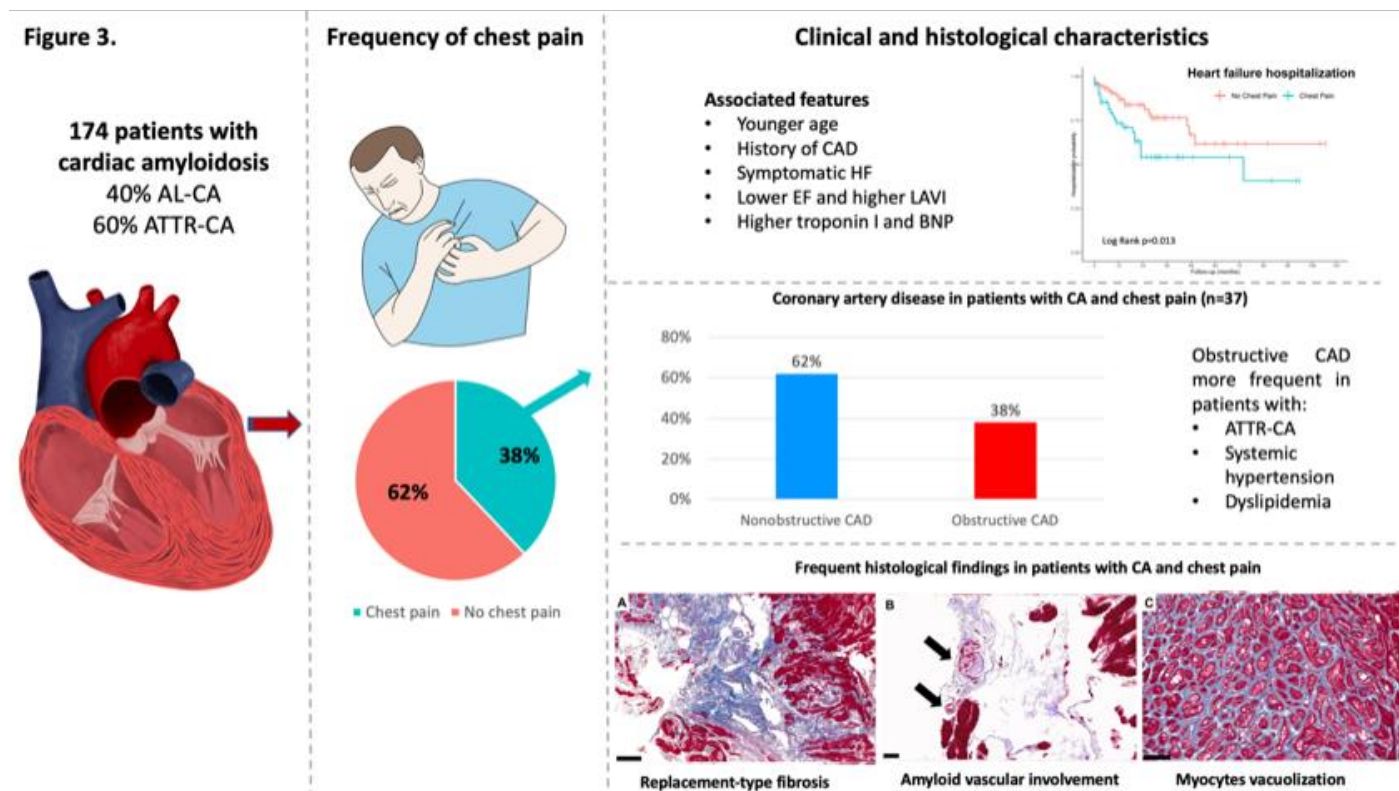
Figure 3. Chest pain in cardiac amyloidosis: occurrence and clinical and histological characteristics. Chest pain was one of the symptoms in 38% of our CA patients, being more frequent in patients with history of coronary artery disease (CAD), heart failure (HF) symptoms, higher high sensitivity cardiac troponin (hs-cTnI) and B-type natriuretic peptide. Among CA patients with chest pain that underwent invasive coronary angiography or coronary computed tomography angiography, obstructive CAD was present in 38%. At histological evaluation of endomyocardial biopsy, replacement-type fibrosis (Panel A, bar 100 µm), amyloid vascular and perivascular involvement (Panel B, bar 20 µm), and myocytes vacuolization (panel C, bar 50 µm) were frequent particularly in light chains cardiac amyloidosis and nonobstructive CAD.
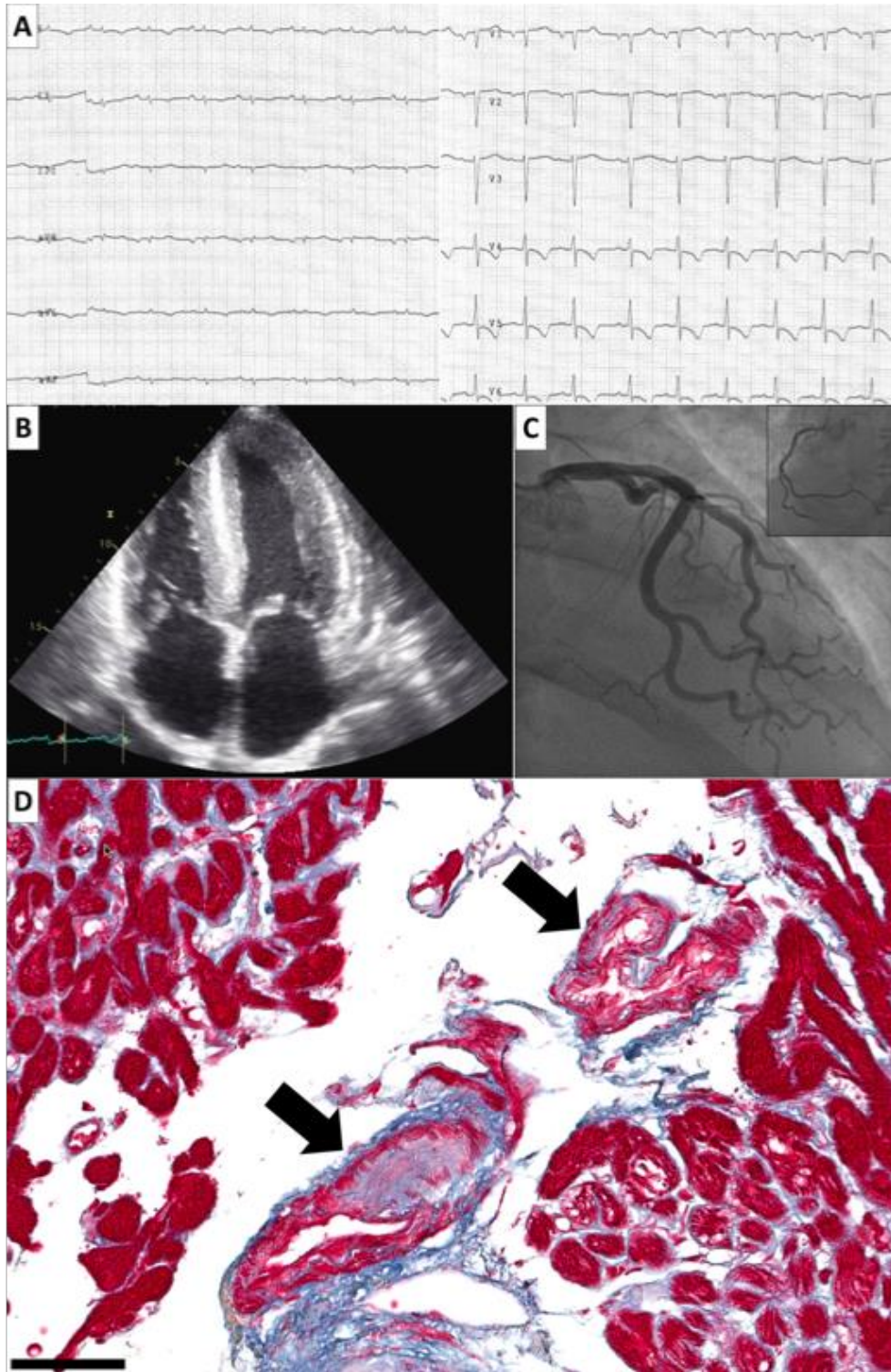
Figure 4. Clinical and histological findings of a 48-year-old light chains cardiac amyloidosis patient presenting with chest pain. Panel A: electrocardiogram at cardiac amyloidosis (CA) diagnosis. **Panel B:** four chamber view at echocardiography at the time of CA diagnosis, showing the classical findings of biventricular pseudohypertrophy with granular sparkling, thickening of the atrio-ventricular valves and of the atrial septum. **Panel C:** invasive coronary angiography showing absence of significant stenoses of the left coronary artery. Similarly, in the smaller panel, the right coronary artery had no significant stenoses. **Panel D:** at endomyocardial biopsy, there was clear involvement of intramyocardial small vessels by amyloid deposition (arrows). Bar 50 μm. Heidenhain's trichrome stain.

9.3.5 Prognostic role of chest pain in cardiac amyloidosis

During a median follow-up of 17 (8-34) months, 48 (28%) patients died (n=19 with ATTR-CA and n=29 with AL-CA) and 43 (25%) had at least one HF hospitalization (n=20 ATTR-CA and n=23 AL-CA). At survival analysis, patients with chest pain had a higher risk of HF hospitalization than those without chest pain (Log rank=0.013, Figure 5 panel A), also considering death as competing risk (Gray's test p value=0.016, Supplemental Figure 2). At competing risk regression analysis, chest pain remained a significant predictor of HF hospitalizations also after adjustment for CA subtype and for history of CAD (HR 1.86, 95% CI 1.02 – 3.39, p=0.042) (Supplemental Table 3). Chest pain (HR 2.32, 95% CI 1.11 – 4.89), p=0.026] and elevated BNP (>463 ng/L) (HR 3.61, 95% CI 1.50 – 8.71), p=0.004] remained independent predictors of HF hospitalizations also after adjustment for CA subtype and reduced LV EF (Supplemental Table 4). Survival analyses for AL-CA and ATTR-CA separately showed the same trend although not significant (Supplemental Figure 3). No significant differences between patients with and without chest pain in terms of all-cause mortality were found (Figure 5 panel B).
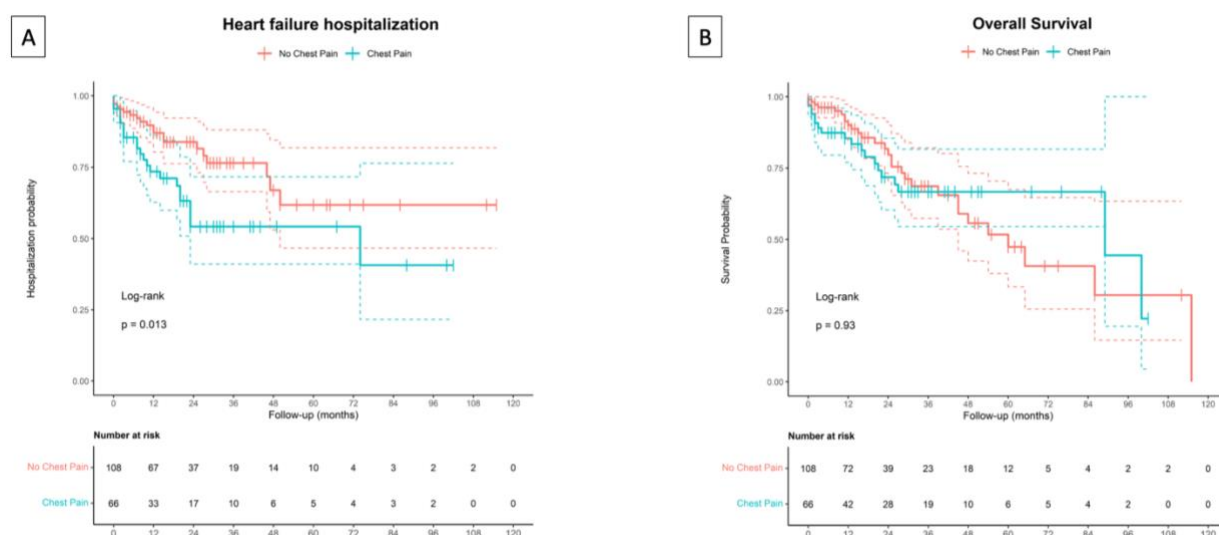

Figure 5. Prognostic significance of chest pain in patients with cardiac amyloidosis. Panel A: Kaplan Meier analysis for the time free from heart failure (HF) hospitalization in patients without and with chest pain. **Panel B**: Kaplan Meier analysis for overall survival in patients without and with chest pain.

9.4 Discussion

This study was designed to assess the prevalence, the possible causes, and the prognostic significance of chest pain in a consecutive cohort of patients with CA. The main findings were the following: (i) chest pain is reported by more than one-third of patients with CA, and in one-third of cases with typical angina characteristics; (ii) patients with CA and chest pain are characterized by a previous history of CAD, HF symptoms such as fatigue and dyspnea, and higher levels of cardiac biomarkers; (iii) in patients with CA and suspected ACS, values of hs-cTnI were similar regardless of the presence of obstructive CAD; (iv) obstructive CAD is present in around one-third of patients with CA and chest pain, and is more frequently detected in those with ATTR-CA and traditional CV risk factors such as systemic hypertension and dyslipidemia; amyloid deposition in the intramyocardial coronary vessels may also explain chest pain occurrence, particularly in patients with AL-CA and unobstructive CAD; (v) chest pain is a significant predictor of HF hospitalization at follow-up, also after adjustment for CA subtype and history of CAD.

Prevalence and clinical characteristics of chest pain in CA

Chest pain is commonly reported in HF patients, both with reduced[21] and preserved EF[22]. In AL-CA and ATTR-CA, symptoms more frequently described are those related to HF[1,23]. Anginal symptoms and signs of ischemia are more rarely reported, although case reports and series described patients presenting with typical or atypical chest pain or mimicking ACS, even with patent epicardial coronary arteries[8,24,25]. Such reports were mainly focused on AL-CA patients with chest pain, while a systematic assessment of this symptom in a comprehensive consecutive cohort of CA patients was still lacking.

Our findings demonstrated how chest pain is indeed common in CA, being reported in 38% of patients in our cohort. Chest pain, besides being more frequent when a history of CAD is present, was also associated with HF symptoms and a more advanced CA involvement[3,4], demonstrated by higher cardiac biomarkers. At subgroup analysis, ATTR-CA patients with chest pain frequently had HF symptoms, association that might be multifactorial also given the relevant burden of CAD in this

subset[10] and the possible coexistence of ischemic and amyloid-related cardiac disease; in AL-CA, patients with chest pain showed a more advanced CA involvement, with higher LV filling pressures even at baseline, and higher cardiac troponin and BNP values.

Possible etiologies of chest pain in CA

Our data provided also new insights on the frequency of obstructive CAD in patients with CA and chest pain undergoing CCTA/ICA. Not surprisingly, it was more frequent in ATTR-CA patients, in keeping with the different clinical profile between ATTR-CA and AL-CA[26], with ATTR-CA patients being older and with more classical CV risk factors such as systemic hypertension and dyslipidemia. When considering just those with typical anginal symptoms undergoing coronary arteries investigations, less than one half of the patients had obstructive CAD. In patients presenting acutely for suspected ACS, obstructive CAD was present in 53% of ATTR-CA patients, while it was not detected in AL-CA patients.

The prevalence of obstructive CAD in our cohort was overall lower than the one of 50% classically reported for HFpEF patients[27], but it raised to 48% when considering just ATTR-CA patients which have a clinical profile more similar to that of HFpEF patients. Our findings are in keeping with the recent literature. In a population-based study[28], history of CAD (regardless of symptoms) was reported in 56% of patients diagnosed with ATTR-CA. Other recent studies have reported a prevalence of CAD between 20%[11,12] and 40%[10] in ATTR-CA, depending on the population studied and on the criteria applied to define relevant CAD.

Our data expand on previous reports, integrating clinical, structural and histological characteristics to ICA/CCTA findings, showing that obstructive CAD certainly contributes to the genesis of chest pain, particularly in ATTR-CA, but other mechanisms play a significant role as well. Dorbala et al.[14] discussed about three possible reasons for anginal symptoms in patients with CA and unobstructive CAD, including structural (amyloid deposition in the vessel), extravascular (extrinsic compression of the microvasculature from amyloid deposits), and functional (autonomic and endothelial dysfunction) mechanisms. Moreover, rarefaction of capillary vessels also might play a role in the genesis of

interstitial fibrosis[29] and, potentially, of chest pain. Finally, increased LV thickness and high LV filling pressure might contribute to hypoperfusion of subendocardial layers and anginal symptoms[30]. Indeed, when we analyzed the histological findings at EMB of CA patients with chest pain, we found that amyloid vascular and perivascular involvement was frequent, in particular in AL-CA patients with nonobstructive epicardial CAD at the index CCTA/ICA (Figure 4). Replacement-type fibrosis and myocyte vacuolization were also frequent in these patients. This is in keeping with previous studies on pathological specimen from autopsy or after cardiac transplantation in AL-CA, in which small vessels obstructive intramural coronary amyloidosis was present in around two thirds of patients, with clinical evidence of myocardial ischemia in 25% of cases[7, 31]. Moreover, patients with AL-CA presenting with angina and with abnormal coronary flow reserve were reported to have amyloid deposition in the intramyocardial coronary vessels[25].

Histological findings in ATTR-CA patients are sparser. Obstructive CAD surely is important in this specific subset; however, microvascular and endothelial dysfunction[32] together with hypertrophy/pseudohypertrophy and increased LV filling pressures might be relevant interplaying actors in the genesis of chest pain. Indeed, diastolic dysfunction worsens in case of ongoing ischemia[33] and, in turn, elevated LV filling pressures contribute to subendocardial ischemia and reduced coronary perfusion pressure.

Cardiac troponin in CA patients with chest pain

When considering laboratory values in patients with suspected ACS, almost all patients had at least one hs-cTnI value above the 99th % URL and no significant differences in hs-cTnI values were found between those with and without obstructive CAD. This might be related to the small study sample and to the retrospective design of the study, with timing of cTn evaluations not standardized among patients. However, this might also be related to the complex pathophysiology of myocardial injury in CA. Indeed, in the first studies investigating cardiac biomarkers in CA patients, cTn increases were similar among those with and without significant atherosclerotic and intramural amyloid-related CAD[34]. In CA, chronic myocardial injury might be due to several mechanisms, including amyloid

interstitial infiltration with cardiomyocytes toxic/mechanical damage, vascular infiltration, diastolic dysfunction and high LV filling pressure. Moreover, multiple triggers, such as arrhythmias[35] or decompensation of HF, can lead to acute myocardial injury or type 2 myocardial infarction[36]. Certainly, serial systematic hs-cTn measurements are crucial to identify acute events[36], but the differential diagnosis on the etiology of such events is challenging and still requires a comprehensive and multiparametric assessment of the potential triggers of myocardial injury.

Prognostic significance of chest pain in CA

In our cohort, the presence of chest pain was associated with a higher risk of HF hospitalization, and it was a significant predictor of HF hospitalization also when CA subtype and history of CAD were included in competing risk models. This is in keeping with the more advanced cardiac impairment demonstrated by patients with chest pain at baseline, with frequent HF symptoms, particularly in ATTR-CA. Moreover, ongoing multifactorial ischemia might contribute to worsen diastolic dysfunction and lead to HF symptoms and higher risk of HF events. Subgroup analysis for AL-CA and ATTR-CA failed to reach statistical significance, possibly due to the relatively small number of patients and of adverse events. We did not find significant differences in terms of overall mortality based on presence or absence of chest pain; this might be related at least in part to the fact that we considered all-cause mortality, not just CV mortality, and this might alter the prognostic value of this symptom and its structural correlates.

Further, larger studies are needed to confirm and expand our findings and to draw conclusions on the best management of patients with CA and chest pain. We showed that in AL-CA patients with chest pain, amyloid vascular/perivascular involvement is a relevant actor and chest pain could be a sign of a more advanced cardiac impairment with higher risk for future HF hospitalization, possibly prompting more aggressive diuretic therapy and closer monitoring and follow-up. For ATTR-CA, given the higher frequency of significant CAD, chest pain should probably prompt dedicated investigations up to ICA, although the prognostic impact of CAD on ATTR-CA patients' survival is yet to be determined.

Limitations

This study has some limitations. First, it is a retrospective single-center study with the limitations related to study design and to the relatively small sample size. Although all patients were asked thoroughly regarding their cardiovascular symptoms and the presence and characteristics of chest pain, a prospective screening was not performed. Moreover, non-anginal equivalents might have been missed. Second, due to the small number of ATTRv patients, a separate analysis for ATTR groups could not be carried out. Third, we did not report cardiac magnetic resonance results, particularly T1 mapping and extracellular volume data, because at the time of enrollment, this technique was not available in our scanner. Fourth, CAD investigations, provocative tests to identify myocardial ischemia and EMB findings were available only in a subset of the initial cohort, limiting the possibility of further adjusted analyses and of stronger conclusions on the genesis of chest pain. However, our data reflect a real-life experience in which not all patients receive comprehensive evaluation for chest pain, particularly those with atypical characteristics. Patients with CA frequently present advanced age, CKD, conduction and rhythm disturbances and several comorbidities which hamper the feasibility and reliability of second-line CAD diagnostic investigations. Nevertheless, recent studies[10,11,12] focusing on ATTR-CA characteristics and diagnostic paths have reported a similar prevalence of obstructive CAD. Fifth, evaluation of endothelial/microvasculature dysfunction was not performed systematically in patients with chest pain, but previous studies already addressed this topic in CA[13,14]. Finally, due to the retrospective design of the study, the timepoints of hs-cTnI measurements were not standardized between patients and further systematic prospective studies are needed to clarify our findings.

Although these limitations may interfere with our ability to draw definitive conclusions on the genesis of chest pain in CA, our findings are based on routine clinical practice and provide new insights into this common symptom and its every-day management in patients with CA.

9.5 Conclusion

Chest pain is frequent in patients with CA, being reported by almost 40% of our sample. Patients with chest pain have more frequently a history of CAD and a more advanced CA clinical phenotype, including HF symptoms and higher cardiac biomarkers. The possible etiology of chest pain seems to differ among CA subtypes, with obstructive CAD being more frequent in ATTR-CA whilst amyloid vascular/perivascular involvement, as well as replacement type fibrosis and myocardial vacuolization, more common in AL-CA. Regardless of the etiology, chest pain identifies patients with CA at higher risk of HF hospitalization and therefore should prompt closer and personalized management to improve patients' outcome.

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Other methods, tables and figures (Supplemental material)

Supplemental methods

Pathology

EMB samples of the right ventricle were obtained at the junction of interventricular septum and right ventricular free wall, through the femoral vein, using the long sheath technique (disposable Cordis bioptome, Miami, FL, USA). The biopsy samples were fixed in 10% buffered formalin (pH 7.35) and processed for histological examination. The paraffin-included sections (5 μm thick) underwent serial cuts and staining by hematoxylin-eosin and Heidenhain trichrome. This last represents the elective staining method for connective tissue (which appears blue) and is particularly indicated to highlight the muscle fibers (which appears red). In addition, Congo red stain was performed to highlight the presence of amyloid deposits with apple green birefringence at polarized light microscopy. For the investigation of signs of myocardial inflammation, the assessment was made at light microscope with the aid of immunohistochemistry (IHC) with specific antibodies against T lymphocytes antigens (anti-CD3, Novocastra, diluition 1:50) and against macrophages antigens (anti-CD68, Dako, diluition 1:50). Before using the first antibody, immunoreactivity was amplified by microwave treatment (750 W, 5 minutes) in buffered citrate solution (10 mM, pH 6.0) and with pressure cooking treatment (3 minutes) in buffered citrate solution. The positivity of the antibody-antigen reaction was tested with the avidine-biotine peroxidase complex (Vector, Burlingame, California, USA).

EMB specimens underwent detailed histopathological analysis to assess the following data.

- Cardiomyocyte diameter: for every case the mean diameter of cardiomyocytes was calculated at 40x on digitally acquired hematoxylin-eosin-stained slides using an image analyzer system and a commercially available software (Image-Pro Plus Version 4.0, Media Cybernetics, MD, USA).

- Cardiomyocyte vacuolization: for every case the presence of myocyte vacuolization (defined as dislocation of contractile elements to the periphery of myocytes with optically clear spaces of the cardiomyocyte's cytoplasm) (1) was assessed.

- Cardiomyocyte necrosis: evidence of myocyte death with membrane scalloping, attenuated fibers or cell remnant was noted in a qualitative evaluation performed in hematoxylin-eosin-stained slides.

- Amyloid load: the amyloid area was calculated with a quantitative score on digitally acquired slides using an image analyzer system and a commercially available software (same as above) as previously described (2). The analysis was performed on trichrome-stained slides and the result was expressed as the percentage of amyloid on the sample that was most severely involved by the deposition.

- Amyloid vascular or peri-vascular involvement: the presence of amyloid deposits in the intramyocardial vessels that were included in the EMB samples was evaluated in a qualitative mode in hematoxylin-eosin-stained slides.

- Replacement-type fibrosis: the area of replacement-type fibrosis was calculated with a quantitative score on digitally acquired slides using an image analyzer system with the method described above, on trichrome-stained slides with the result expressed as the percentage of fibrosis on the same sample evaluated for the assessment of the amyloid load.

- Edema and inflammation: the presence of interstitial edema and of clusters of inflammatory cells (lymphocytes, granulocytes, macrophages or plasma cells) was assessed on hematoxylin-eosin-stained slides and rendered in a qualitative fashion (presence or absence).

- IHC evaluation: in addition to the qualitative assessment of inflammatory cells, the presence of T lymphocytes and macrophages in the EMB samples was performed with the morphometric analysis of CD3 and CD68 positive cells. Firstly, the area of the most involved

sample was calculated with the above-mentioned software in mm^2. Then, for each case the number of positive cells per the selected fragment was digitally calculated with the color threshold function. Finally, the ratio between the number of CD3+ and CD68+ cells and the calculated area was obtained (the final result was expressed in CD3+/mm^2 CD68+/mm^2).

Two cardiovascular pathologists performed the histopathological analysis being blinded to the patients' clinical data.

Amyloid typing was performed by immune electron microscopy on formalin-fixed paraffin-embedded blocks after dewaxing and resin-embedding. Selected sections were then processed for post-embedding immunogold as previously reported (3). The primary antibodies used were Anti Human Kappa Light Chains, Anti Human Lambda Light Chains and Anti Human TTR.

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	ATTR – CA			AL - CA		
	No chest pain (n=68)	Chest pain (n=36)	p	No chest pain (n=40)	Chest pain (n=30)	p
Age at diagnosis, years	79 (73-82)	77 (71-82)	0.27	73 (60-77)	65 (60-74)	0.23
Sex	M:63 (93) F: 5 (7.4)	M:32 (89) F: 4 (11)	0.72	M: 25 (63) F: 15 (37)	M: 20 (67) F: 10 (33)	0.72
Systemic hypertension	49 (72)	24 (67)	0.57	22 (56)	12 (40)	0.18
Diabetes mellitus	10 (15)	7 (19)	0.53	6 (15)	1 (3)	0.13
History of CAD	13 (19)	13 (36)	0.06	3 (7.7)	5 (17)	0.28
Atrial fibrillation/flutter	43 (63)	20 (56)	0.45	7 (18)	8 (27)	0.38
Symptomatic HF	32 (47)	27 (75)	0.006	14 (36)	14 (47)	0.36
Electrocardiography						
Low QRS voltages	23 (34)	8 (22)	0.22	16 (41)	15 (50)	0.46
Anterior pseudonecrosis	12 (18)	9 (25)	0.37	5 (13)	8 (27)	0.15
LBBB	5 (7.4)	2 (5.6)	1.00	0 (0)	2 (6.7)	0.19
RBBB	13 (19)	7 (19)	0.97	6 (15)	4 (13)	1.00
T-wave inversion	12 (18)	6 (17)	0.90	5 (13)	6 (20)	0.51
Echocardiography						
IVSd mm	18 (16-19)	18 (16-20)	0.39	15 (13-18)	17 (14-19)	0.51
PWd mm	15 (14-17)	16 (14-17)	0.84	13 (12-15)	15 (13-17)	0.27
LV EDV ml/m^2	55 (44-66)	57 (42-73)	0.48	55 (44-59)	51 (44-64)	0.93
LV mass index	166 (142-189)	169 (141-208)	0.43	131 (110-165)	148 (118-184)	0.17
LVEF, %	55 (45-60)	49 (42-58)	0.19	56 (52-61)	53 (48-57)	0.07
GLS, %	-12 (-13,-9)	-11 (-13, -6)	0.21	-11 (-15,-7)	-9 (-14, -8)	0.97
E/e'	15 (12-20)	16 (13-18)	0.66	15 (9-22)	18 (14-24)	0.048
Restrictive pattern	14 (21)	7 (19)	0.86	10 (26)	9 (31)	0.67
LAvolume ml/mq	48 (38-54)	51 (44-62)	0.06	37 (31-50)	44 (33-51)	0.15
sPAP, mmHg,	36 (29-44)	32 (25-39)	0.10	31 (25-39)	33 (25-36)	0.37
TAPSE mm	17 (14-20)	16 (15-20)	0.99	19 (16-24)	18 (16-22)	0.12
Pericardial effusion	12 (19)	9 (26)	0.40	13 (34)	14 (50)	0.20
≥moderate valve disease	12 (18)	9 (25)	0.39	5 (13)	6 (21)	0.38
Laboratory value						
Hb, g/dL	14 (12-15)	14 (13-15)	0.71	13 (11-14)	13 (11-14)	0.79
Creatinine umol/L	99 (82-112)	108 (78-124)	0.35	105 (73-145)	106 (78-126)	0.92
eGFR, ml/min	61 (53-78)	57 (48-75)	0.36	57 (34-85)	57 (44-73)	0.87
dFLC mg/L	NA	NA	NA	285 (101-609)	270 (122-606)	0.91
Hs-cTnI ng/L (Abbott assay)	66 (42-129)	82 (36-191)	0.75	65 (32-130)	141 (56-253)	0.015
Hs-cTnI ng/L*	65 (41-124)	85 (36-198)	0.53	65 (32-130)	141 (56-253)	0.015
BNP ng/L	364 (200-509)	342 (160-762)	0.46	504 (205-970)	1320 (542-3634)	0.022

Supplemental table 1. Baseline characteristics of the overall population divided into subgroups according to CA subtype and to the presence of LQRSV. Categorical variables are reported as n (%), continuous variables are reported as median (Q1-Q3). Abbreviations: AL, light chains amyloidosis; ATTR, transthyretin amyloidosis; BNP, brain natriuretic peptide; CA, cardiac amyloidosis; CAD, coronary artery disease; dFLC, differential free light chains; EDV, end diastolic volume; EF, ejection fraction; eGFR, estimated glomerular filtration rate; F, females; GLS, global longitudinal strain; Hb, hemoglobin; HF, heart failure; hs-cTnI, high sensitivity cardiac troponin I; IVS, interventricular septum; LA, left atrium; LBBB, left bundle branch block; LV, left ventricle; M, males; PW, posterior wall; RBBB, right bundle branch block; sPAP, systolic pulmonary artery pressure; TAPSE, tricuspid annular plane systolic excursion. *Includes also 17 hs-cTnI values measured with different assays in different Institutions.

	Overall n=36	No amyloid vascular/perivascular involvement n=19	Amyloid vascular/perivascular involvement n=17	P value
Age at diagnosis, years	70 (61-77)	70 (60-74)	69 (59-78)	0.90
Sex	M: 24 (67) F: 12 (33)	M: 12 (63) F: 7 (37)	M: 12 (71) F: 5 (29)	0.64
CA subtype	AL: 23 (64) ATTR: 13 (36)	AL: 9 (47) ATTR: 10 (53)	AL: 14 (82) ATTR: 3 (18)	0.029
Chest pain	19 (53)	7 (37)	12 (71)	0.043
Systemic hypertension	18 (50)	11 (64)	7 (44)	0.23
Diabetes mellitus	3 (8)	2 (12)	1 (6.7)	1.00
Known CAD/previous MI	8 (22)	4 (21)	4 (24)	1.00
AF/flutter	13 (36)	8 (42)	5 (29)	0.43
LV EF, %	52 (44-58)	51 (44-59)	52 (42-57)	0.50
Hs-cTnI, ng/L*	141 (27-209)	88 (14-189)	162 (79-274)	0.24

Supplemental Table 2. Clinical characteristics of patients undergoing endomyocardial biopsy according to the presence or absence of amyloid vascular/perivascular involvement. Categoric variables are reported as n (%), continuous variables are reported as median (Q1-Q3). Abbreviations: AF, atrial fibrillation; AL: light chain amyloidosis; ATTR: transthyretin amyloidosis; CA: cardiac amyloidosis; CAD: coronary artery disease; F: females; hs-cTnI, high sensitivity cardiac troponin I; LVEF, left ventricular ejection fraction; M: males; MI, myocardial infarction. * Hs-cTnI values were available in 17 patients. The difference in hs-cTnI values between patients with and without amyloid vascular/perivascular involvement was significant when considering only AL-CA patients [23 (7-127) ng/L vs 188 (144-321), p=0.038].

	Competing Risk Regression	
	HR (95% CI)	p value
MODEL 1		
Chest pain	2.03 (1.12 – 3.67)	**0.019**
Cardiac amyloidosis subtype (AL-CA)	1.47 (0.79 – 2.73)	0.22
MODEL 2		
Chest pain	1.92 (1.06 - 3.47)	**0.032**
History of CAD	1.70 (0.87 – 3.32)	0.12
MODEL 3		
Chest pain	1.86 (1.02 – 3.39)	**0.042**
Cardiac amyloidosis subtype (AL-CA)	1.73 (0.91 – 3.28)	0.09
History of CAD	2.04 (1.02 – 4.07)	**0.043**

Supplemental Table 3. Competing risk regression to evaluate the determinants of HF hospitalization considering death as competing risk. Abbreviations. AL-CA, light chains cardiac amyloidosis; CAD, coronary artery disease; CI, confidence interval; HF, heart failure; HR, hazard ratio.

	HR (95% CI)	P VALUE
Chest pain	2.32 (1.11 – 4.89)	**0.026**
Cardiac amyloidosis subtype (AL-CA)	1.16 (0.53 – 2.56)	0.71
LV EF < 50%	0.87 (0.38 – 1.98)	0.73
Elevated BNP (>463 ng/L)	3.61 (1.50 – 8.71)	**0.004**

Supplemental Table 4. Cox regression analysis for HF hospitalization in the overall cohort. Abbreviations. AL-CA, light chains cardiac amyloidosis; BNP, bran natriuretic peptide; CI, confidence interval; EF, ejection fraction; HF, heart failure; HR, hazard ratio; LV, left ventricle.
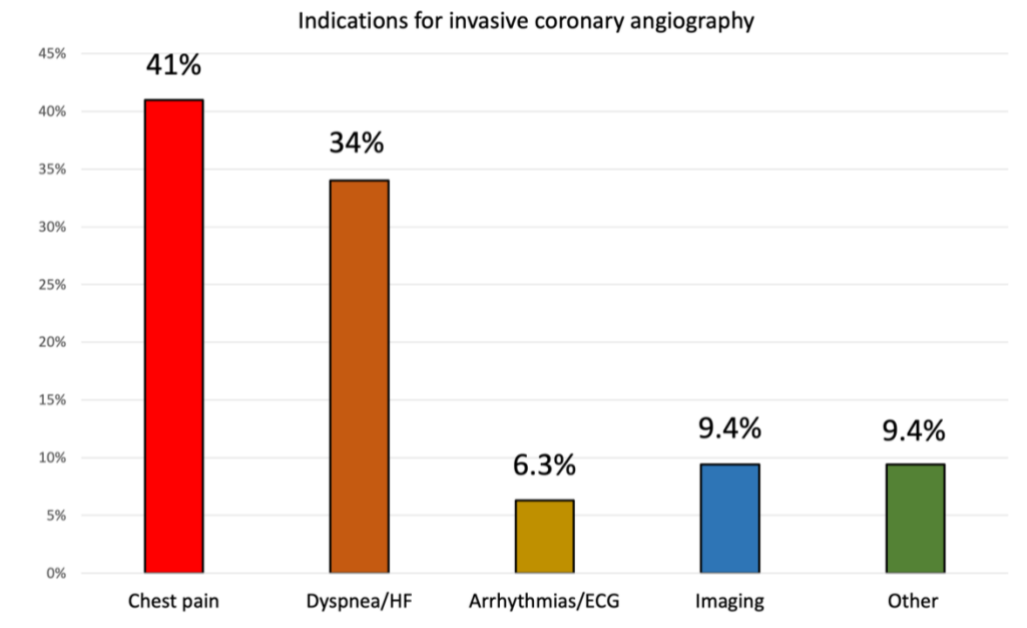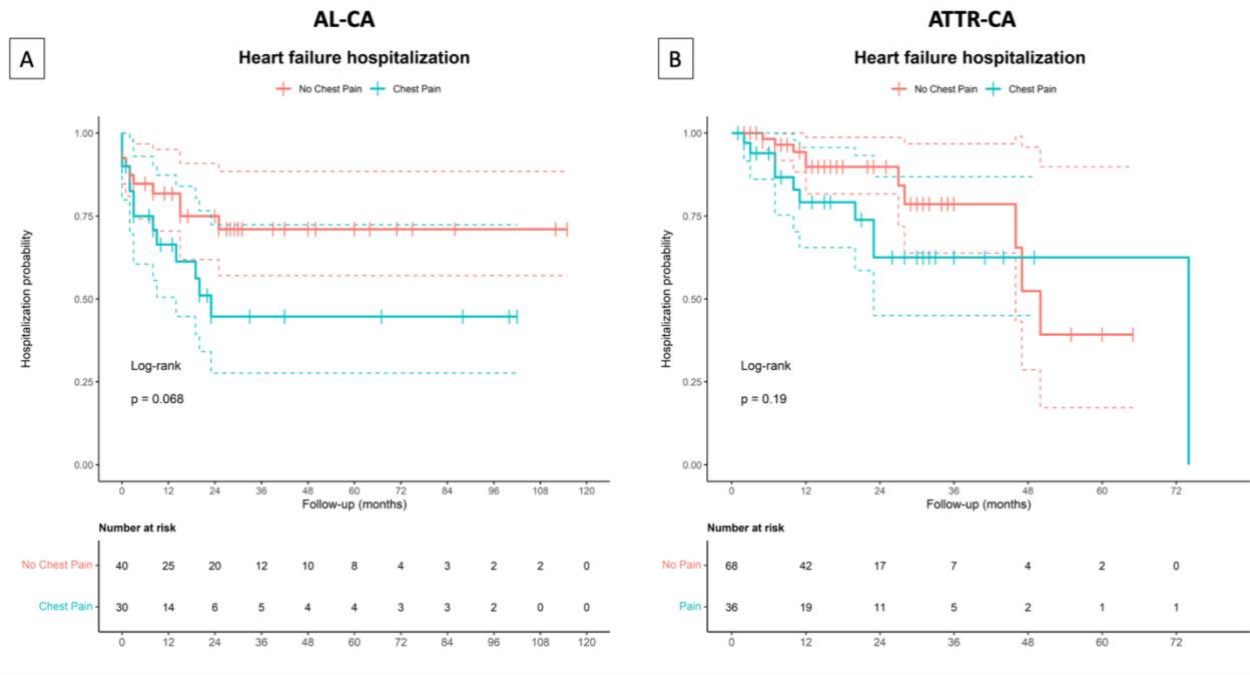
Supplemental Figure 1. Clinical indications for coronary angiography in the overall cohort. Abbreviations: ECG, electrocardiogram; HF, heart failure.
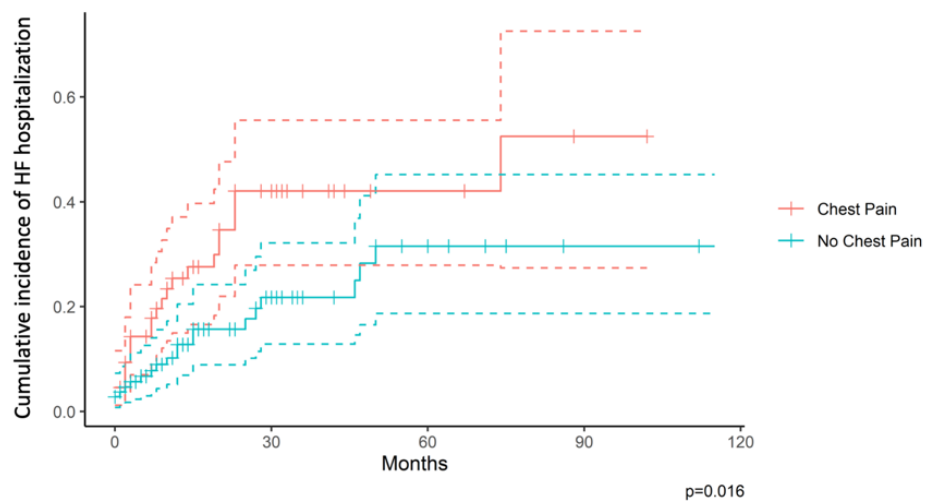
Supplemental Figure 2. Cumulative incidence of heart failure hospitalization in patients with and without chest pain considering death as competing risk.

152

Supplemental Figure 3. Prognostic significance of chest pain in patients with cardiac amyloidosis.
Kaplan Meier analysis for the time free from heart failure (HF) hospitalization in patients without and with chest pain in light chains cardiac amyloidosis (AL-CA, Panel A) and transthyretin cardiac amyloidosis (ATTR-CA, Panel B).

Chapter 10. Summary of conclusions from original contributions

1) By analysing hospital discharge summaries in a 5-million inhabitants region of Italy, we demonstrated an increased incidence of AH, up to 33.8 cases/million in 2020, and a 10-year period prevalence in 2020 of 124.5 cases/million. Moreover, up to 7% of men undergoing multiple CTS release surgeries may represent cases of suspected CA, possibly deserving further testing. Our data confirm that systemic amyloidosis is an emerging condition whose epidemiological dimensions are increasing in terms of patient care. Further studies on tailored screening of selected populations, such as patients undergoing multiple CTS surgeries, are necessary.

2) In patients undergoing PYP imaging for suspected ATTR-CM, very low hs-cTnT values can be of help in ruling out the diagnosis. At increasing thresholds, false negative results are present, and this should be taken into consideration in clinical practice. On the other hand, high hs-cTnT has limited predictive value in ruling-in disease. Similar results were found for NT-proBNP and for the combination of the two biomarkers.

3) In patients with ATTR-CA, particularly ATTRwt-CA, hs-cTnI measured with three different assays in different cohorts has a strong and independent prognostic role for mortality, even after adjustment for variables known to influence survival in these patients. A threshold of hs-cTnI of 80 ng/L (for Abbott and Beckman assay) and of 56 ng/L (for Siemens Centaur assay) appears to be useful for risk stratification of ATTRwt-CA patients in clinical practice. One staging system based on hs-cTnI and NP and one based on hs-cTnI, NP and eGFR were tested and demonstrated good performance for risk stratification of patients with ATTR-CA, particular ATTRwt-CA. These biomarkers-based systems maintained their prognostic significance also when adjusted for disease-modifying therapy. Therefore, our findings suggest that staging models for ATTRwt-CA based on cTn can be applied also in Institutions utilizing hs-cTnI measured with these three assays.

4) Chest pain is frequent in patients with CA, being reported by almost 40% of our sample. Patients with chest pain have more frequently a history of CAD and a more advanced CA clinical phenotype, including HF symptoms and higher cardiac biomarkers. The possible etiology of chest pain seems to differ among CA subtypes, with obstructive CAD being more frequent in ATTR-CA whilst amyloid vascular/perivascular involvement, as well as replacement type fibrosis and myocardial vacuolization, more common in AL-CA. Regardless of the etiology, chest pain identifies patients with CA at higher risk of HF hospitalization and therefore should prompt closer and personalized management to improve patients' outcome.