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Proceedings and predictions in cardiac amyloidosis: Unsolved mysteries and challenges for the future

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Proceedings and predictions in cardiac amyloidosis: Unsolved mysteries and challenges for the future

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Editorial: Proceedings and predictions in cardiac amyloidosis: unsolved mysteries and challenges for the future

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Editorial on the Research Topic
Proceedings and predictions in cardiac amyloidosis: unsolved mysteries and challenges for the future

> *"Sic parvis magna" – Sir Francis Drake*

Perceptions of amyloidosis have changed dramatically over the recent years following major advances in diagnosis and therapeutic strategies (1), especially in the field of cardiac amyloidosis (2). Recognition of disease and confirmation of final diagnosis, clinical management and treatment approaches represent ongoing challenges (3). The article Research Topic on "*Proceedings and predictions in cardiac amyloidosis: unsolved mysteries and challenges for the future*" has been conceived to discuss the latest advances and gray areas to address (4, 5) in amyloidosis from the perspective of international experts in the field.

Cardiac amyloidosis is commonly caused by systemic light chain amyloidosis or transthyretin amyloidosis (6). Systemic light chain amyloidosis is an acquired condition which is caused by a clonal plasma or B-cell expansion (monoclonal gammopathy) producing amyloidogenic immunoglobulin (Ig) light chains (7). Transthyretin amyloidosis is caused by deposition of misfolded or cleaved transthyretin protein in organs. This condition may be hereditary, in presence of destabilizing genetic mutations, or non-hereditary (wild type form) in the absence of genetic mutations (1, 8). Advances in cardiac magnetic resonance imaging and cardiac scintigraphy with bone tracers have revolutionized the diagnosis of cardiac amyloidosis (9, 10). Porcari et al. address the change in epidemiology of light chain and transthyretin cardiac amyloidosis following the development of non-invasive diagnostic criteria, highlighting the difference in patient characteristics at presentation and outcome compared to the past. Receiving a diagnosis of cardiac amyloidosis or carrying a gene mutation with the associated risk of developing disease in the future has a psychological impact on patients and their families, which is investigated (Smorti et al.).

With the exception of transthyretin cardiac amyloidosis which can now be diagnosed non-invasively in ∼70% of cases, histological analysis remains the definitive method for diagnosis and typing of amyloid (11). Clinical decision-making relies on histological information to provide patient-tailored clinical management, which includes Congo red staining used in combination with polarized light microscopy and immunohistochemistry (6), along with a mass spectrometry-based proteomic approach for amyloid typing (12). Riefolo et al. provide a thoughtful review on the contemporary histological approach to amyloidosis, with a focus on endomyocardial biopsy. The essential contribution of histology for typing amyloid fibril proteins is discussed in depth, especially in rare forms and challenging scenarios to orient treatment strategies.

Although histological studies reports a remarkable prevalence of disease (6), the epidemiology of amyloidosis, especially cardiac amyloidosis, is unknown worldwide (13). Caponetti et al. provide a comprehensive perspective on the association between underlying cardiac amyloidosis and specific clinical setting, potentially serving as tool to redefine screening approaches to amyloidosis in the real world (13). The authors underline the unique contribution of carpal tunnel syndrome that can precede the diagnosis of cardiac amyloidosis by 5 to 9 years (14). Among patients with bilateral surgery and ventricular thickening, the prevalence of transthyretin cardiac amyloidosis ranges from 14% in patients ≥60 years without occupational risk factors, to 33% considering only men (13, 15, 16). The authors emphasize how the adoption of sex-specific criteria to define cardiac amyloidosis might potentially result in earlier diagnoses (17, 18), and they discuss the results of a national survey of prevalence and accuracy of echocardiographic red flags of cardiac amyloidosis in consecutive patients undergoing routine echocardiography (AC-TIVE study) (19, 20), demonstrating a prevalence of transthyretin cardiac amyloidosis of 1% in the Italian population. Echocardiography is essential to raise suspicion of cardiac amyloidosis and provides multi-parametric assessment of systolic and diastolic cardiac. However, The value of left ventricle ejection fraction is limited in the setting of cardiac amyloidosis as discussed by Matteo et al.. The authors underline the potential advantages of other parameters for assessing cardiac contraction such as stroke volume (21), which might be an earlier marker of systolic dysfunction.

Cardiac amyloidosis has been traditionally considered an invariably fatal disease, but the scenario has changed following the development of disease-modifying treatments. The contemporary clinical course of transthyretin cardiac amyloidosis is characterized by progressive heart failure and arrhythmias (22). Scirpa et al. and Razvi et al. summarize the strengths and limitations of the main disease-specific staging systems developed to estimate survival and address the gray area of pre-symptomatic stage (23, 24) and foresee that a multi-parametric approach will result in a more accurate risk prediction (25). Tomasoni et al. address the advances in therapeutic strategies aimed at reducing the deposition of transthyretin amyloid in organs or hepatic synthesis of transthyretin through (26):

- Stabilization of the transthyretin tetramer to prevent dissociation (27, 28);
- Disruption of the messenger RNA (mRNA) in the hepatocyte with either small interfering RNA (29) or antisense oligonucleotides (30);
- Transthyretin gene editing to prevent the production of the relevant mRNA (31);
- Recognition of the transthyretin fibrils by the immune system and acceleration of their removal from vital organs with monoclonal antibodies (1);

Quarta et al. provides an expert opinion on the latest anti-fibril therapy in both light chain and transthyretin amyloidosis, which holds great potential for the application of depletion therapy in the foreseeable future.

Although amyloidosis is considered a contraindication to cardiac transplant, partly due to a perceived risk of amyloid recurrence in the allograft, cardiac transplant is a concrete therapeutic option for patients with light chain and transthyretin amyloidosis. Razvi et al. provide novel finding that "*Cardiac transplant is well-tolerated, restores functional capacity and improves prognosis in transthyretin cardiac amyloidosis. Post-cardiac transplant 1-year survival was 100%, 3-year survival was 92%, and 5-year survival was 90%. All but one surviving patient were New York Heart Association functional class I. Bone scintigraphy did not show evidence of cardiac allograft amyloid infiltration at 12 years post cardiac transplant.*" Notably, the authors provide the first evidence that the risk of amyloid recurrence in the cardiac allograft is low, thus suggesting that transthyretin amyloidosis does not represent a contraindication to cardiac transplant and that this treatment option should not be denied in patients with feasible characteristics. Future dedicated research is required to select the best candidates to cardiac transplant.

The epidemiology of cardiac amyloidosis is rapidly changing due to enhanced recognition of disease and the possibility of non-invasive diagnosis for transthyretin amyloidosis. Many gray areas have to be addressed (32). An exciting horizon of possibilities awaits to be explored in amyloidosis.

Author contributions

AP, GS, and JG contributed to the conception, design, manuscript preparation, and revision. All authors read and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evolving trends in epidemiology and natural history of cardiac amyloidosis: 30-year experience from a tertiary referral center for cardiomyopathies

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Objective: Natural history of cardiac amyloidosis (CA) is poorly understood. We aimed to examine the changing mortality of different types of CA over a 30-year period.

Patients and methods: Consecutive patients included in the "Trieste CA Registry" from January 1, 1990 through December 31, 2021 were divided into a historical cohort (diagnosed before 2016) and a contemporary cohort (diagnosed after 2016). Light chain (AL), transthyretin (ATTR) and other forms of CA were defined according to international recommendations. The primary and secondary outcome measures were all-cause mortality and cardiac death, respectively.

Results: We enrolled 182 patients: 47.3% AL-CA, 44.5% ATTR-CA, 8.2% other etiologies. The number of patients diagnosed with AL and ATTR-CA progressively increased over time, mostly ATTR-CA patients (from 21% before 2016 to 67% after 2016) diagnosed non-invasively. The more consistent increase in event-rate was observed in the long-term (after 50 months)

in ATTR-CA compared to the early increase in mortality in AL-CA. In the contemporary cohort, during a median follow up of 16 [4–30] months, ATTR-CA was associated with improved overall and cardiac survival compared to AL-CA. At multivariable analysis, ATTR-CA (HR 0.42, $p = 0.03$), eGFR (HR 0.98, $p = 0.033$) and ACE-inhibitor therapy (HR 0.24, $p < 0.001$) predicted overall survival in the contemporary cohort.

Conclusion: Incidence and prevalence rates of ATTR-CA and, to a less extent, of AL-CA have been increasing over time, with significant improvements in 2-year survival of ATTR-CA patients from the contemporary cohort. Reaching an early diagnosis and starting disease-modifying treatments will improve long-term survival in CA.

KEYWORDS

cardiac amyloidosis, epidemiology, diagnosis, prognostic stratification, non-invasive cardiac imaging

Introduction

Although previously considered as a rare and orphan disease, cardiac amyloidosis (CA) is increasingly recognized as frequent cause of heart failure (HF) and mortality in recent years (1). Light chain (AL) amyloidosis has an estimated prevalence of 1–2 in every 100.000 subjects (2). Although the exact epidemiological figure of transthyretin (ATTR) amyloidosis is still under scrutiny, this condition is more prevalent than traditionally thought, being reported in the heart of 25–40% of unselected adults > 75 years (3, 4). In contemporary years, the identification of populations at higher prevalence of CA (5–10) and major advances in non-invasive techniques such as cardiac magnetic resonance imaging and bone scintigraphy for the non-biopsy diagnosis of disease have led to a considerable increase in CA recognition, redefining the paradigm of cardiac involvement in amyloidosis (11, 12). The impact of these diagnostic advancements on clinical profiles at presentation and on the natural history of patients with CA has played a crucial role in ATTR-CA (13), but a comparison between AL and ATTR-CA has not been addressed so far. This is a crucial knowledge-gap to be covered as AL and ATTR-CA are completely different forms of amyloidosis in terms of pathophysiology, management and treatment options. AL amyloidosis is a treatable condition with different chemotherapy regimens and autologous stem cell transplantation, while ATTR-CA has become treatable after the first disease-modifying treatment has been tested in the ATTR-ACT trial in 2018 (14–16).

Therefore, we aimed to analyze the trends in epidemiology and natural history of patients with CA diagnosed over a 30-year period at a tertiary referral center for cardiomyopathies.

Materials and methods

This is a single-center, retrospective, observational study performed at the Cardiovascular Department, Cattinara University Hospital, Azienda Sanitaria Universitaria Giuliano-Isontina (ASUGI) and University of Trieste, Trieste, Italy. The local Regional Institutional Review Board approved the study (identifier 43_2009). The study was conducted according to the Declaration of Helsinki and informed consent was obtained under the institutional review board policies of the hospital administration.

Study population and definitions

Consecutive patients diagnosed with CA at the Cardiovascular Department, Azienda Sanitaria Universitaria Giuliano-Isontina (ASUGI), between January 1st, 1990 and December 31th, 2021 from the Trieste CA Registry were included in the study population and their data were retrospectively reviewed. The diagnosis of CA was made in presence of "invasive" or "non-invasive" criteria, according to the position statement on diagnosis and treatment of CA of the European Society of Cardiology (12). In detail, ATTR-CA was diagnosed in presence of a Perugini grade 2 or 3 myocardial uptake at cardiac scintigraphy with bone tracers and absence of monoclonal protein at urine and serum tests. Histological confirmation of amyloid deposition in the heart by endomyocardial biopsy (EMB) or in other affected tissues was obtained in all patients suspected of AL-CA with monoclonal proteins and in those not fulfilling non-invasive criteria (12). For the purpose of the study, patients were divided into an "historical cohort" (enrolled < 2016) and a "contemporary

cohort" (enrolled ≥ 2016) according to the validation of non-invasive diagnostic work-up for ATTR-CA (17). None of the patients with ATTR-CA received disease-modifying treatments during the study period.

Characterization of patients

Patients' baseline was set at the time of CA diagnosis and clinical data performed within 1 month were collected from electronic medical records, including all the following: (i) clinical history and examination, (ii) electrocardiogram (ECG), (iii) echocardiography, and, (iv) blood tests. ECG and echocardiographic images stored on our electronic database were systematically reviewed offline for this specific study by three cardiologists (A.P., M.M., L.P.), blinded to patients' outcome. Twelve-lead ECG was performed using standard equipment and retrospectively reviewed for heart rate, rhythm, QRS voltage, depolarization and repolarization abnormalities. Low voltages were defined as a QRS amplitude ≤ 0.5 mV in all limb leads or ≤ 1 mV in all precordial leads (18). All echocardiographic parameters were measured according to standard international definitions (19). Left ventricle (LV) volumes and LV ejection fraction (LVEF) were calculated using the Simpson's biplane method. Restrictive filling pattern (RFP) was defined as E-wave deceleration time < 120 ms or ≤ 150 ms in the presence of E/A ≥ 2. Right ventricle (RV) systolic dysfunction was defined as a tricuspid annular plane systolic excursion (TAPSE) < 17 mm and/or fractional area contraction (FAC) < 35% (19). The presence and severity of valve disease was defined according to current recommendations (20).

Cardiac scintigraphy with technetium pyrophosphate (99mTc-PYP) was performed with acquisition of planar and single photon emission computed tomography. A semi-quantitative score for the LV was obtained based on results of planar images as described by Perugini (21). The cameras and acquisition protocol used at our Institution is shown in **Supplementary Table 1**.

Histological evaluation of cardiac and extra-cardiac tissues was performed by the chief of our Institute of Pathological Anatomy and Histology (R.B.) with a specific expertise in the cardiovascular (CV) field, according to the standards and definitions proposed by the Committee of the Society for Cardiovascular Pathology and the Association for European Cardiovascular Pathology (22). In particular, histological sections were stained with hematoxylin and eosin (H&E) and Congo red, carefully analyzed for the presence of amyloid infiltration in the myocardium and vessels and evaluated under a polarized light microscope (23). Immunohistochemistry with kappa and lambda light chains antibodies, anti-TTR antibodies, anti-apolipoprotein AI and anti-serum amyloid A antibodies was performed on the most representative sample for each patient to characterize the amyloid deposits (4).

Outcome

The primary outcome of the study was all-cause mortality. The secondary outcome measure was cardiac death. The events were collected from the dedicated electronic databases of our center and, if needed, from patients' general practitioners and/or telephone contacts with patients and their relatives. At our institution, protocols of coroner referral and post-mortem analysis were constant over time. Events were independently assessed by three cardiologists (G.G.V., R.S, F.L.), blinded to patients' characteristics.

Statistical analysis

Descriptive statistics were measured as median with interquartile range (IQR) [25°; 75°] for continuous variables as data were not normally distributed according to the results of Kolmogorov-Smirnov test; categorical variables were expressed as absolute numbers and percentages. Differences between groups were evaluated using Mann–Whitney test for continuous variables, while Chi square ($\chi 2$) or Fisher's exact test were used for dichotomous variables. The Kaplan-Meier method was used to estimate overall survival, and the log rank test was used to compare the curves. In the case of secondary end points, to account for the presence of competing risks, cumulative incidence curves were estimated and compared using appropriate methods (24). Univariable and multivariable analyses were performed for the primary and secondary study outcomes in patients from the contemporary cohort. Each variable was evaluated at univariable cause-specific Cox regression and, when a p-value < 0.1 was found, was included into a multivariable Cox model. The number of events was taken into account to estimate an adjusted HR with an event per variable (epv) ratio of 10. The end of follow-up was set at 31th December, 2021. We defined a p-value < 0.05 as statistically significant. All statistical analyses were performed using IBM SPSS Statistics 24.0 package (New York, NY) statistical software version 20 and R (R Foundation for Statistical Computing, Vienna, Austria)[1], packages "cmprsk" and "crrSC."

Results

Increasing prevalence of cardiac amyloidosis over time

The study population included 182 patients diagnosed with CA: 49.5% (n = 90) from the "historical cohort" and 50.5% (n = 92) from the "contemporary cohort." CA was related to

1 https://www.r-project.org/

the following etiologies: 47.3% (n = 86) AL, 44.5% (n = 81) ATTR, and 8.2% (n = 15) other etiologies (5 dialysis-related amyloidosis, 10 undetermined etiologies) (**Figure 1**).

Over time, the consistent increase in non-invasive CA diagnosis by cardiac scintigraphy (59.8 vs. 3.3%, $p < 0.001$) was paralleled by a decrease in the number of CA diagnosed by biopsy (96.7 vs. 40.2%, $p < 0.001$), which still was adopted in a significant quota of cases in the contemporary cohort (i.e., mostly for AL-CA confirmation). Mortality in patients with CA diagnosed in recent years was lower compared to the past (p = 0.002, **Figure 2**). Chemotherapy regimens used in patients with AL-CA are shown in **Supplementary Table 2**.

Characterization of cardiac amyloidosis over time

Table 1 shows the baseline characteristics of the study population according to the enrolment period. All patients presented with HF. Compared to the historical cohort, patients of the contemporary cohort were predominantly males (75 vs. 58.9%, p = 0.021), more affected by ATTR-CA (67.4 vs. 21.1%, $p < 0.001$), presented less frequently in NYHA $\geq$ 3 (36.3 vs. 53.5%, p = 0.021), and had greater rates of AF (53.8 vs. 19.1%, $p < 0.001$), LVEF < 50% (36.8 vs. 22.1%, p = 0.034) and aortic valve stenosis (any degree of severity) (21.1 vs. 6%, p = 0.005) (**Table 1**).

Compared to the historical ATTR-CA cohort, ATTR-CA patients in the recent cohort had a similar age at diagnosis (mean age 78 vs. 80 years, p = 0.174), higher frequency of history of AF (67.2 vs. 38.9%, p = 0.030) and increased E/E' ratio (21 vs. 17, p = 0.046) (**Supplementary Table 3**). Of note, none of the patients before 2016 presented with NYHA 1, while none of the patients after 2016 presented with NYHA 4. Similar cardiological characteristics were found for AL-CA patients.

Prognostic implications of amyloidosis type in the contemporary and historical cohorts

Overall mortality of patients with ATTR-CA was significantly higher in the contemporary compared to the historical cohort (p = 0.006), while no difference was found in cardiac mortality rates (p = 0.26) (**Figure 3**, left). AL-CA patients from the contemporary cohort had higher frequency of all-cause death (p = 0.044) and similar rates of cardiac mortality (p = 0.3) compared to those from the historical cohort (**Figure 3**, right). During a median follow up of 65 [8–118] months, at survival analysis, patients with AL-CA and ATTR-CA from

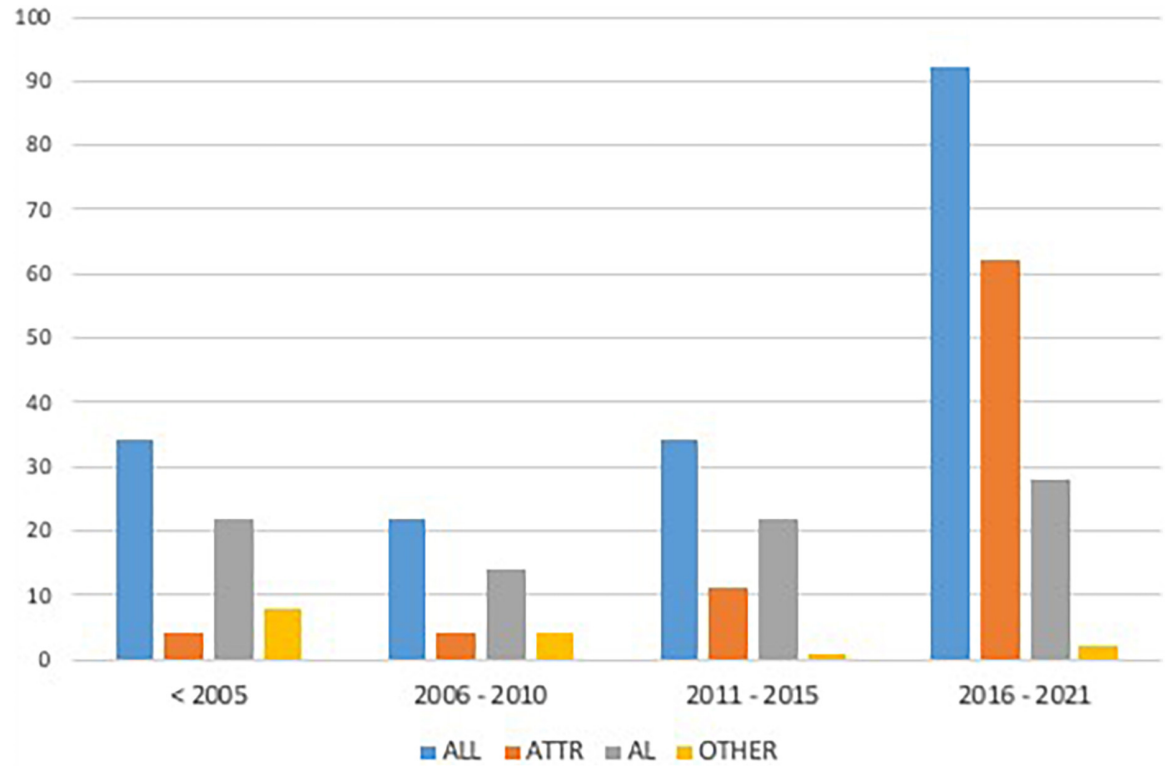

FIGURE 1
Enrolment rate of the study population according to different periods. AL, Light Chain Amyloidosis; ATTR, Transthyretin Amyloidosis.

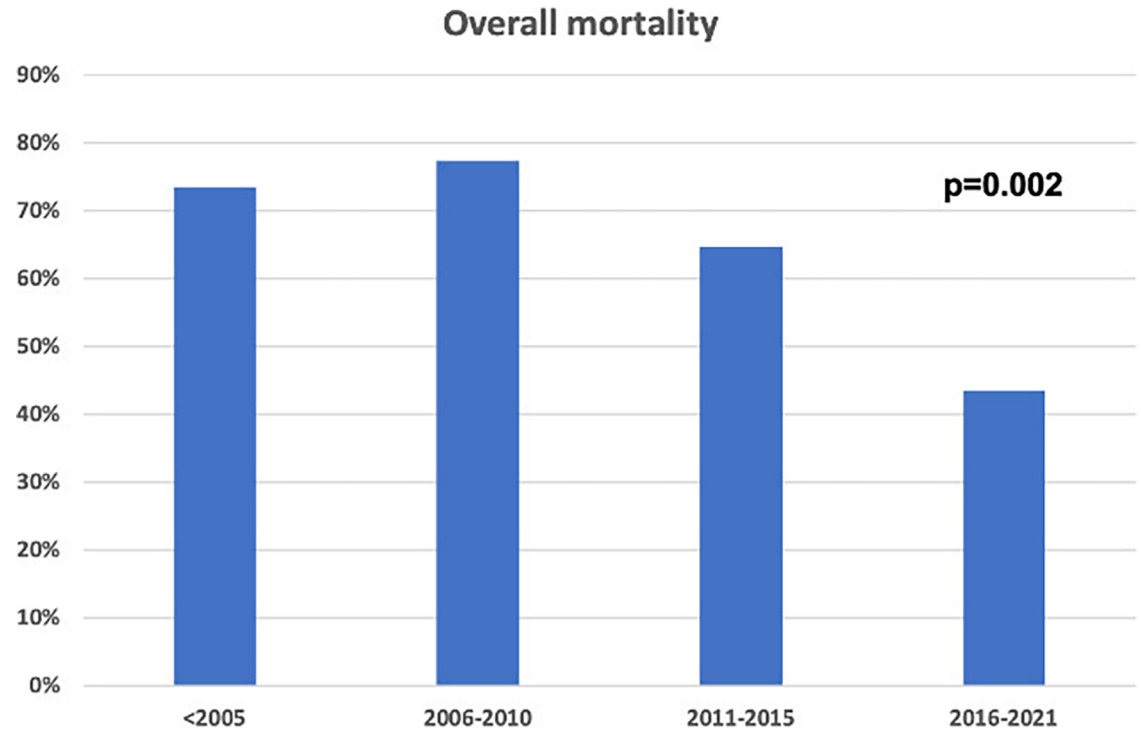

FIGURE 2
Overall mortality in the study population according to enrolment periods.

the historical cohort had similar rates of all-cause death (67 and 74% respectively, $p = 0.5$) and cardiac death (57 and 42% respectively, $p = 0.1$) (**Figure 4**, left). In the historical cohort, an early separation of the survival curves was observed due to a higher event-rate in patients with AL-CA that was paralleled over time by a progressive increase in the event-rate in patients with ATTR-CA, with similar long-term outcome in both types of amyloidosis. In the contemporary cohort, over a median follow up of 16 [4–30] months, ATTR-CA was associated with more favorable outcome compared to AL-CA, with an observed overall survival of 80 vs. 40% at 24 months ($p = 0.002$; **Figure 4**, right). At multivariable analysis using significant covariates emerged at univariable analysis, in patients from the contemporary cohort, ATTR-CA (overall survival HR 0.42, $p = 0.03$; cardiac death HR 0.39, $p = 0.042$), ACE-inhibitor therapy (overall survival HR 0.24, $p < 0.001$; cardiac death HR 0.25, $p = 0.07$) and eGFR (overall survival HR 0.98, $p = 0.033$; cardiac death HR 0.96, $p = 0.041$) were associated with both study outcomes (**Table 2**).

Discussion

The present study describes the changes in epidemiology, clinical profiles and natural history of CA over the last 30 years in a third-level referral Center for cardiomyopathies. To the best of our knowledge, this is the first report including comprehensive cardiological characterization and investigating baseline predictors for global outcomes and cardiovascular outcomes in a combined cohort of AL and ATTR-CA patients across different time periods.

The major findings of the present study are that (a) the number of patients diagnosed with CA progressively increased over time, mostly related to ATTR-CA; (b) CA patients from the contemporary cohort presented with less symptomatic HF compared to those from the historical cohort; (c) although an early increase in the event-rate was found in AL-CA, long-term survival rates among ATTR-CA and AL-CA were similar in the historical cohort, while, in the contemporary cohort, ATTR-CA was associated with reduced all-cause mortality and cardiac mortality compared to AL-CA; and, (d) in the contemporary cohort, ATTR-CA, higher eGFR and therapy with ACE-inhibitors were associated with a more favorable global and cardiovascular outcome. Rather than a change in natural history of the disease, we believe that the observed increase in incidence and prevalence of CA is likely related to a number of factors including (1) heightened awareness of disease, (2) recognition of clinical and instrumental red-flags and subgroups of patients at higher risk of CA, and (3) development of non-invasive criteria for the diagnosis and specific treatments (25, 26).

TABLE 1 Baseline characteristics of the study population according to the enrolment period.

	Available (*n*)	Study population (*n* = 182)	< 2016 (*n* = 90)	> 2016 (*n* = 92)	*P*-value
Age, y	**182**	**73 (63–80)**	**70 (58–77)**	**77 (69–82)**	**<0.001**
Male	**182**	**122 (67%)**	**53 (58.9%)**	**69 (75%)**	**0.021**
AL amyloidosis	**182**	**86 (47.3%)**	**58 (64.4%)**	**28 (30.4%)**	**0.001**
ATTR amyloidosis	**182**	**81 (44.5%)**	**19 (21.1%)**	**62 (67.4%)**	**0.001**
Other etiologies	**182**	**15 (8.2%)**	**13 (14.4%)**	**2 (2.2%)**	**0.001**
Biopsy-proven diagnosis	**182**	**124 (68.1%)**	**87 (96.7%)**	**37 (40.2%)**	**<0.001**
Scintigraphy-proven diagnosis	**182**	**58 (32%)**	**3 (3.3%)**	**55 (59.8%)**	**<0.001**
NYHA ≥ 3	**177**	**79 (44.6%)**	**46 (53.5%)**	**33 (36.3%)**	**0.021**
Systolic BP, mmHg	169	120 (110–140)	120 (110–140)	120 (110–140)	0.176
Syncope	182	19 (10.4%)	12 (13.3%)	7 (7.6%)	0.20
Hypertension	**182**	**99 (54.4%)**	**40 (44.4%)**	**59 (64.1%)**	**0.008**
eGFR < 60 mL/min	151	77 (51%)	33 (50%)	42 (45.6%)	0.83
eGFR, mL/min	151	50 (36–75)	59 (34–75)	59 (40–76)	0.484
IHD	182	12 (6.6%)	5 (5.6%)	7 (7.6%)	0.58
History of AF	**180**	**66 (36.7%)**	**17 (19.1%)**	**49 (53.8%)**	**<0.001**
Carpal tunnel	**181**	**44 (24.3%)**	**6 (6.7%)**	**38 (41.3%)**	**<0.001**
Medications					
BBs	171	82 (48%)	36 43.4%)	46 (52.3%)	0.244
ACEi/ARBs	171	86 (50.3%)	36 (43.4%)	50 (56.8%)	0.079
Diuretics	171	143 (83.6%)	69 (83.1%)	74 (84.1%)	0.866
MRAs	171	68 (39.8%)	30 (36.1%)	38 (43.2%)	0.347
Electrocardiography					
Rhythm at baseline	182				0.145
Sinus rhythm		109 (59.9%)	60 (66.7%)	49 (53.3%)	
AF		61 (33.7%)	24 (26.7%)	37 (40.2%)	
PM		12 (6.6%)	6 (6.7%)	6 (6.5%)	
HR, bpm	145	75 (65–88)	76 (68–90)	70 (65–85)	0.110
RBBB	182	30 (16.5%)	10 (11.1%)	20 (21.7%)	0.053
LBBB	182	27 (14.8%)	10 (11.1%)	17 (18.5%)	0.162
LFAB	182	31 (17%)	12 (13.3%)	19 (20.7%)	0.189
LVH	182	21 (11.5%)	9 (10%)	12 (13%)	0.521
Q wave	182	55 (30.2%)	32 (35.6%)	23 (25%)	0.121
Low QRS voltages	182	64 (35.2%)	37 (41.1%)	27 (29.3%)	0.097
Echocardiography					
LVEDVi, mL/m2	**155**	**42 (34–55)**	**38 (30–51)**	**45 (38–57)**	**0.002**
IVS, mm	**174**	**16 (14–19)**	**15 (14–18)**	**17 (15–20)**	**0.016**
PW, mm	167	14 (12–16)	14 (12–16)	14 (12–17)	0.347
LVEF	**167**	**55% (47–63)**	**57% (51–65)**	**53% (41–62)**	**0.021**
LVEF < 50%	**173**	**51 (29.5%)**	**19 (22.1%)**	**32 (36.8%)**	**0.034**
E/E'	**112**	**20 (14–27)**	**20 (11–23)**	**21.5 (16–28)**	**0.035**
RFP	124	61 (49.2%)	32 (47.8%)	29 (50.9%)	0.729
LA diameter, mm	142	44 (39–50)	43 (39–49)	46 (40–51)	0.101
RA area, cm2	131	23 (18–26)	22 (18–26)	23 (19–27)	0.466
RV dysfunction	165	91 (55.2%)	46 (56.1%)	45 (54.2%)	0.808
Biventricular dysfunction	161	40 (24.8%)	17 (21.3%)	23 (28.4%)	0.294

(Continued)

TABLE 1 (Continued)

	Available (*n*)	Study population (*n* = 182)	< 2016 (*n* = 90)	> 2016 (*n* = 92)	*P*-value
Moderate-severe MR	149	30 (20.1%)	14 (20%)	16 (20.3%)	0.969
Aortic stenosis	**159**	**21 (13.2%)**	**5 (6%)**	**16 (21.1%)**	**0.005**
RV hypertrophy	174	69 (39.7%)	31 (36%)	38 (43.3%)	0.336
Thickened IAS	**174**	**26 (14.9%)**	**8 (9.3%)**	**18 (20.5%)**	**0.039**
Pericardial effusion	174	69 (39.7%)	37 (43%)	32 (36.4%)	0.369

ACEi, Angiotensin-converting enzyme inhibitor; AF, Atrial Fibrillation; AL, Amyloid light chain; ARBs, Angiotensin II Receptor Blockers; ATTR, Transthyretin Amyloidosis; BBs, Beta Blockers; BP, Blood Pressure; eGFR, estimated Glomerular Filtration Rate; HR, Heart Rate; IAS, Interatrial Septum; IHD, Ischemic Heart Disease; IVS, Interventricular septum; LA, Left Atrium; LBBB, Left Bundle Branch Block; LFAB, Left Fascicular Anterior Block; LVEDVi, Left Ventricular End Diastolic Volume index; LVEF, Left Ventricular Ejection Fraction; LVH, Left Ventricular Hypertrophy; MR, Mitral regurgitation; MRAs, Mineralocorticoid Receptors Antagonists; NYHA, New York Heart Association; PM, Pacemaker; PW, Posterior Wall; RA, Right Atrium; RBBB, Right Bundle Branch Block; RFP, Restrictive Filling Pattern; RV, Right Ventricle. Bold identifies parameters with *p*-value <0.05.

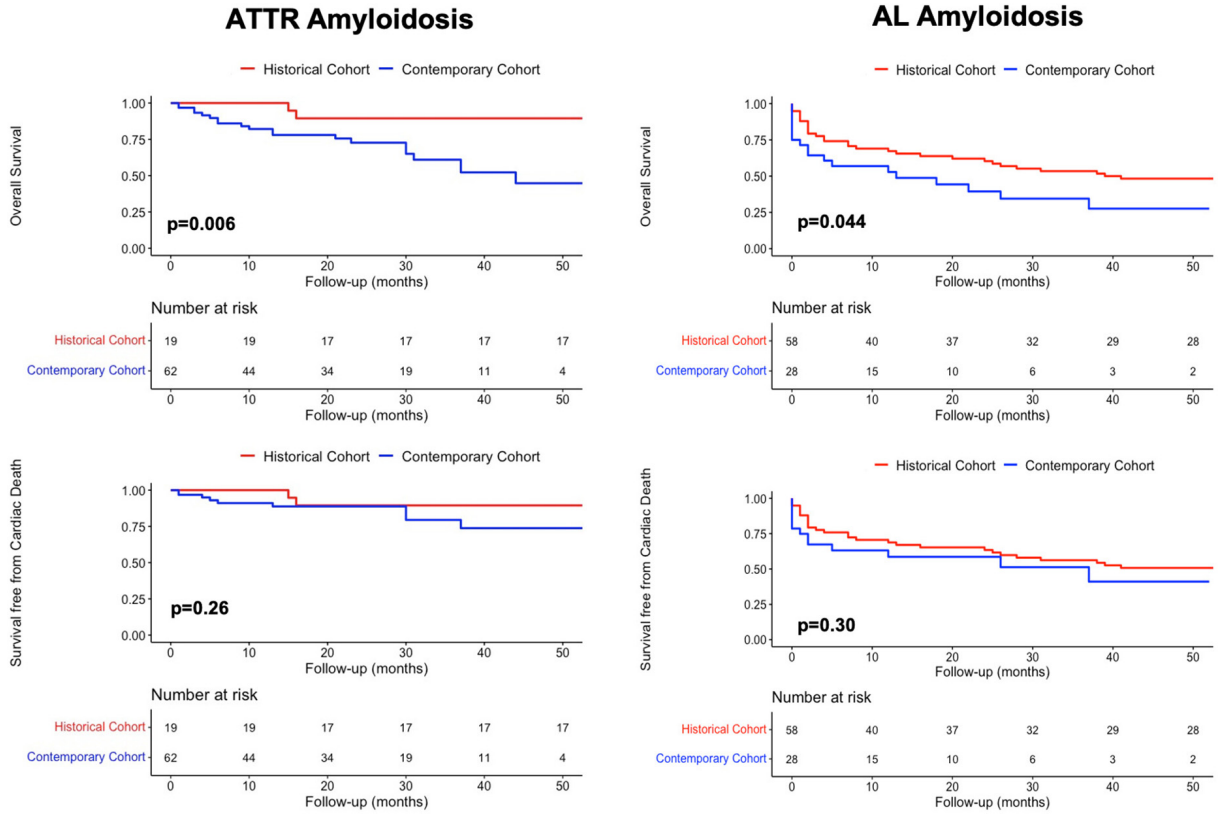

FIGURE 3
Kaplan-Meier survival analysis in patients from the historical and the contemporary cohort with **(Left column)** ATTR amyloidosis and **(Right column)** AL amyloidosis for overall mortality and cardiac death. AL, Light Chain Amyloidosis; ATTR, Transthyretin Amyloidosis.

The evolving epidemiology of cardiac amyloidosis

In the present analysis, the number of patients diagnosed with AL and ATTR-CA progressively increased over time. In recent years, bone scintigraphy has become the predominant mode of diagnosis for CA, resulting in a significant increase of patients diagnosed with ATTR-CA and, to a lesser extent, with AL-CA (**Figure 1**) (5–8, 12). Of note, our results suggest that the improved diagnostic yield has led to the identification of more patients with early as well as advanced CA (**Table 1**). Notably, current approach to CA resulted in increased recognition of AL-CA as patients with suspicion of ATTR-CA undergo a comprehensive assessment, including search for monoclonal proteins in urine and serum, thus leading to identification of otherwise undiagnosed patients with AL-CA (27). However, the diagnostic approach to AL-CA has not significantly changed over the years as reflected in similar cardiac phenotype at

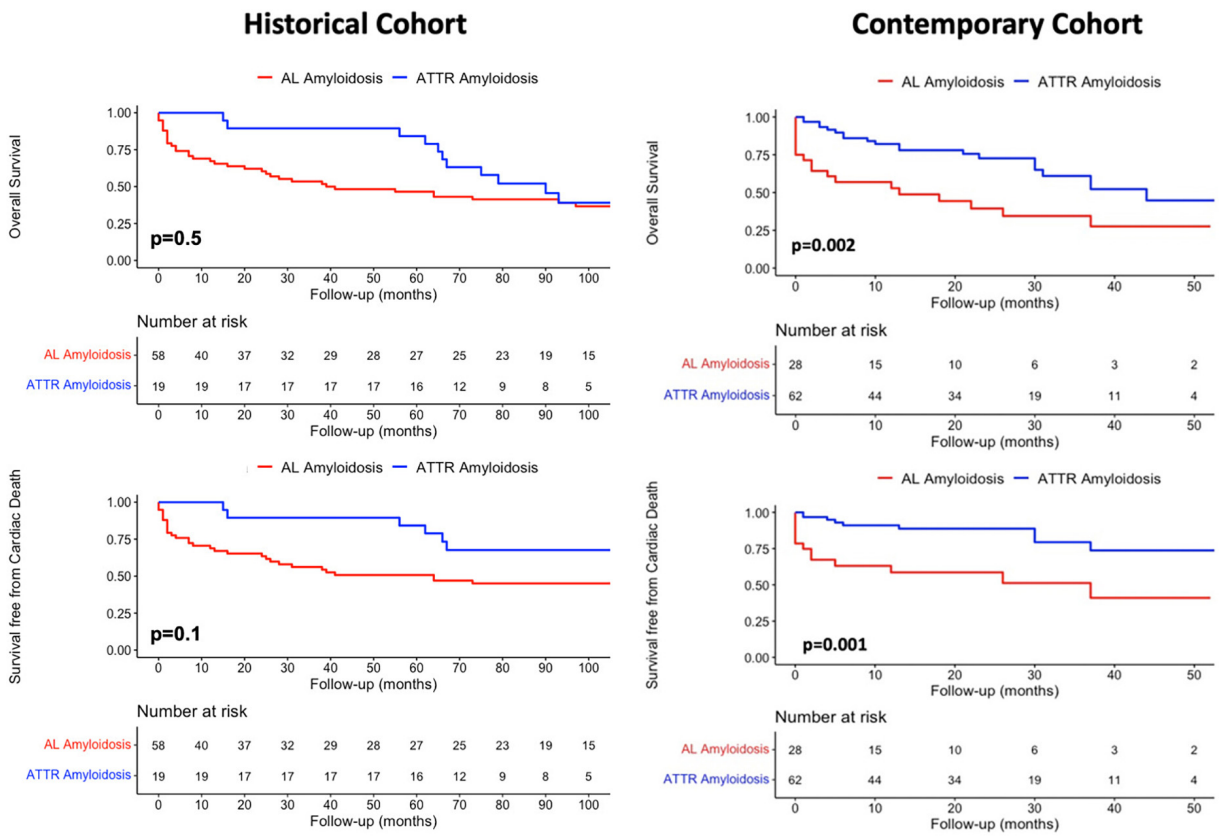

FIGURE 4
Kaplan-Meier survival analysis for **(Top row)** overall mortality and competing risk analysis for **(Bottom row)** cardiac death according to amyloidosis etiology in the historical and contemporary cohorts. AL, Light Chain Amyloidosis; ATTR, Transthyretin Amyloidosis.

presentation among AL-CA patients from the historical and contemporary cohort These findings are in line with recent data from referral centers for amyloidosis reporting evidence of substantially greater recognition of both AL and ATTR amyloidosis (28). Although AL amyloidosis still remains the most common type of amyloidosis in national referral centers, accounting for 55% of all cases (29), differences in the structure of national health systems, referral patterns and populations' ethnicities may explain this discrepancy among different centers (1, 30).

The natural history of transthyretin and light chain-cardiac amyloidosis across ages

Patients with CA from the historical cohort had very poor outcomes regardless of type of amyloidosis, reasonably related to recognition of disease in late stages and lack of effective therapies. In our cohort, the natural history of AL-CA was characterized by an early increase in all-cause mortality rate, especially in the months following initiation of chemotherapy (**Figure 4**). Interestingly, in ATTR-CA patients from the historical cohort, the consistent increase in event-rate was observed in the long-term (after 50 months; **Figure 4**, top, left), supporting the possibility to effectively change the natural history of disease with disease-modifying therapies. In the contemporary cohort, ATTR-CA was associated with more favorable global and cardiovascular outcomes compared to AL-CA, reflecting the major advances in earlier diagnosis and treatment, especially for ATTR amyloidosis (**Figure 4**). In 2016, a landmark study by Gillmore et al. (17) paved the way for the clinical application of bone scintigraphy for the non-invasive diagnosis of ATTR-CA, demonstrating that the positive predictive value of a moderate-high myocardial uptake approaches 100% in the absence of a monoclonal protein in serum and urine, thus limiting the need for EMB to selected cases (31, 32). Broadening the diagnostic horizon of CA, predominantly ATTR-CA (9), resulted in recognition of more patients in different stages of cardiac disease and in improved overall survival in the contemporary cohort compared to AL-CA patients (**Figure 3**). The observed lower overall survival of patients with ATTR-CA and AL-CA from the contemporary compared to the historical cohort, in spite of

TABLE 2 Univariable and multivariable analyses for overall mortality and cardiac death in the contemporary cohort.

Parameters	Univariable analysis		Multivariable analysis (OM)		Multivariable analysis (CD)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age at diagnosis, for every year increase	1.001 (0.96–1.03)	0.953				
Male sex	**0.49 (0.25–0.95)**	**0.035**	0.62 (0.29–1.34)	0.08	0.78 (0.26–2.30)	0.66
Scintigraphy vs. biopsy	**0.23 (0.12–0.46)**	**<0.001**				
ATTR vs. AL	**0.39 (0.20–0.73)**	**0.004**	**0.42 (0.18–0.93)**	**0.038**	**0.39 (0.15–0.96)**	**0.042**
History of AF	0.76 (0.41–1.42)	0.394				
Syncope	2.44 (0.87–5.75)	0.126				
Hypertension	1.08 (0.56–2.07)	0.812				
SBP, mmHg	0.98 (0.96–1.007)	0.173				
Carpal tunnel	0.54 (0.27–1.07)	0.151				
NYHA ≥ 3	1.76 (0.94–3.29)	0.110				
eGFR < 60 ml/min	1.15 (0.61–2.18)	0.655				
eGFR, ml/min	0.98 (0.97–1.001)	0.07	**0.98 (0.97–0.99)**	**0.033**	**0.96 (0.94–0.98)**	**0.041**
Low QRS voltages	1.81 (0.95–3.45)	0.123				
BBs	0.58 (0.30–1.11)	0.104				
ACEi/ARBs	**0.24 (0.12–0.48)**	**<0.001**	**0.24 (0.11–0.50)**	**<0.001**	**0.25 (0.09–0.69)**	**0.007**
IVS, per every mm increase	1.006 (0.94–1.07)	0.866				
LVEF, per every% increase	1.004 (0.98–1.02)	0.748				
LVEF < 50%	1.02 (0.52–1.99)	0.943				
E/E', for every point increase	0.99 (0.96–1.03)	0.903				
RFP	0.47 (0.21–1.05)	0.112				
LA diameter, mm	0.99 (0.96–1.02)	0.858				
RV dysfunction	0.89 (0.46–1.73)	0.741				
Biventricular dysfunction	0.69 (0.31–1.52)	0.360				
Moderate-severe MR	1.37 (0.61–3.04)	0.438				
Aortic Stenosis	1.41 (0.68–2.92)	0.355				
Pericardial effusion	0.99 (0.50–1.93)	0.979				

ACEi, Angiotensin-converting enzyme inhibitor; AF, Atrial Fibrillation; AL, Amyloid light chain; ARBs, Angiotensin II Receptor Blockers; ATTR, Transthyretin Amyloidosis; BBs, Beta Blockers; CD, Cardiac Death; eGFR, estimated Glomerular Filtration Rate; IVS, Interventricular septum; LA, Left Atrium; LVEF, Left Ventricular Ejection Fraction; LVH, Left Ventricular Hypertrophy; MR, Mitral regurgitation; NYHA, New York Heart Association; OM, Overall Mortality; RFP, Restrictive Filling Pattern; RV, Right Ventricle, SBP, Systolic Blood Pressure. Bold identifies parameters with *p*-value <0.05.

similar rates of cardiac death, results from the increasing competing risks of non-cardiac death that is typical of elderly patients (25) (**Figure 3**). In the past, many patients with CA, especially ATTR amyloidosis, were not recognized or diagnosed at post-mortem examination, as reflected by the lower absolute number of ATTR-CA patients in the historical compared to the contemporary cohort (**Table 1**). Our results are in line with recent reports from nationwide studies that shows a progressive reduction in overall mortality of CA over years (30).

Tools for prognostic stratification in the contemporary era

In this study, the type of amyloidosis (AL vs. ATTR amyloidosis), renal function and tolerability of ACE-inhibitor therapy were associated with a better global and cardiac outcome in the contemporary cohort (**Table 2**). The prognostic role of renal function has been largely investigated and this parameter is included in validated prognostic scores (26, 33).

Although CA confers increased risk of mortality and morbidity, the more favorable natural history of ATTR-CA compared to AL-CA was expected based on the heterogeneity and severity of organ involvement found in patients presenting with AL amyloidosis in clinical practice, whose survival is largely dependent on the tolerability and efficacy of chemotherapy. These findings further underline that AL and ATTR amyloidosis are 2 different diseases. The goal of early initiation of specific treatment would be to obtain an increase in survival with net and persistent separation of the curves in the contemporary cohort rather than a progressive decline in survival observed in untreated patient from the historical cohort (**Figure 3**). Of note, the association between ACE-inhibitor therapy and survival is

of particular interest. In our opinion, patients tolerating these drugs might have a less advanced systemic and cardiac amyloid burden rather than having direct survival advantages from this treatment. According to recent studies, ACE-inhibitors and beta-blockers might be safely prescribed in CA, starting from low doses, then slowly up-titrated with frequently re-evaluation of treatment tolerance (34, 35). Dedicated studies are required to understand whether these drugs have a prognostic impact in patients with HF due to amyloidotic etiology.

Limitations

This is a single-center retrospective study conducted in a third-level referral center for the diagnosis and management of cardiomyopathies. Therefore, the expertise of our Center in this field is a potential bias to consider. NT-proBNP and troponin could not be systematically included in the analysis as they were routinely evaluated from 2018 on (for NT-proBNP) or because of a change in the assay sensitivity over time (high-sensitive troponin evaluated from 2019 on). CMR data were not available in this analysis. Etiology-specific prognostic predictors on multivariable analysis could not be investigated in the present study because of a limited number of events in the contemporary cohort; however, this is an important issue to investigate in future dedicated studies. Finally, at our institution, ATTR-CA patients underwent systematically genetic testing for transthyretin mutations after 2016 and all of them were diagnosed with wild-type form.

Conclusion

Recent years have been characterized by an exponential increase in incidence and prevalence rates of CA, especially ATTR amyloidosis. In the modern era, patients with ATTR-CA have more favorable global and cardiovascular outcome compared to those with AL-CA. In the contemporary cohort, a diagnosis of ATTR-CA, renal function and ACE-inhibitor therapy at presentation were associated with a more favorable global and cardiovascular outcome.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Regional Institutional Review Board approved the study (identifier 43_2009). The patients/participants provided their written informed consent to participate in this study.

Author contributions

APo, MM, and GS contributed to conception and design of the study. VA, RS, GV, LP, MR, AL, and FL organized the database. APo performed the statistical analysis. APo, VA, and MM wrote the first draft of the manuscript. MD, RK, APe, FD, RB, GD, and FZ wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1026440/full#supplementary-material

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Amyloidosis: What does pathology offer? The evolving field of tissue biopsy

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Since the mid-nineteenth century pathology has followed the convoluted story of amyloidosis, recognized its morphology in tissues and made identification possible using specific staining. Since then, pathology studies have made a significant contribution and advanced knowledge of the disease, so providing valuable information on the pathophysiology of amyloid aggregation and opening the way to clinical studies and non-invasive diagnostic techniques. As amyloidosis is a heterogeneous disease with various organ and tissue deposition patterns, histology evaluation, far from offering a simple yes/no indication of amyloid presence, can provide a wide spectrum of qualitative and quantitative information related to and changing with the etiology of the disease, the comorbidities and the clinical characteristics of patients. With the exception of cardiac transthyretin related amyloidosis cases, which today can be diagnosed using non-biopsy algorithms when stringent clinical criteria are met, tissue biopsy is still an essential tool for a definitive diagnosis in doubtful cases and also to define etiology by typing amyloid fibrils. This review describes the histologic approach to amyloidosis today and the current role of tissue screening biopsy or targeted organ biopsy protocols in the light of present diagnostic algorithms and various clinical situations, with particular focus on endomyocardial and renal biopsies. Special attention is given to techniques for typing amyloid fibril proteins, necessary for the new therapies available today for cardiac transthyretin related amyloidosis and to avoid patients receiving inappropriate chemotherapy in presence of plasma cell dyscrasia unrelated to amyloidosis. As the disease is still burdened with high mortality, the role of tissue biopsy in early diagnosis to assure prompt treatment is also mentioned.

KEYWORDS

cardiac amyloidosis (CA), pathology, diagnosis, biopsy, proteomics

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Introduction

Biopsy is the most reliable method to show up amyloid fibrillar deposits within organs and tissues. These deposits derive from a variety of abnormally aggregated precursor proteins, and can cause cytotoxicity-mediated lesions, distortion of tissue architecture and organ dysfunction. The term amyloidosis indicates the disease that can result from pathologic protein aggregation and includes a wide range of systemic or localized disorders, highly heterogeneous in terms of cause, clinical manifestation, anatomic distribution, progression, and prognosis (1).

Although the first mentions of amyloidosis in the spleen date back to autopsy reports of the seventeenth century, the term "amyloid" was introduced in medical literature and popularized in 1854 by the German pathologist Rudolph Virchow (2, 3). He used this term for a substance found in human tissue, similar to that previously described at autopsy as "stony," "gelatinous," "lardaceous," or "waxy" visceral material. Virchow most likely considered amyloid to be similar to starch, a kind of animal cellulose, although at that time the distinction between starch and cellulose was unclear. In 1859 the German chemist August Kekule presumed that the material infiltrating organs was mainly constituted by "albumoid compounds," but, nonetheless, the name "amyloid" did not change and the use of a unified nomenclature opened the way to biological and clinical research through multidisciplinary collaboration between pathologists, chemists, physicists, and clinical researchers, collaboration still active today (3, 4).

From the turn of the century there were major advances in amyloid studies with the ever more common use of light microscopy, which identified its amorphous structure, and the use of histopathologic dyes such as Congo red (CR) and thioflavin. CR was found to bind avidly to amyloid (5) and to show apple-green birefringence when viewed under polarized light (6, 7).

More detailed submicroscopic and physical studies in the second half of 20th century demonstrated the fibrillary ultrastructure of amyloid (8) and that fibrils were composed of polypeptide chains with generic cross-beta conformation (9, 10). Amino acid sequence analyses gradually led to the discovery that each type of amyloid consists of a different fibril protein and, in the 2000s, the term "amylome" was introduced to describe the multitude of proteins potentially able to generate amyloid-like fibrils, many of which, however, do so only under certain *in vitro* conditions. It is therefore mandatory to clarify exactly what we mean by the term amyloid (11, 12).

To date 40 proteins have been identified in humans as amyloidogenic, 18 of which associated with systemic amyloidosis and 22 with localized disease (1).

In order to achieve a proper classification of amyloidosis, the current goals for clinical management are first to identify amyloid deposits in tissue, then to understand fibril distribution and the anatomical structures involved and, most importantly, to determine protein composition, i.e., to type amyloid.

Following the pioneering methods published around a decade ago, a mass spectrometry-based proteomic approach to amyloid typing revolutionized diagnostic protocols and placed renewed value on information obtained from histology and immunohistochemistry.

The review describes the histologic approach to amyloidosis today, and the current role of tissue screening or targeted organ biopsy protocols in the light of present diagnostic algorithms and various clinical situations, with particular focus on histopathologic patterns in endomyocardial and renal biopsies. Special attention is given to techniques for typing amyloid fibril proteins, necessary for the new therapies available today for cardiac transthyretin amyloidosis and to avoid patients receiving inappropriate chemotherapy in presence of plasma cell dyscrasia unrelated to amyloidosis. As the disease is still burdened with high mortality, the role of early diagnosis using tissue biopsy to assure prompt treatment is also mentioned.

Pathology examination of amyloidosis

Anatomo-pathological study of organs and tissues is essential in a complex disease like amyloidosis, characterized by a wide spectrum of acquired and hereditary etiologies, various pathogenetic mechanisms, involvement of many organs and tissues and considerable phenotypic heterogeneity (13) (**Table 1**). Pathology investigation involves various steps, each of which can provide major diagnostic, therapeutic, and prognostic information. Gross and histology examination are performed as well as identification of the precursor proteins in the tissue samples, using various typing methods, ranging from immune-biochemical techniques to molecular proteomic analysis.

Histology is particularly crucial for various reasons (14–16):

1. making a definite diagnosis in cases of clinically unexpected amyloidosis or in dubious cases which require a broader differential diagnosis for organ diseases, something still not infrequent, especially in spoke Hospitals;
2. determining organ and tissue involvement in order to define the systemic or localized nature of the disease;
3. describing type of distribution and anatomical structures involved in single organs;
4. defining organ disease burden;
5. indicating the most pertinent sample for optimal amyloid fibril typing.

According to the 2020 recommendations of the International Society of Amyloidosis nomenclature committee, which state that "in medical practice amyloid is recognized

TABLE 1 Some characteristics of main types of amyloidosis.

Type	Underlying pathologic conditions
AL	Monoclonal protein-secreting disorders (usually clonal plasma cell).
AA	• Associated to long-standing inflammatory process: chronic infections, rheumatological/autoimmune/inflammatory disorders, hereditary auto-inflammatory disease. • Benign tumors. • Various hematological and solid cancers. • Unknown etiology.
ATTRv, AGel AApoAI, AApoAII, AApoCII, AApoCIII, AFib, ALys	Hereditary amyloidoses due to mutations of various gene proteins.
ATTRwt	Aging-related amyloidosis
Aβ2M derived from β2 microglobulin, associated to long-term dialysis. Amyloid deposits derived from insulin and injection of enfuvirtide.	Iatrogenic amyloidosis
Organ-specific amyloidosis	Localized amyloid deposits derived from hormones or local protein precursors of endocrine organs or tumors (e.g., thyroid medullary carcinoma; isolated atrial amyloidosis).
Main pathogenetic mechanisms	
• Excess protein production favoring abnormal folding of proteins and their aggregation into insoluble aggregates. • Mutated protein with a higher tendency to misfold. • Intrinsic propensity of normal wild-type protein to misfold and form amyloid fibrils. • Proteolytic remodeling of a wild-type protein into an amyloidogenic fragment.	

microscopically by its amorphous structure, affinity for the dye Congo red and its increased birefringence under polarized light after such staining," the cornerstone for diagnosis still rests on identification in tissues of amyloid deposits with their typical microscopic structure and histochemical properties (1).

The following sections describe the standardized step-by step approach that the pathologist should follow to reach a diagnosis of amyloidosis and to provide as much information as possible when examining tissues in this context. For a correct approach the pathologist should have a thorough knowledge of the technical aspects and staining protocols, should be aware of the characteristics of specific tissues when analyzing amyloid deposits and should be properly trained in amyloid morphological findings and in the other alterations and diseases to be considered for a differential diagnosis.

Main technical aspects to bear in mind when examining amyloid deposits in tissues

The usual formalin-fixed and paraffin-embedded (FFPE) specimens can be used for complete examination and characterization of tissue amyloid deposits. These samples are suitable not only for optimal histological, histochemical, and immunohistochemical investigation, but are also for molecular analysis, as Mayo Clinic proteomic researchers showed (17). Frozen tissue is needed only in those Centers which traditionally use immunofluorescence for amyloid typing.

Tissue fragments fixed in glutaraldehyde solutions are required for ultrastructural examination, although for centers which make use of immunoelectron microscopy for amyloid protein typing this fixative is not always adequate.

Targeted organ biopsies (heart, kidney, liver, bone marrow) are prepared and sectioned according to respective guide-line or consensus document protocols, which already cover serial or multiple sections and unstained slides for further investigation (18–20). Screening biopsies (labial salivary glands, gastrointestinal tract, subcutaneous abdominal fat) are managed according to routine techniques. In all cases close attention is necessary to preserve material for proteomic analysis.

With regard to abdominal fat tissue, the authors suggest skin punch biopsy or surgical subcutaneous fat biopsy rather than fine needle aspiration biopsy or needle biopsy with wider diameter, in order to obtain more material. Moreover it is advisable that fat tissue arrives fresh at the Laboratory, although some centers prefer it to be immediately immersed in the fixative.

In some centers thicker sections (5–10 μm) for CR are used in order to increase sensitivity in detection of amyloid deposits, but it should be said that automated stainers eliminate the problem (14).

Standard histopathologic examination

The initial step in tissue amyloidosis diagnostics is morphologic identification or a suspicion of amyloid deposits in routine Hematoxylin-Eosin slides.

All amyloid deposits consist of fibrillary proteins with similar structure, which at histology appear as extracellular eosinophilic acellular, amorphous, and homogeneous material: this morphology is identical in each organ and in any form of amyloidosis and should principally be differentiated from collagen deposition, especially in the form of hyalin fibrosis, and from elastin (21) (**Figure 1**).

The subsequent step is using CR stain, which displays a classic birefringence of amyloid fibrils when placed between two polarizers, thus confirming deposition. CR is the universal staining performed in pathology for amyloid assessment and the current gold standard for generic diagnosis of amyloidosis (**Figure 2**). When CR is negative in presence of a well-founded morphologic suspicion, the stain should be repeated in two or more sections in order to exclude technical problems. Including CR stain in protocols of major organ biopsies may be recommendable to reveal early, not yet morphologically evident, amyloid deposits or to identify limited deposits in clinically unsuspected cases, especially in organs such as kidneys where the clinical symptoms may not be very clear. It should, however, be stressed that a diagnosis of amyloidosis based only on CR birefringence in absence of histologic evidence should be proposed with caution (see below).

Congo red interpretation requires experience because dye results can vary considerably: high-quality microscopic optics, adequate observation conditions (strong light source, room darkness, quality of polarizers), and standardized staining protocols, both manual and automated, are required. Scant versus extensive deposits, pathologist's experience, interobserver variability, tissue source (fat pad biopsy or aspirate vs. organ biopsy) are other factors which can substantially affect the results (22–25). The most frequent definitions of amyloid fibril birefringence are "typical green or apple-green birefringence" (**Figure 2**), but several papers express reservations about this terminology. In clinical practice, especially when the above-mentioned requisites are not observed, a mixture of colors, more commonly green, yellow, orange-red, blue-green, and whitish may be seen at microscope (7, 14, 26).

To cope with this diagnostic difficulty, which may not be confined to CR, the best approach is to carefully compare morphological findings, i.e., the deposits identified at histology, and birefringence tissue sites.

Types and characteristics of amyloid fibrils (such as full-length, thickness, truncation) can also influence intensity of birefringence, thus making it more difficult to diagnose some types of amyloidosis (for example, transthyretin amyloidosis) (27–29).

Other stains such as metachromatic dyes, Alcian blue (which binds to the ever-present glycosaminoglycans in fibrils) and Thioflavin T (or S), are not generally used in referral pathology laboratories, or may be used as additional staining.

Azan Mallory trichrome stain shows amyloid as bluish-gray and helps to identify the deposits, to distinguish them from collagen and to evaluate the disease extent in bright field microscope (**Figures 3**, **4**).

In our pathology center a consolidated quality control (QC) system ensures that CR and other staining protocols fall within quality specifications. Expert pathologists and technicians work together to regularly check the QC trends.

Further information provided by histology evaluation

General points

Although amyloidosis may be found in a localized form, it is most frequently a systemic disease, which involves numerous organs and tissues, more commonly heart, kidneys, nervous system, liver and gastrointestinal tract and, less commonly, lung, muscle, and soft tissue. Amyloidosis diagnosis may involve a general pathologist, but the disease is basically organ-specific with organ-related histopathological patterns, and ideally requires specialized pathologists (cardiopathologist, hematopathologists, nefropathologist, neuropathologist).

In addition to a definite diagnosis of organ involvement, histology evaluation of an organ biopsy can provide a wide spectrum of findings relating to disease etiology and comorbidities, as well as the pathobiology of deposition and acute and chronic organ damage.

Focus on the heart

In the most common forms of amyloidosis in Western Countries, the heart is frequently involved: immunoglobulin light chain amyloidosis (AL) due to clonal plasma cell dyscrasia, and transthyretin amyloidosis (ATTR), due to anomalies of transthyretin (TTR), which includes the most frequent acquired wild-type form (ATTRwt), where protein misfolding is age-related, and the hereditary variant form (ATTRv) where transthyretin is mutated. These forms account for 98% of cases with significant cardiac disease, which is known to be the main determinant of adverse clinical outcomes (29–32). Cardiac involvement is also clinically critical in Apolipoprotein AI amyloidosis (AApoA1), while it is very rare in patients with reactive systemic amyloidosis (AA) where fibrils are composed of serum amyloid A protein (SAA) (33).

In our Center we performed various pathological studies of whole hearts, which showed the clinical value of assessing morphological variability of amyloid infiltration in terms of anatomical structures involved and different distribution patterns in cardiac walls or along the base-apex axis (34–36).

Histomorphology clearly shows that cardiac amyloidosis is both a myocardial and a vascular/microvascular disease, each with different deposition patterns.

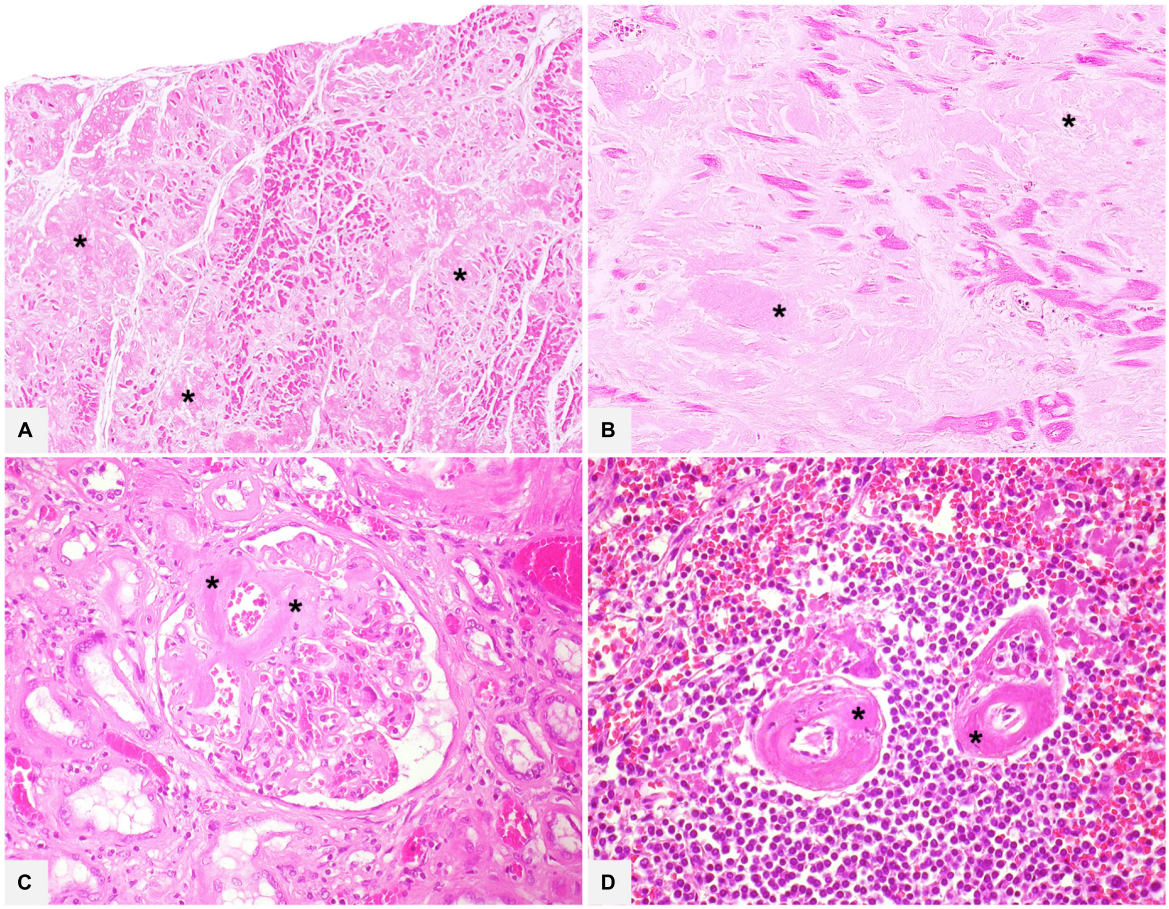

FIGURE 1
Various organ specimens (**A:** heart left atrium; **B:** heart left ventricle; **C:** kidney; **D:** spleen) in cases of systemic amyloidosis showing extracellular eosinophilic, amorphous, and homogeneous amyloid deposits (asterisks). Hematoxylin-Eosin: **(A)** 50×; **(B)** 200×; **(C,D)** 400×.

There are two main myocardial interstitial patterns: pericellular and nodular/replacement. In the former, amyloid deposits are distributed around individual cardiomyocytes, vary in thickness and can involve areas of varying extent, thus producing a lace-like aspect; in the latter, nodular or micronodular amyloid aggregates, whether large or small, can distort the myocardial architecture or replace the myocardium. The two patterns are frequently mixed and deposit extent can be graded as mild-focal, moderate-multifocal and severe-diffuse (37) (**Figures 4A–F**). Deposit extent can also be morphometrically evaluated.

Amyloid deposits can be seen in vessels of various size, both arteries and veins, at epicardial and intramyocardial sites, and more extensively in mural vessels (37). Deposits may entirely or partially involve vessel circumference, be localized only in the intima or the medial layer or in the whole wall and cause various degrees of stenosis to the point of obstruction. Capillary networks too may be affected and show reduced density (**Figures 4G–K**).

Amyloid deposition also occurs in the subendocardium, usually as nodular aggregates associated or not with fibrosis, and in the epicardial tissue (34) (**Figure 4L**).

In amyloid cardiomyopathy histological examination can also give important information on myocardial injury. Most frequently chronic damage in a remodeling myocardium is found, characterized by various morphological alterations, such as attenuation/atrophy, vacuolization, or reactive hypertrophy of cardiomyocytes. In cases with significant microvascular involvement, it is occasionally possible to find myocyte ischemic-like damage at histology (**Figure 5**).

Although incidence of myocardial inflammation in cardiac amyloidosis is unknown, in our referral center, which handles many endomyocardial biopsies (EMBs) and numerous whole transplanted hearts of patients with amyloidosis, we had the opportunity to study myocardial inflammatory infiltrates associated to amyloid deposits, varying from simply lymphocytic or macrophagic-lymphocytic to giant cell granulomatous inflammation (**Figure 5**). Data are still scanty, but literature documented that amyloid may play a role in

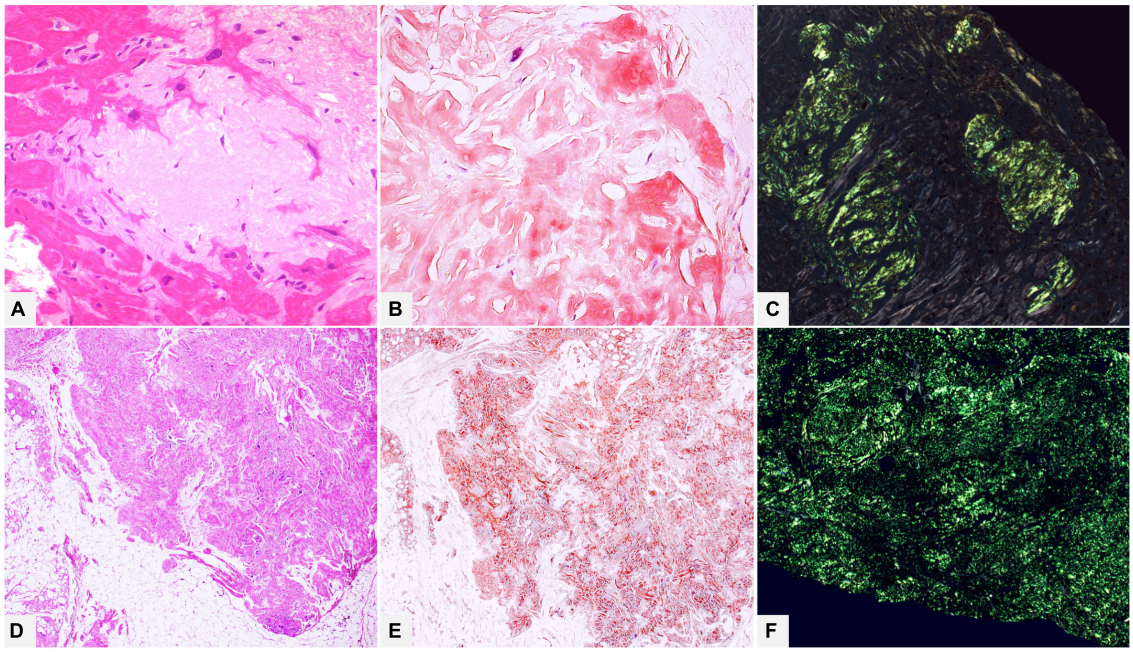

FIGURE 2
(A–C) Endomyocardial biopsy of a 52-year-old male patient with monoclonal gammopathy of undetermined significance: histology shows interstitial myocardial nodular amyloid deposits. **(D–F)** Patient of 15 years suffering from type I diabetes with insulin replacement therapy, who underwent surgical removal of abdominal fibro-lipomatous mass with diffuse interstitial nodular amyloid deposits. With Congo red, amyloid appears orange-red when viewed under transmitted-light microscope **(B,E)** and shows brilliant green birefringence under polarized light **(C,F)**. **(A)** Hematoxylin-Eosin 400×. **(B)** Congo red 400×. **(C)** Congo red under polarized light 200×. **(D)** Hematoxylin-Eosin 100×. **(E)** Congo red 100×. **(F)** Congo red under polarized light 200×.

immune/autoimmune response (38). The issue of amyloidosis and inflammation is yet based on isolated and preliminary observations and research is needed to expand knowledge on pathogenetic mechanisms, possible role of inflammatory infiltrates on deposits, interactions with specific amyloidosis type and impact on disease progression and survival or on future therapeutic implication (39–41).

Finally, the presence of subendocardial and myocardial fibrosis should also be assessed to obtain information on overall morphologic alterations in the heart.

In conclusion histologic changes can provide substantial information on the multifactorial origin of cardiac damage and the complex pathophysiology of amyloidosis, whose varied clinical observations cannot be completely explained by the extracellular deposition of amyloid fibrils within the heart and the mechanical stress of deposits on myocytes. Direct cardiac toxicity of light chain precursor proteins in AL had been called into question, although the underlying mechanisms are not be clearly elucidated as well as a possible histologic expression of this type of damage (42, 43). Correlating systematically detailed morphologic patterns with clinical characteristics could provide further information to elucidate the spectrum of cardiac dysfunction, from altered ventricle relaxation to restrictive disease or to progressive systolic heart failure, and the mechanisms of myocardial ischemia or microvascular dysfunction-induced heart failure (44). It might also throw light on prognostic implications of cardiac disease burden and support the rapidly evolving field of therapeutic and drug efficacy research (45–48).

Focus on the kidney

The kidney is the organ most commonly affected by amyloidosis; the associated renal dysfunction contributes to morbidity and mortality. Almost all the recognized amyloidogenic proteins can involve the kidney (49).

The prevalence of amyloidosis is estimated at 1.6% in native kidneys (50). The most common cause are AL (81%), AA (7%), and Leukocyte chemotactic factor-2 amyloidosis (ALECT2) (3%) (51, 52); by contrast with cardiac involvement, ATTR is an uncommon cause of renal disease (53).

Generally, all kidney biopsies are examined in light microscopy, immunofluorescence and electron microscopy. Under light microscopy, amyloid can involve any compartment of the kidney (glomeruli, tubules, interstitium, vessels) and distribution of deposits could vary with amyloid type (53, 54). Immunofluorescence is the most common method for AL diagnosis, where negativity for immunoglobulins and

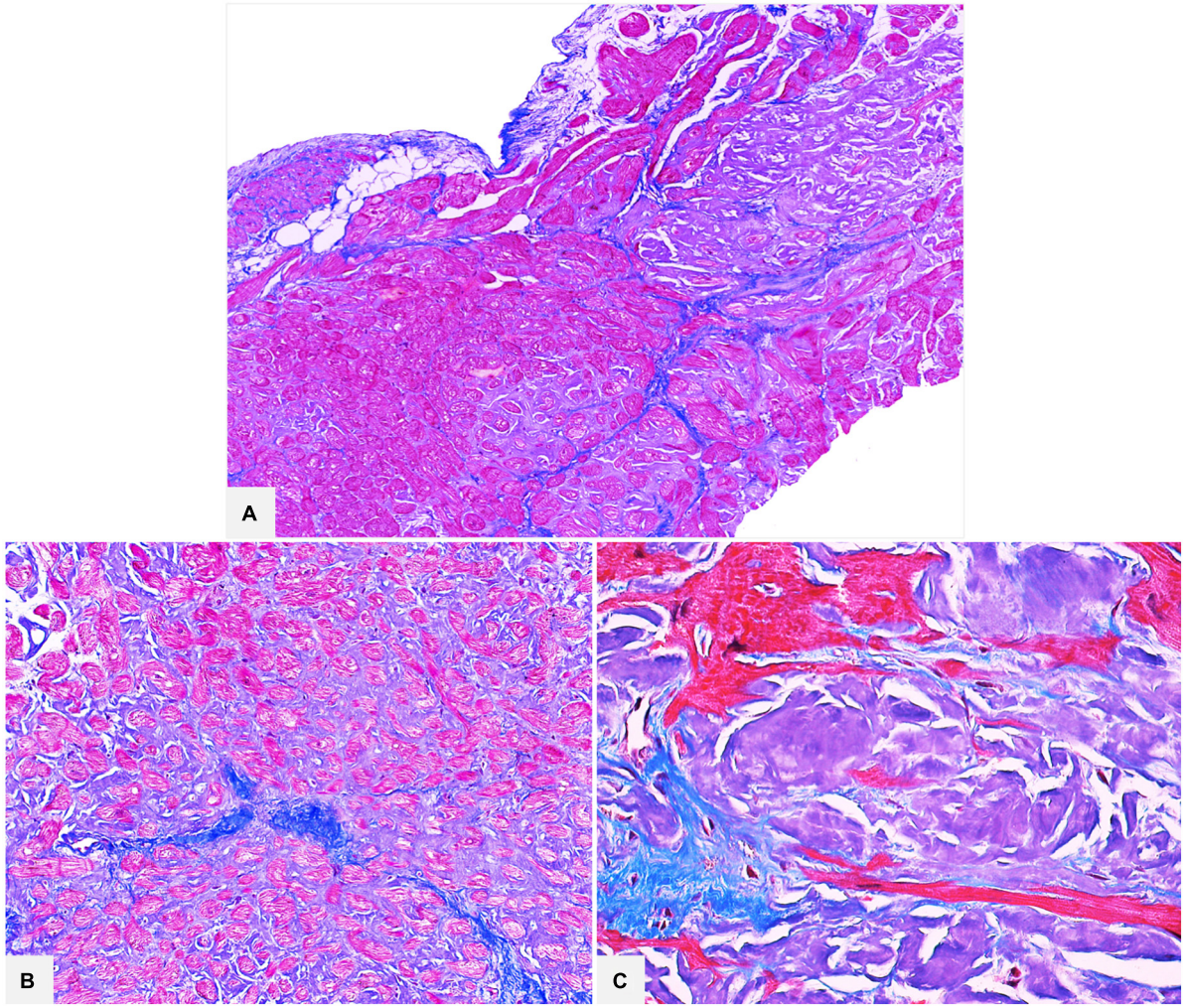

FIGURE 3
Cardiac specimens from native hearts of patients transplanted for ATTR. The myocardial interstitial pericellular and nodular amyloid deposits are stained bluish-gray with Azan Mallory trichrome and are clearly distinguishable from the brilliant blue collagen deposition (**A:** 50×; **B:** 100×; **C:** 400×).

complement and positivity for one of the light chains is generally observed, with a sensitivity of 65–85% (55).

Several histological grading scores for renal amyloidosis have been proposed. One of the most used was suggested by Sen et al. in 2010 (1), based only on the glomerular pattern of injury. In 2017 Rubinstein et al. (56) proposed another score, validated in an AL cohort including glomerular, interstitial and vascular deposits, which was found to be predictive of end stage renal disease. More recently this score was also validated in an AA cohort (57).

Typing amyloid

After histological diagnosis and description of organ-related morphologic findings, amyloid tissue typing, i.e., identification of fibril protein, is required as a guide to therapy, especially today when targeted therapeutic strategies are available for the two main types, AL and ATTR.

Immunohistochemistry and immunofluorescence

The most common methods worldwide to determine fibril type are immunohistochemistry (IHC) on FFPE tissue, and immunofluorescence (IF) on fresh-frozen tissue, although they can be inconclusive or misleading, particularly outside centers of expertise (14, 16, 58, 59).

Immunofluorescence is the diagnostic gold standard in renal biopsies; for other organs IHC on FFPE sections is commonly used (14).

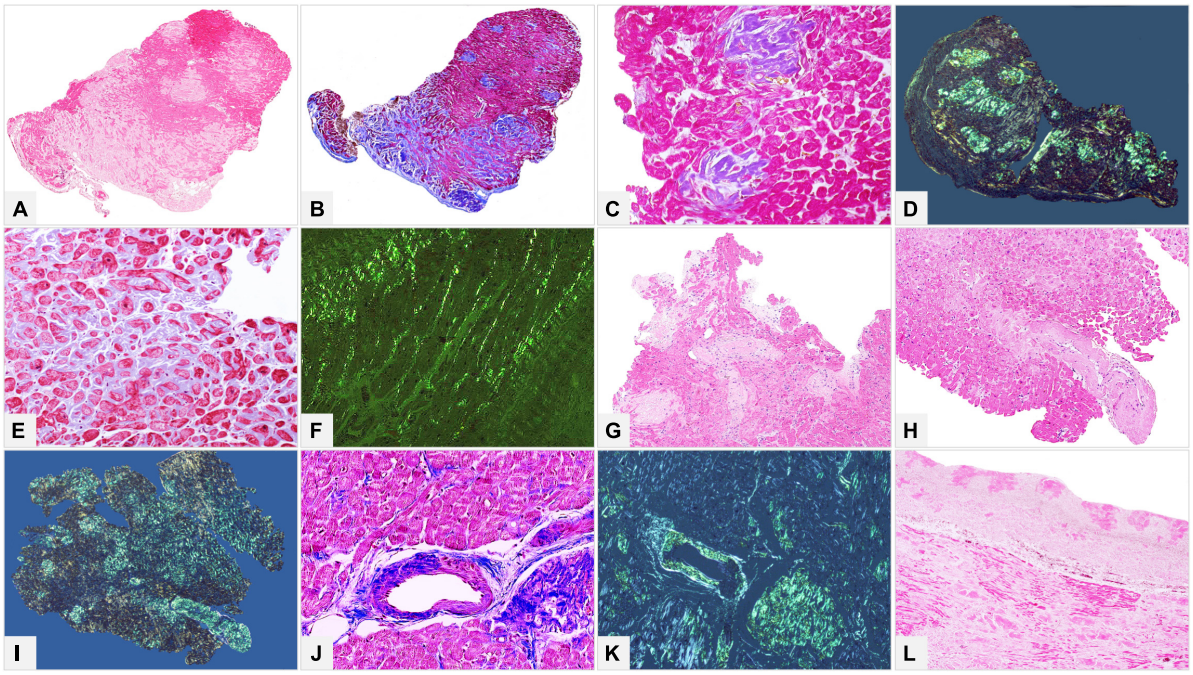

FIGURE 4
(A–K) Endomyocardial biopsies of patients with AL and ATTR. **(L)** Atrial sample of native heart of a patient transplanted for ATTR. **(A,B)** Myocardial interstitial mixed pericellular and nodular patterns diffusely distributed throughout the biopsy fragment. **(C,D)** Myocardial micronodular pattern, where amyloid aggregates replace the myocardium. **(E,F)** Myocardial interstitial pericellular pattern, made up of thin amyloid deposits around cardiomyocytes. **(G–K)** Amyloid deposits within mural arteries involving both intima and medial layers with circumferential **(G–I)** or focal **(J,K)** distribution. **(L)** Nodular amyloid deposits within subendocardial fibrous thickening. **(A)** Hematoxylin-Eosin 50×. **(B)** Azan Mallory trichrome 50×. **(C)** Azan Mallory trichrome 200×. **(D)** Congo red under polarized light 50×. **(E)** Azan Mallory trichrome 200×. **(F)** Congo red under polarized light 200×. **(G,H)** Hematoxylin-Eosin 100×. **(I)** Congo red under polarized light 50×. **(J)** Azan Mallory trichrome 200×. **(K)** Congo red under polarized light 100×. **(L)** Hematoxylin-Eosin 50×.

Optimal results with immunohistochemical stains are largely dependent on (58, 60, 61):

1. quality of antibodies;
2. experience of the pathologist;
3. standardized technical methods in local laboratories.

Commercially available antibodies

A number of antibodies and antibody panels are generally used in pathology diagnostics. Commercial antibodies for the most common fibril proteins (kappa and lambda immunoglobulin light chains, TTR, SAA) are usually employed, especially in non-specialized laboratories.

The main limitation of these antibodies is that they are produced against the native proteins, which have a regular length and conformation. Amyloid fibrillary proteins, however, are anomalous, often fragmented, and may present conformational and post-translational alterations, which can generate altered epitopes, or genetic mutations of amino acid sequence, which cause epitope loss and result in antibody-binding loss. In particular, the specificity of commercial antibodies against immunoglobulin light chains (IgLCs) is low, because they are produced against the constant regions and usually react with entire immunoglobulin; AL fibrils stem from mutations of the hypervariable region and require recognition of various antigenic specificities (62). Other factors such as non-specific signal interference due to tissue contamination from serum proteins, and cross-linking of proteins due to fixation in formalin can contribute to false-negatives or false-positives and to increased background. Lack of staining or, more frequently, multiple reaction of a single amyloid deposit with various antibodies can occur, especially with anti- TTR, anti-lambda and anti-kappa IgLCs, and even anti-AApoAI; these make it impossible to define amyloidosis type or can pose a problem for interpretation (63).

As the literature data on sensitivity and specificity of commercial antibodies vary considerably, it is really difficult to estimate their true accuracy in identifying fibril type in local laboratory routine practice (59, 64–67): special attention is required for IgLC staining low sensitivity and concomitant false-positive staining for TTR (67).

Amyloid-type specific antibodies

For these reasons, since early reports of unreliability of antibodies against proteins of origin (68, 69), some

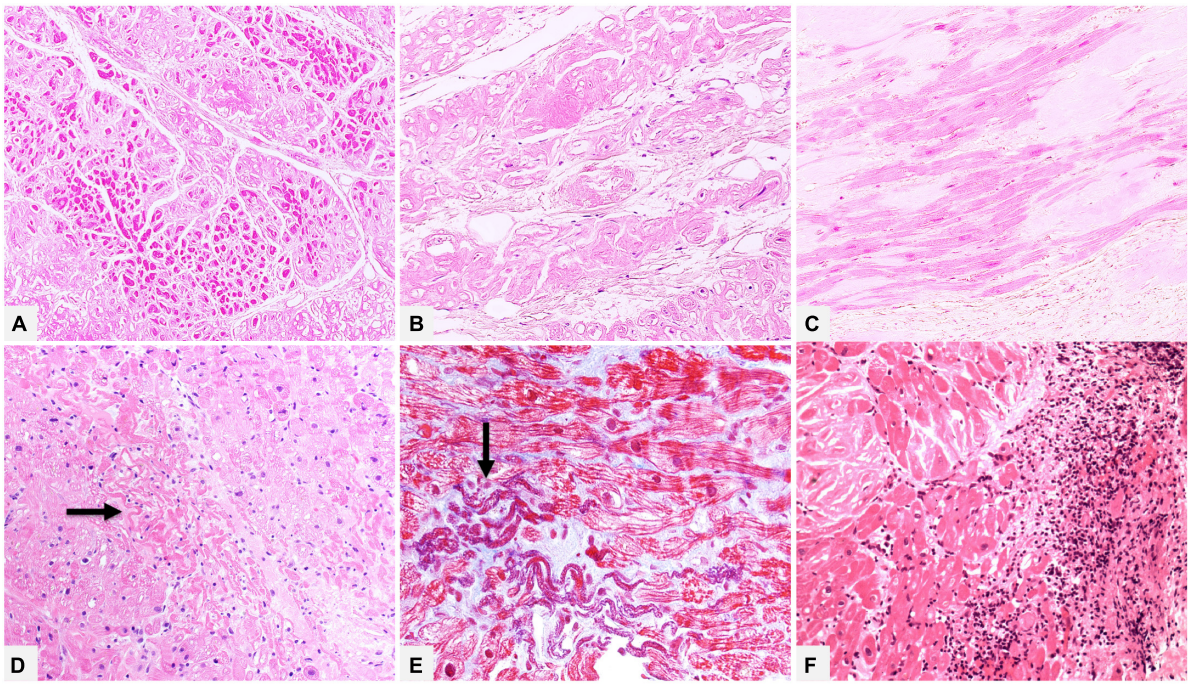

FIGURE 5
(A–C) Native heart of a 61 year-old male patient transplanted for ATTRwt. In the myocardial interstitium we can see amyloid deposits and morphologic chronic remodeling of cardiomyocytes, which show attenuation/atrophy (**A**, Hematoxylin-Eosin 100×), cytoplasmic vacuolization (**B**, Hematoxylin-Eosin 200×) or reactive hypertrophy (**C**, Hematoxylin-Eosin 200×). **(D,E)** Endomyocardial biopsy of a 55 year-old male patient with AL, where deposits prevalently involve vessel wall (not shown). There are foci of recent ischemic-like myocardial damage (thinned and wavy cardiomyocytes, hypereosinophilic and/or coagulated cytoplasm) (arrows). **(D)** Hematoxylin-Eosin 200×. **(E)** Azan Mallory trichrome 400×. Panel **(F)** shows an endomyocardial biopsy of a patient affected by ATTRv with extensive myocardial interstitial inflammatory infiltrates, mainly consisting of lymphocytes, associated with amyloid deposits and myocyte inflammatory damage (Hematoxylin-Eosin, 200×).

laboratories specializing in amyloidosis diagnostics and research have developed their own amyloid type-specific monoclonal and polyclonal antibodies to determine the amyloidogenic protein (70).

The most complete data in the literature is the work of Linke and colleagues who, over a number of years, developed a set of specific antibodies using a large number of tissues with chemically or immunochemically typed amyloids as prototypes, i.e., antibodies directed not against the precursor, but against the purified fibril protein (70). Linke et al. verified antibody performance by serial controls on a large number of prototype amyloids at their own and in other institutes, and also by mass spectrometry, so achieving high diagnostic accuracy on FFPE tissue with 97.9% sensitivity and 99.3% specificity (71). Using a reduced kit (anti-AA, anti-lambda and anti-kappa IgLCs, anti-TTR) for confirmation of a supposed amyloid, correct typing of these most common forms decreases to 90% (61) because IHC can find only the targeted amyloid types.

Similar excellent results with these antibodies, now commercially available in the form of kits for various purposes, are also reported by Lassner and Schonland (59, 71).

The use of specific and standardized antibodies considerably increases sensitivity and specificity of the IHC method, and allows correct fibril typing in a greater number of cases (72), although immunostaining of amyloid deposits by more than one antibody is not fully resolved with these antibodies (63, 73).

Despite these advantages it should be mentioned that it is difficult to use non-validated and non-commercialized specific antibodies in certified laboratories.

Exemplary clinical cases are shown in **Figures 6–8**.

Expertise of the pathologist and the laboratory

As for CR, interpretation of immunostainings requires a pathologist with extensive experience, who should also be familiar with different immunolabeling patterns (69).

Potential pitfalls include not only discerning non-specific background but also amyloid specific staining, which can vary in terms of distribution both over all deposits and within single deposits. Immunolabeling can appear uniform or non-homogeneous, equally distributed or spotty and widespread or limited; reactivity intensity may be strong or weak.

A caution approach is needed for inconsistent and variable immunolabelling patterns; strong, uniform and coherent immunostaining can usually be considered diagnostic, as long as the results are correlated with clinical data (69).

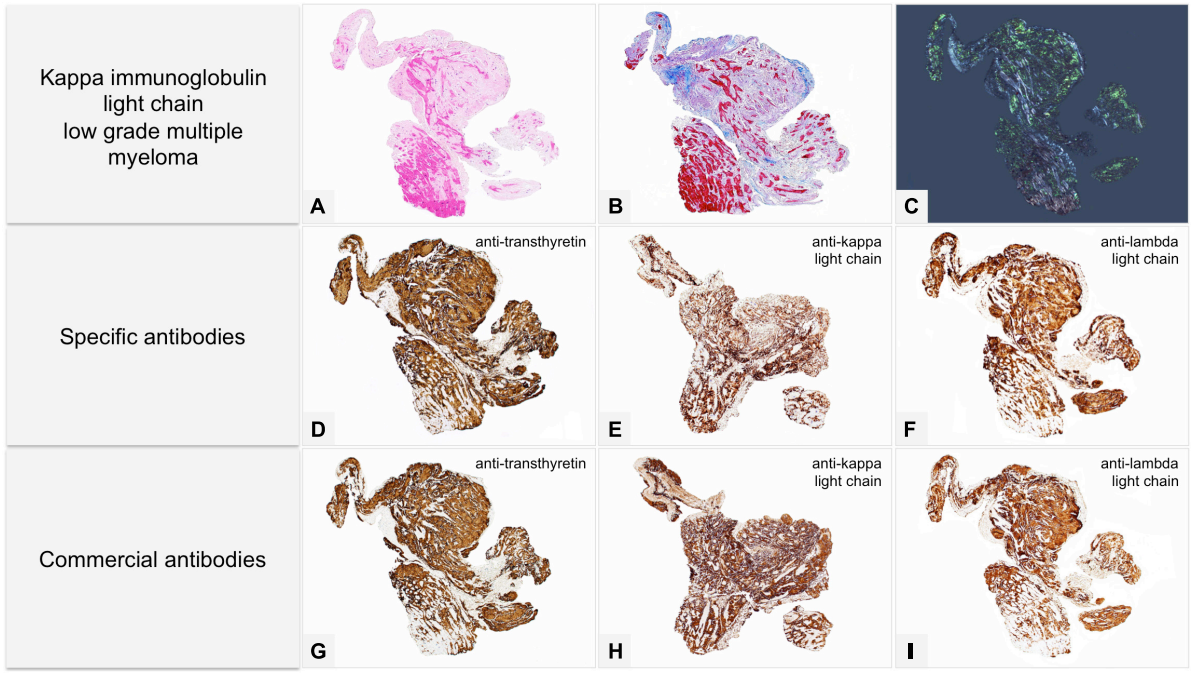

FIGURE 6
Male of 81 years affected by kappa light chain low grade multiple myeloma, with score 2 cardiac uptake on scintigraphy with bone tracers. Endomyocardial biopsy was performed for suspected cardiac amyloidosis and showed slight to moderate myocardial interstitial and subendocardial amyloid deposits. Proteomic analysis with mass spectrometry in tandem mode was positive for TTR proteotypic peptides. IHC failed to type amyloid with both commercial and specific antibodies. Immunolabeling was strong and diffuse with all antibodies with no significant differences in intensity and distribution. **(A–C)** Histology of a biopsy fragment showing subendocardial and myocardial eosinophilic amorphous deposits with Hematoxylin-Eosin (**A:** 50×), bluish-gray deposits with Azan Mallory trichrome (**B:** 50×) and green birefringent deposits with Congo red under polarized light (**C:** 50×). Specific antibodies (amY-kit reduced PeloBiotech). **(D)** Anti-ATTR-TIE (50×). **(E)** Anti-kappa-KRA/KUN (50×). **(F)** Anti-lambda-UTI/LAT (50×). Commercial antibodies against native proteins. **(G)** Anti-ATTR AbCam, clone EP2929Y (50×). **(H)** Anti-kappa Roche-Ventana (50×). **(I)** Anti-lambda Roche-Ventana (50×).

Pathology laboratory technicians should be able to perform all technical steps to guarantee the best performance of antibodies and methodology should be standardized and reproducible. The aims of the IHC standardization process are to optimize specific versus background staining and to select the intensity of immunolabeling using positive and negative controls.

Today's automated platforms are an additional guarantee for adequate standardization as compared to manual platforms (60).

Proteomics

Mass spectrometry (MS)-based proteomic analysis of amyloid deposits has been shown to identify fibril subtype with a high degree of accuracy, and is therefore considered the gold standard technique in amyloidosis typing (74). Vrana et al. first developed the procedure for FFPE organ biopsy specimens (75), and then for unfixed fat aspirate specimens (76).

The procedure uses the shotgun proteomics approach to analyze specific areas of CR positive tissue viewed under polarized light. The areas are first resected by laser microdissection (LMD), then collected in a microvial, solubilized and further processed to obtain a peptide solution mixture, which is in turn analyzed by nano-flow liquid chromatography (nf-LC) coupled to high-resolution and high-accuracy mass spectrometry in tandem mode (hr-MS/MS). The collected spectra are checked through database matching software, such as Sequest (77), Tandem (78), or Mascot (79), in order to identify proteins.

The software usually uses tryptic peptide databases containing sequences for the Swiss-Prot human canonical proteome, that is sufficient to identify virtually all amyloid proteins in specimens. But in cases of hereditary amyloidosis, where mutated proteins are present, special databases have been developed to identify mutated peptide sequences, although these are only available in a few specialized centers worldwide (74, 76).

The shotgun proteomics approach usually detects amyloidogenic fibril proteins in samples, together with many additional proteins (17). As chaperone proteins involved in the amyloidogenesis process have commonly been found (80), independently of the specific fibril forming protein which

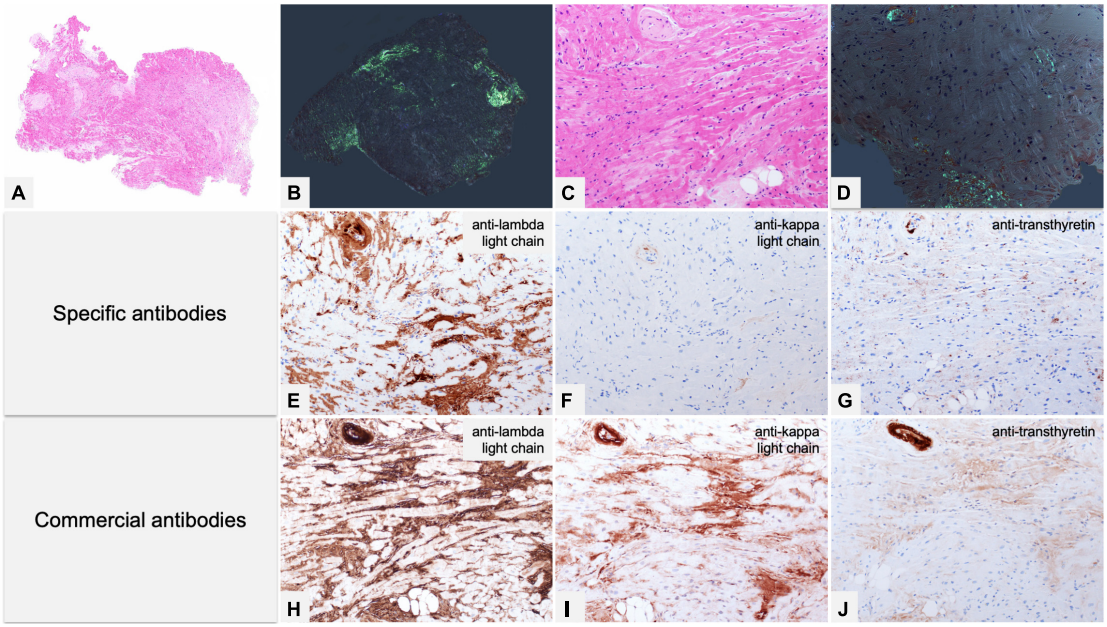

FIGURE 7
Endomyocardial biopsy of a 55-year-old male patient performed for clinically unexplained cardiopathy/cardiomyopathy. **(A–D)** Histology shows amyloid deposits within vessels and myocardial interstitium, clearly evident with Congo red birefringence **(B,D)**. **(E–G)** Immunohistochemistry with specific antibodies was suggestive of AL, as it showed strong immunostaining for lambda light chain in vessel and myocardial deposits, and substantial negativity for kappa light chain and TTR. **(H–J)** Immunohistochemistry with commercial antibodies was inconclusive: all three antibodies showed strong immunostaining of the artery, although myocardial interstitial amyloid deposits were particularly strong only with anti-lambda light chain, partially due to background. Fibril protein typing with mass spectrometry in tandem mode was positive for lambda light chain proteotypic peptides. Subsequent hematological study led to diagnosis of lambda light chain monoclonal gammopathy of undetermined significance and related AL principally localized in the heart. **(A)** Hematoxylin-Eosin 50×. **(B)** Congo red under polarized light, 50×. **(C)** Hematoxylin-Eosin 200×. **(D)** Congo red under polarized light, 200×. Specific antibodies (amY-kit reduced PeloBiotech): **(E)** Anti-lambda-UTI/LAT (200×). **(F)** Anti-kappa-KRA/KUN (200×). **(G)** Anti-ATTR-TIE (200×). Commercial antibodies against native proteins. **(H)** Anti-lambda Roche-Ventana (200×). **(I)** Anti-kappa Roche-Ventana (200×). **(J)** Anti-ATTR AbCam clone EP2929Y (200×).

changes in various amyloidosis types (17), their presence has been proposed as an amyloid molecular signature.

In the diagnostic evaluation of bioptic specimens, the general strategy would be: when the signature is detected, an amyloid type is identified by correlating patient clinical factors with the most abundant amyloidogenic protein consistently found in a series of repeated proteomics analyses.

However, when a CR-positive sample contains the biochemical signature of amyloidosis but not a known amyloid type, the proteome can be further scrutinized for potential novel amyloid fibril forming proteins. Using this approach, various previously unknown fibril proteins, such as leukocyte chemotactic factor-2, apolipoprotein A4, apolipoprotein C2, liraglutide, and epidermal growth factor containing fibulin-like extracellular matrix protein, have been identified as novel amyloid types with very different clinical presentations and outcomes (17).

After scrutinizing more than 16,000 cases, Vrana et al. (76) were able to indicate a universal amyloid signature composed of Apolipoprotein E (ApoE), Serum amyloid P component (SAP), and Apolipoprotein A-IV (ApoA-IV), since these were invariably present in their pathological deposits. They went on to assert that, when found together, these three proteins constitute a biochemical signature and support the general diagnosis of amyloidosis, even independently of CR staining results.

Recently, however, other authors have proposed slightly different amyloid signatures: Misra et al. (81) indicated ApoE, SAP, and glycosaminoglycans; Benson et al. (1) proposed ApoE, SAP and heparan sulfate proteoglycan; Schumann et al. (82), using a new proteomics approach based on MALDI imaging, have found an even more elaborate signature composed of ApoE, SAP, Apolipoprotein A-1, Vitronectin, and SAA.

Although the shotgun proteomics approach is rightly considered the "gold standard" method in the diagnosis of amyloidosis type, in practice technical complexities, sample recovery issues, processing, microdissection, data analysis, and the availability of expensive instrumentation and plus multidisciplinary professional team restrict this approach to only a few reference centers throughout the world (54, 83).

Hence, in an attempt to implement MS-based amyloid typing in our center, we developed a targeted proteomic approach based on standard liquid chromatography and mass spectrometry (LC-MS)/MS instrumentation by limiting

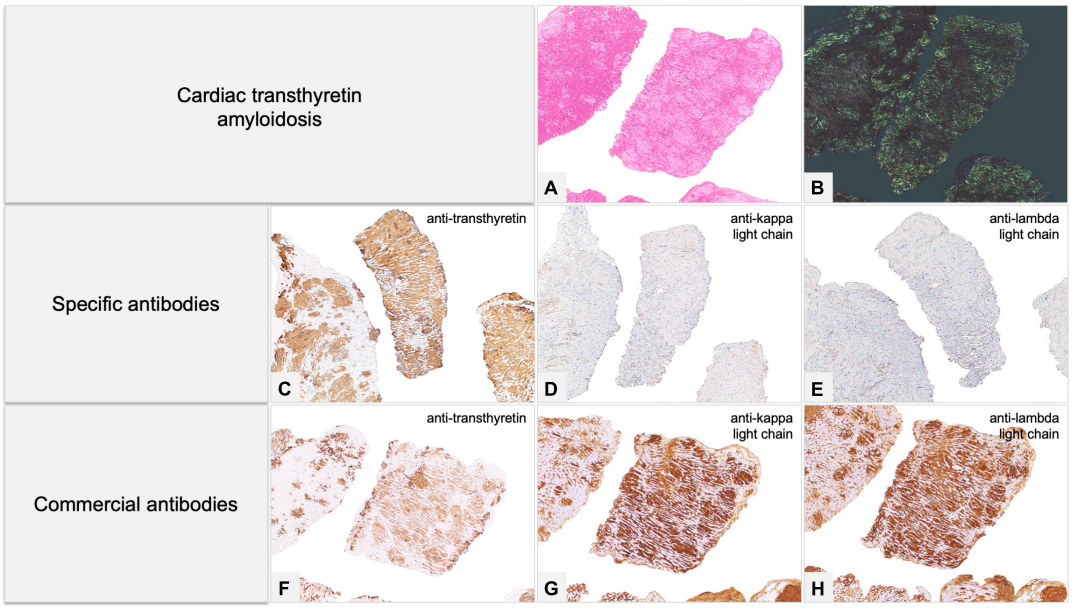

FIGURE 8
Endomyocardial biopsy of a 68-year-old male patient, performed for suspected cardiac amyloidosis in absence of hematological disease. **(A,B)** Histology shows extensive amyloid deposits involving myocardial interstitium, with a prevalent nodular pattern, and the vessels. **(C–E)** Immunohistochemistry with specific antibodies clearly favored an ATTR form, as it showed diffuse homogeneous immunostaining for TTR **(C)** and negativity for anti-kappa **(D)** and anti-lambda **(E)** immunoglobulin light chains. The result was confirmed by proteomics with mass spectrometry in tandem mode, which was positive for TTR proteotypic peptides. **(F–H)** Immunohistochemistry with commercial antibodies, however, showed weak immunostaining for TTR **(F)** and strong, diffuse positivity using anti-kappa **(G)** and anti-lambda **(H)** light chains. Genotyping allowed diagnosis of ATTRwt. **(A)** Hematoxylin-Eosin 50×. **(B)** Congo red under polarized light, 50×. Specific antibodies (amY-kit reduced PeloBiotech): **(C)** Anti-ATTR-TIE (50×). **(D)** Anti-kappa-KRA/KUN (50×). **(E)** Anti-lambda-UTI/LAT (50×). Commercial antibodies against native proteins. **(F)** Anti-ATTR AbCam clone EP2929Y (50×). **(G)** Anti-kappa Roche-Ventana (50×). **(H)** Anti-lambda Roche-Ventana (50×).

the number of proteins sought in both FFPE biopsies and subcutaneous fat aspirated (SFA) samples (84). In addition to proteotypic transitions of fibril-forming proteins, we included Cardiac Actin for EMBs and Fatty Acid Binding Protein-4 for SFA specimens. Identifying these last tissue specific proteins served not only as a tissue marker, but also to define relative cut-off values of fibril proteins in positive samples and to rule out false positivity due to monomeric forms circulating physiologically in the human body and accidentally included in bioptic specimens (84). We have recently started to include the amyloid signature in our method involving the presence of three chaperone proteins (ApoE, SAP, ApoA-IV): as these are, however, physiologically present in the patients' bloodstream, their presence is not necessarily an unequivocal marker of amyloidosis, but could merely indicate blood inclusions in bioptic specimens.

Our method can be performed on standard 2–5 um thick sections of FFPE tissues (or small chunks of SFA specimens) positively stained with CR. Without using LMD the sections are transferred whole from the glass slits to an eppendorf. The tissue is then solubilized, proteins are extracted, denatured and digested by trypsin, using a commercial kit of reagents for shotgun proteomics (Easypep mini, Thermo-fisher, Waltham, MA, USA). The resulting peptide mixture is analyzed by a standard LC approach, on 2 × 150 mm, 1.8 um particle size peptide specific columns, coupled with triple quadruple tandem mass detection in multiple-reaction-monitoring (MRM) mode. Peptide specific MRM transitions for proteotypic peptides are obtained *via* an open access database: the SRMAtlas.[1] We developed the method with two different systems, with similar successful results: Nexera-2 UPLC (Shimadzu, Kyoto, Japan) coupled with an API5500 mass spectrometer (Sciex, Toronto, Canada); and 1295C UPLC (Agilent, Santa Clara, CA, USA) coupled with a 6495C mass spectrometer (Agilent, Santa Clara, CA, USA).

In our targeted approach, the presence of chromatographic peaks for specific mass transitions in ion extraction chromatograms is used to verify the presence of proteotypic peptides. At least three positive proteotypic peptides is indicative of the presence of a target protein in a sample.

However, since the SRMAtlas database contains only sequences for SwissProt canonical human proteome sequences and does not contemplate amino acid substitutions, less conserved canonical proteotypic peptide signals can be used to presume identification in expected hereditary forms, despite reduced identification confidence.

1 www.srmatlas.org

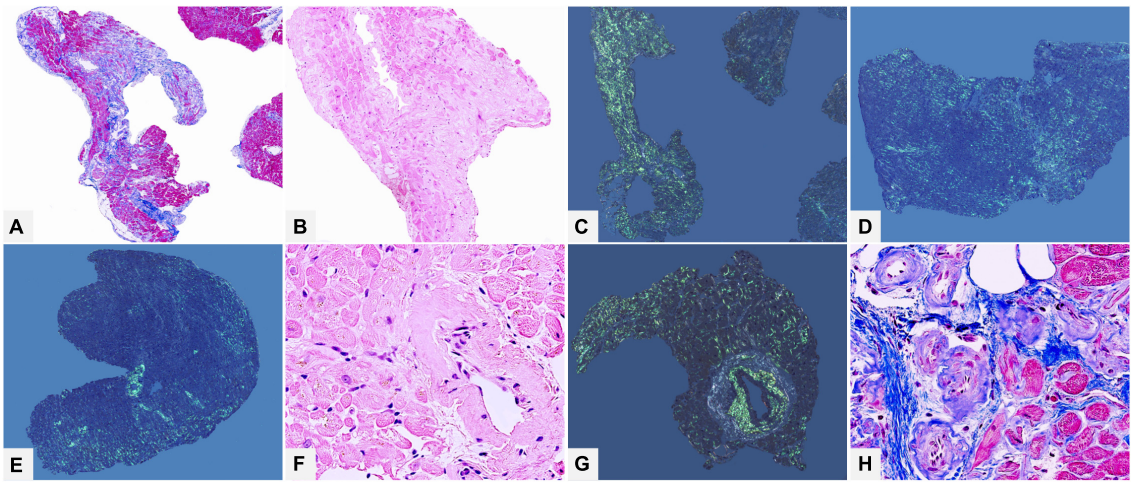

FIGURE 9
Male of 62 years affected by clinically not well defined cardiopathy with hypertrophic phenotype. Thorax computed tomography showed mediastinal lympho-adenomegaly and a pulmonary picture suggestive of grade 2 sarcoidosis. Endomyocardial biopsy identified cardiac amyloidosis with diffuse myocardial interstitial **(A–D)** and vascular **(E–H)** deposits. Immunohistochemistry typing (not shown) suggested AL, most probably related to lambda light chain, later confirmed by proteomics. Subsequent hematological tests confirmed the disease and a bone marrow biopsy defined the underlying plasma cell dyscrasia: low grade multiple myeloma. The final diagnosis was systemic lambda light chain amyloidosis with lymph node and cardiac involvement in clinical onset stage I. **(A)** Azan Mallory trichrome 50×. **(B)** Hematoxylin-Eosin 100×. **(C,D)** Congo red under polarized light, 50×. **(E)** Congo red under polarized light, 50×. **(F)** Hematoxylin-Eosin 400×. **(G)** Congo red under polarized light, 100×. **(H)** Azan Mallory trichrome 400×.

In our Center, for the last year, amyloid typing protocol has included a first IHC screening level, using commercial antibodies and two sets of specific ones (one marketed by Pelobiotech GmbH-Germania and the other developed by the University of Uppsala not currently marketed) and, in inconclusive or dubious cases, LC-MS/MS as a second tier test in order to pinpoint any false IHC results (17). At the end of the typing process, data from the two levels are compared and matched with clinical data. Preliminary results of this sequential approach show that using specific rather than commercial antibodies substantially increases correct amyloid typing by as much as 70% of cases, so reducing the need to proceed to proteomics.

In perspective, Mass Spectrometry Imaging (MSI), a technique where an ion or a laser beam is raster scanned over the tissue surface to vaporize it into molecules that are then immediately transferred to the mass spectrometer, has the potential to go beyond shotgun proteomics in amyloid typing (85, 86) thanks to its ability to preserve spatial distribution of proteins in tissues and therefore allow direct observation of the molecular composition of amyloid deposits (87). In addition, the MSI approach can permit direct localization of other types of biomolecule, such as lipids (88) or metabolites in bioptic specimens (89), and could provide new insights into the process of fibrillar protein aggregation, today still largely misunderstood (90).

Nanometric spatial resolution, however, is still impossible with MSI at its present level of development and technical improvements are needed for its successful application in clinical pathology (82).

The question of immunoelectron microscopy

Immunoelectron microscopy (IEM) combines IHC and electron microscopy (EM). This technique is based on extreme microscopic magnification that allows amyloid fibrils to be visualized. With colloidal gold-labeled specific antibodies, it is possible to see whether the antibodies bind specifically or un-specifically to the amyloid fibrils, so overcoming the low specificity of standard IHC. IEM has been successfully established at some amyloidosis centers (91), but its use requires great caution by both clinicians and pathologists. To obtain optimal results, the sample cannot be fixed in 2,5% Glutaraldehyde (as for transmission EM), but in 0.5% Karnovsky's solution (0.5% glutaraldehyde, 2% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.3), as described by Arbustini et al. (92).

Although the availability of IEM is limited, in experienced centers it seems to obtain excellent results. In a single-center study of 423 cases of systemic amyloidosis, IEM identified the amyloid type in over 99% of cases (93). A recent study compared IEM and MS for amyloid subtyping: IEM defined amyloid type in 91.6% of cases and MS 88.8%, over 106 biopsies from different organs; the authors also support the combined use

of both methods to increase the sensitivity of defined amyloid type and mention the important issue of tissue amount in a diagnostic routine, indicating that MS requires a very small amount (0.1 mm^2) and IEM a little more tissue (1 mm^2) (94).

As usual, the perfect method does not exist and few papers in the literature give a comparison between IEM and MS (95).

Potentially both methods can be performed on FFPE material, although it is particularly challenging for IEM outside expert centers which use this technique routinely. Very few laboratories are able to use the paraffin recovery technique, even fewer from a stained slide (96), partly due to the present reduction of clinical indications for EM in other pathologies. Like IHC, IEM uses antibodies, so it can identify only the fibrils present in the antibody panel, while MS can potentially identify all proteins. MS requires as little as two working days laboratory time, whereas IEM needs a minimum of 7. Both techniques are expensive and depend on equipment and staff. It must be emphasized that, as clinicians request biopsies less frequently, it is very difficult to find technicians and pathologists trained in EM, so many hospitals are abandoning its use.

Summary

We believe that the targeted sequential IHC with specific antibodies/LC-MS approach is most probably the gold standard for amyloid typing for many reasons (16, 61, 72):

1. Immunohistochemistry is a simple, quick, inexpensive method, available in most pathology laboratories and can obtain excellent results when using "good" antibodies in expert Centers;

LC/MS is the most sensitive method, able to provide accurate protein information and, using an extended database, to identify mutations and, potentially, novel forms.

In not so rare cases with a real coexistence of a mixed protein population, immunohistochemical data are essential to proteomics, which can have difficulty in recognizing whether proteins stem from amyloid. This may occur in ATTR cases where circulating kappa IgLCs can contaminate TTR fibrils, or in AL cases where kappa or lambda IgLCs can produce a nest effect and attract circulating wild-type TTR, or especially when a monoclonal gammopathy of undetermined significance (MGUS) coexists with ATTR, which can occur in 10–49% of patients (97).

Finally, when a diagnosis of ATTR is reached, genotyping is mandatory.

Histologic evaluation: When and where

Histologic identification of amyloid deposits on tissue specimens is the most sensitive method for definitive amyloidosis diagnosis, and a sequential approach with IHC and proteomics the most sensitive method for amyloid fibril typing and diagnosis of amyloidosis type. Thus in such a clinically complex disease having tissue available is of great importance.

When amyloidosis is suspected, tissue biopsy is always required except for cases of cardiac ATTR, which may be diagnosed by non-invasive methods when the following stringent criteria are met (98): patient with signs and symptoms, electrocardiography, echocardiography, or cardiac magnetic resonance suggestive of cardiac amyloidosis, Perugini score 2 or 3 (99) cardiac uptake on scintigraphy with bone tracers, and absence of monoclonal proteins examined with serum free light chain quantification and serum and urine immunofixation (21, 100). In the absence of these criteria, histological confirmation of diagnosis and/or typing is required (21, 100).

In the last decade the evolution of non-invasive diagnostic methods, specifically cardiac scintigraphy with bone tracers, has gradually changed the diagnostic approach to and clinical management of cardiac ATTR. So, although from the 1990s until the early 2000s EMB was frequently used in referral centers, today it is less common (41, 101).

It should be remembered that a bone scintigraphy scan alone is not enough to distinguish ATTR from AL cardiomyopathy without also testing for IgLCs (102).

In AL histological confirmation is mandatory as in all neoplastic diseases, where complex therapeutic protocols with many side effects are necessary (103).

Biopsy sites

The most sensitive amyloidosis diagnostic method is a biopsy of a clinically involved organ, such as kidney (sensitivity: 99%) or heart (sensitivity: 100%): organ biopsy is the first choice in localized amyloidosis forms or when other diseases must be excluded in clinically unclear cases (**Figure 9**).

Although organ biopsy is probably the most used, but for some authors overused (104), in screening of systemic amyloidosis a surrogate biopsy site may be useful, to avoid the invasiveness of organ biopsy and possible additional bleeding diathesis in patients (98, 104, 105).

The choice of the correct site is crucial because tissue sensitivity in amyloid detection depends on type of suspected amyloidosis, as AL deposits are more likely to be identified than ATTR ones.

The most used alternative sites are: subcutaneous abdominal fat, gastrointestinal tract (usually rectal biopsy) and minor salivary gland biopsy.

Abdominal fat tissue aspirate or biopsy is the most used for screening, especially in AL forms where sensitivity is quite high, ranging from 70 to 90%; fat tissue is inadvisable when ATTR is suspected because sensitivity is only 67% in ATTRv and as low as 14% in ATTRwt (106–110). Apart from the small amount, the main problem of abdominal fat tissue is the presence of

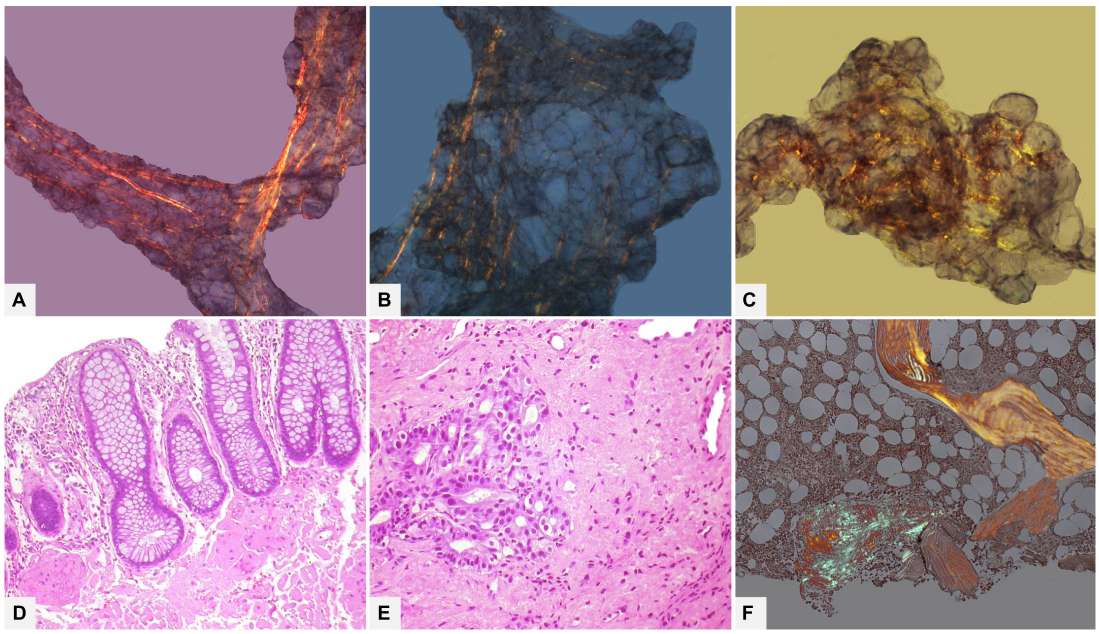

FIGURE 10
Various tissue and organ biopsies of various patients with AL systemic amyloidosis. **(A–C)** (100×): Subcutaneous abdominal fat biopsy showing ambiguous birefringence, yellow and focally green, with Congo red under polarized light. **(D)** Rectal biopsy with mucosal and submucosal amyloid deposits (Hematoxylin-Eosin 200×). **(E)** Gastric biopsy with extensive submucosal amyloid deposits (Hematoxylin-Eosin 200×). **(F)** Bone marrow biopsy with interstitial nodular amyloid deposit with congo red under polarized light (50×).

fibrous strands, where CR birefringence is yellow or yellowish-green, making staining interpretation ambiguous and difficult (**Figure 10**).

For labial salivary gland biopsy high sensitivity (from 81 to 89%) is reported in AL as well as in familial amyloid polyneuropathy (91% of cases) (106, 111–113).

The sensitivity of gastrointestinal biopsies in general ranges from 70 to 90% (**Figure 10**). In particular, with rectal biopsy, sensitivity is high for AL (85%) (**Figure 10**) and ATTRv (81%) and low for ATTRwt (50%) (106, 107, 114).

Bone marrow biopsy deserves separate discussion. In the context of a clinical syndrome compatible with AL and of light chain abnormal findings, the screening work-up includes an iliac crest bone marrow biopsy with the main aim of checking for possible clonal plasma cell disorders. In such cases the biopsy protocol also includes Congo red stain to screen possible amyloid deposits, with a diagnostic yield of 50–60% of AL cases (but remember only of 30–40% of ATTR cases!). If deposits are found (**Figure 10**) the nature of localized or systemic amyloidosis should be determined: the characteristics of clinical syndrome and the pattern of organ involvement are the main guides to deciding whether proceed with a biopsy of extra-bone marrow tissue, keeping in mind that the probability of Congo red positive bone marrow developing systemic amyloidosis is very low (106, 115–117).

Another separate discussion is required for transverse carpal ligament biopsies obtained during carpal tunnel syndrome (CTS) surgery. Although CTS is a recognized red flag for ATTR cardiac amyloidosis, which can precede diagnosis by 5–9 years (118), there are very few studies that use the carpal ligament biopsy for screening purposes. One of these studies found 10.2% of amyloid positive specimens, but just 2% of these were ATTRv and a further 2% had cardiac amyloidosis (119); different results were reported in a Japanese cohort (120). Nowadays the histological diagnosis of carpal ligament biopsies is performed by only a few centers, partly because the presence of recent and older collagen fibrous tissue in these samples makes the analysis challenging (121).

Conclusion

In amyloidosis, pathology study offers much key information in both diagnostics and research. Notably histology has been and continues to be an essential tool for reaching a definite diagnosis, excluding other diseases, classifying systemic and localized forms, describing organ involvement patterns and disease burden.

Screening or organ biopsies have been crucial to increasing knowledge of the disease since the 1990s and, today, are still essential for fibril protein typing and meeting the increasing clinical need for early diagnosis and treatment within a multidisciplinary collaboration scenario.

Author contributions

MR and OL contributed to the conception and design of the study and wrote most of the manuscript. MC and BF wrote sections of the manuscript. SL critically revised the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Changing paradigm in the treatment of amyloidosis: From disease-modifying drugs to anti-fibril therapy

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Cardiac amyloidosis is a rare, debilitating, and usually fatal disease increasingly recognized in clinical practice despite patients presenting with non-specific symptoms of cardiomyopathy. The current standard of care (SoC) focuses on preventing further amyloid formation and deposition, either with anti-plasma cell dyscrasia (anti-PCD) therapies in light-chain (AL) amyloidosis or stabilizers of transthyretin (TTR) in transthyretin amyloidosis (ATTR). The SoC is supplemented by therapies to treat the complications arising from organ dysfunction; for example, heart failure, arrhythmia, and proteinuria. Advancements in treatments have improved patient survival, especially for those whose disease is detected and for whom treatment is initiated at an early stage. However, there still are many unmet medical needs, particularly for patients with severe disease for whom morbidity and mortality remain high. There currently are no approved treatments to reverse amyloid infiltration and deplete the amyloid fibrils already deposited in organs, which can continue to cause progressive dysfunction. Anti-fibril therapies aimed at removing the deposited fibrils are being investigated for safety and efficacy in improving outcomes for patients with severe disease. However, there is no clinical evidence yet that removing deposited amyloid fibrils will improve organ function, thereby improving quality of life or extending life. Nevertheless, anti-fibril therapies are actively being investigated in clinical trials to evaluate their ability to complement and synergize with current SoC.

KEYWORDS

cardiac light chain amyloidosis, cardiac amyloidosis (CA), cardiac amyloidosis—ATTR, standard of care (SoC), treatment gaps, future treatments

Introduction

Amyloidosis is a rare and debilitating disease caused by misfolded proteins that self-aggregate into amyloid fibrils and deposit into various organs (1, 2). Cardiac amyloidosis (CA) results when amyloid fibrils deposit in the interstitial spaces of the myocardium (1, 2). CA is associated with long delays in diagnosis and may be followed by short period between diagnosis and death, especially among patients with advanced disease (3). Progressive deposition of amyloid fibrils in the myocardium results in a loss of cardiac architecture and function, leading to poorer quality of life, increased hospitalizations, and death (1, 2, 4, 5). In light chain (AL) amyloidosis, the circulating amyloid precursor also contributes to cardiac dysfunction through direct toxicity (6). Although the epidemiology of CA is not fully established, it is believed that CA is underrepresented as a cause of heart failure (7, 8).

The 2 main forms of CA are AL and transthyretin (TTR) amyloidosis (ATTR; Figure 1A) (1–4, 6–8). The fibrils in AL amyloidosis consist of misfolded immunoglobulin light chains resulting from clonal B-cell proliferation or plasma cell dyscrasia (PCD) originating in the bone marrow (1–4, 6–8). The fibrils in ATTR amyloidosis consist of misfolded TTR forming due to dissociation of either the wild-type protein (ATTRwt) or facilitated by mutations in the TTR gene (ATTRv) (1–4, 6–8). Both forms of CA may be difficult to diagnose due to non-specific symptoms and overlap with other cardiomyopathies causing delayed initiation of treatment and, consequently, poorer prognosis (9, 10). Survival was significantly better among patients with AL amyloidosis diagnosed in <6 months from symptom onset with >52% of patients surviving over the 5-year period of the study than for those whose diagnosis took longer who also had significantly increased risk of death as shown by >63% of patients dying during the study period (9). Undiagnosed and delayed diagnosis of CA results in high morbidity and high mortality whereas early diagnosis has both clinical and quality of life (QoL) benefits for patients with AL, ATTRwt, or ATTRv CA (10–12). Using a disease simulation model, early diagnosis and timely treatment have been shown to extend the calculated life expectancy from the onset of symptoms by more than 5 and more than 7 years among patients with ATTRwt and ATTRv amyloidosis, respectively (12).

Standard of care and limitations

Treatments for AL, ATTRwt, and ATTRv amyloidosis are very different. Thus, it is critical to identify the amyloidosis type and characterize the fibrils prior to treatment initiation to ensure patients receive the correct treatment (7, 8, 13). Treatment of CA is largely risk-adapted based on the disease burden and stratification of patients. For both AL and ATTR amyloidosis, the standard of care (SoC) focuses on preventing further generation and deposition of amyloid fibrils combined with supportive care (Figure 1B) (1, 7, 8, 13, 14). In addition, both AL and ATTR amyloidosis need a multidisciplinary team of specialists, specific to each patient, to manage the primary disease and the myriad comorbidities that occur (15–17).

AL amyloidosis

The basis of SoC is increased overall survival and improved organ function when amyloidogenic light chain synthesis is suppressed or stopped (18). Therefore, the focus of current, risk-adapted therapy is to prevent more amyloid fibril generation (1, 7, 8, 13, 14). The source of amyloidogenic light chains is a clonal expansion of a plasma cell, similar to multiple myeloma (1, 3, 8, 13, 14). Therefore, most therapeutic agents currently used to treat AL amyloidosis are those with proven efficacy in treating multiple myeloma (19). Validated criteria for hematologic response, classified as complete response (CR), very good partial response (VGPR), and partial response (PR), and organ response have been published previously (20). The main options are autologous stem cell transplant (ASCT) or anti-PCD chemotherapy/immunotherapy to eliminate the underlying PCD (21, 22). However, very few patients are candidates for ASCT as strict eligibility criteria, including age <70 years, low troponin and natriuretic peptides values, preserved cardiac, hepatic and renal function, are essential to reduce transplant-related mortality (22–27). Many patients eligible for ASCT benefit from induction therapy with anti-PCD regimens to prepare them for transplant and to improve outcomes (23, 25, 28). Patients obtaining good hematologic response with induction therapy may not need ASCT (26, 29).

For patients who are ineligible for ASCT, and those who decline the procedure, anti-PCD chemotherapy is the only option (23, 28). Current guidelines recommend a combination of cyclophosphamide, bortezomib, dexamethasone (CyBorD), and daratumumab as first-line therapy for newly diagnosed patients with AL amyloidosis (27, 28). Cyclophosphamide is an alkylating agent that causes damage to DNA strands resulting in apoptosis of the cell (30). Bortezomib is a proteasome inhibitor (31). Proteasomes are a multi-subunit enzyme complexes found in large numbers in the cell and are involved in reducing proteotoxicity and regulating proteins that control cell-cycle progression and apoptosis (32, 33). Inhibition of the catalytic core of proteasomes results in accumulation of ubiquitinated proteins and cellular apoptosis (31). Amyloid-generating plasma cells are particularly sensitive to proteasome inhibition because they rely on the proteasome to reduce the toxic effects of the amyloidogenic light chains and prevent apoptosis (34). Dexamethasone induces cellular apoptosis *via* the nuclear glucocorticoid receptor (35). However, dexamethasone use is associated with an increased risk of a cardiac event and death

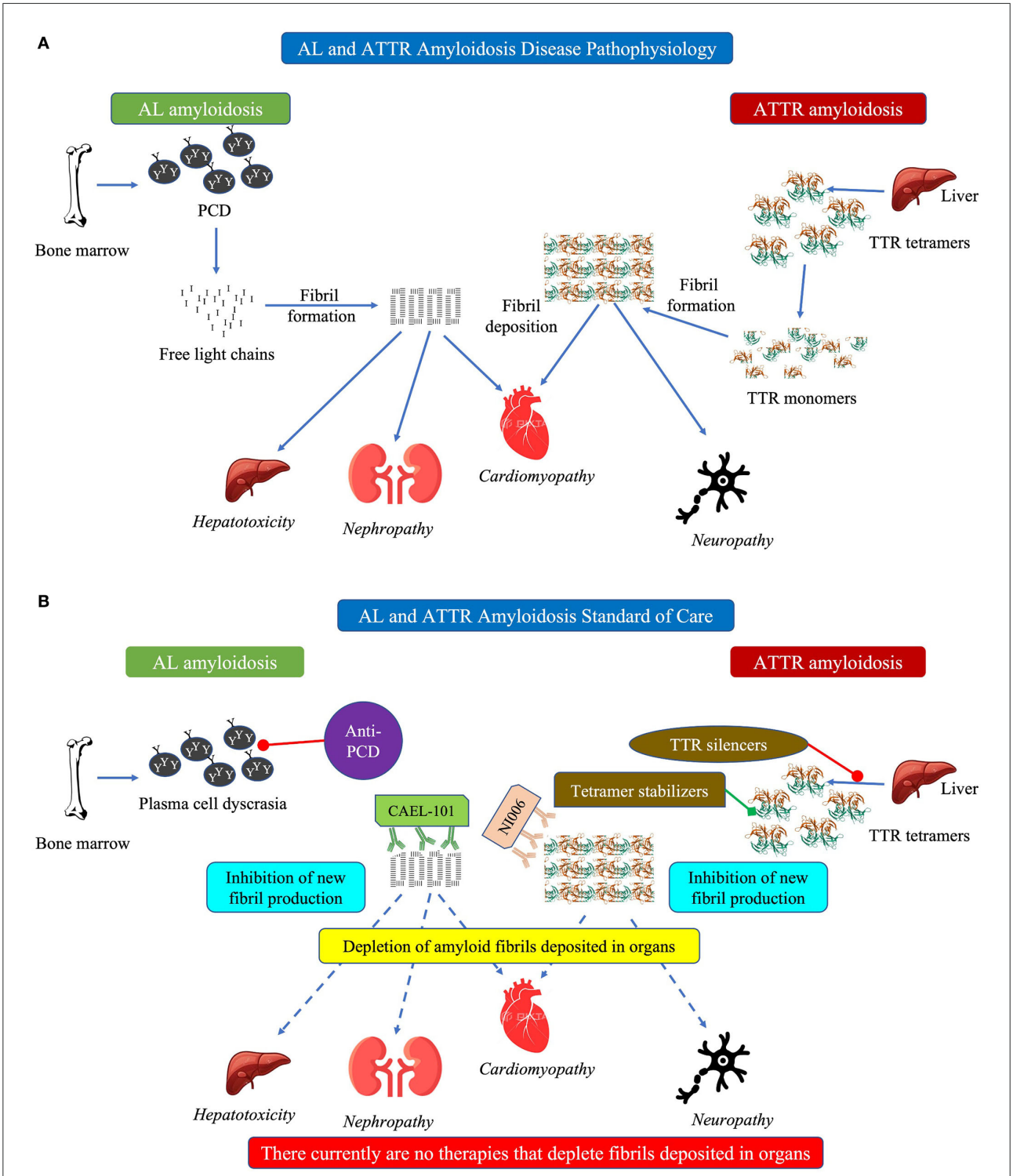

FIGURE 1
(A) Schematic diagram showing pathophysiology of cardiac amyloidosis and **(B)** schematic diagram showing current standard of care in cardiac amyloidosis. In **(A)** this schematic figure shows the pathophysiology of how both AL (left side) and ATTR (right side) amyloidosis can cause cardiomyopathy. In **(B)** this schematic figure shows the current standard of care for AL amyloidosis (anti-PCD) and ATTR amyloidosis (TTR silencers, tetramer stabilizers). Text in italics indicate manifestations of the disease. AL, light chain amyloidosis; ATTR, transthyretin amyloidosis; PCD, plasma cell dyscrasia; TTR, transthyretin.

among patients with severe CA (stage IIIb per the European modification of the Mayo 2004 staging system) (36, 37). There is the potential need for monitoring in an intensive care unit for all high-risk patients, e.g., those in Stage IIIb and IV, receiving chemotherapy (38, 39). Daratumumab is a monoclonal antibody (mAb) that binds to CD38, a transmembrane glycoprotein expressed on the surface of plasma cells, causing apoptosis (40). It is the only agent specifically approved for treating AL amyloidosis when administered with CyBorD. Efficacy of CyBorD-daratumumab is very high, with 78% of patients achieving significant hematologic response, defined CR or VGPR (41–43). In a small group of patients with AL amyloidosis, median survival was 655 days for those treated with CyBorD ($n = 15$) compared with 178 days for those treated with melphalan-dexamethasone ($n = 10$) (44).

However, a survey of patients with AL amyloidosis reported more than 30% reducing treatments and more than 20% discontinuing at least 1 treatment due to adverse events (AE), requiring patients to receive other drug combinations (25, 45). In regions where daratumumab or bortezomib are not available, other combinations of alkylating agents, steroids, and immunomodulatory agents often are used as first-line therapy (46). Patients who are refractory to first-line anti-PCD therapy or who relapse are treated with immunomodulatory agents (e.g., thalidomide, lenalidomide, and pomalidomide), usually in combination with dexamethasone, to overcome resistance to alkylating agents and proteasome inhibitors (23, 47). Regardless of the anti-PCD treatment regimen, all patients require comprehensive supportive care from diagnosis onward to maintain organ function as best possible (48).

Of note, all anti-PCD therapeutic agents are chemotherapeutic agents that cause cell death and consequently can have relevant toxicity. Alkylating agents like cyclophosphamide and melphalan can have severe side effects, including hematopoietic, gastrointestinal, hepatic, gonadal, pulmonary, renal, cardiac, and neural toxicity (30). Treatment with bortezomib can result in peripheral neuropathy (49). The combination of bortezomib and dexamethasone can increase plasma levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), a biomarker for cardiomyopathy, and risk of death especially among patients with advanced disease (36, 37). Immunomodulators are associated with increased cardiomyopathy, thromboembolic complications, myelosuppression, immunosuppression, renal failure, and may aggravate heart failure (18, 23, 25, 47, 50). Despite recent advances, only about 50% of patients achieve complete hematologic response with the currently available therapies. Unless effective rescue treatment is employed, the disease can continue to progress in many patients, particularly those who do not attain at least hematologic VGPR, indicating a huge therapeutic gap. Isatuximab, an anti-CD38 antibody similar to daratumumab, is under investigation to treat the PCD underlying AL amyloidosis (51). However, like all other anti-PCD therapies, it does not address removal of the fibrils already deposited in organs (51).

ATTR amyloidosis

The current SoC for ATTR amyloidosis involves disease-modifying therapy to address the underlying disease, symptomatic therapy to manage cardiovascular and neurologic complications, supportive care, and genetic counseling (52–54). The goal of specific treatment in ATTR amyloidosis is to stabilize the TTR tetramer or stop amyloid fibril production (55).

The liver produces about 95% of TTR measured in the serum (52, 55). Hence, liver transplantation historically has been the first-line therapy to eliminate the main source of amyloidogenic TTR (52, 53, 55, 56). However, progression of CA after liver transplantation limits its utility, especially among patients with advanced disease (53, 55–57). This may be due to the continued presence of small amyloid fibril fragments that stimulate the aggregation of larger, pathogenic fibrils—a process termed "amyloid seeding" (58).

Silencers

TTR expression can also be decreased pharmacologically using agents that "silence" or block the synthesis of the TTR protein (Figure 1B) (59). Antisense oligonucleotides (ASO), such as inotersen, are single-stranded deoxyribonucleotide strands that are complementary to the mRNA target and block protein production of the target, TTR in this case (59, 60). Inotersen, administered subcutaneously, stabilized cardiac symptoms in patients with ATTR cardiomyopathy (ATTR-CM) (61). Whereas treatment with inotersen significantly improves neurological symptoms, in rare cases it can cause severe thrombocytopenia and glomerulonephritis resulting in a boxed warning (53, 56, 61, 62). To prescribe inotersen in the USA, physicians must be trained and certified in Risk Evaluation and Mitigation Strategy (REMS) of the drug and their patients must be enrolled in the REMS program and undergo regular monitoring.

Small interfering RNA (siRNA), such as patisiran, are a class of short double-stranded non-coding RNA molecules that recognize and degrade target mRNA, TTR mRNA in this case (59, 63, 64). Treatment with patisiran, currently only approved for treating ATTR polyneuropathy (ATTR-PN), also might be associated with cardiac amyloid regression in a proportion of patients, as evidenced by reduced extracellular volume and disease stabilization with significant differences in NT-proBNP, left ventricular wall thickness, global longitudinal strain, and cardiac output. In these patients, changes also were associated with improved overall survival and lower cardiovascular-related hospitalizations compared with placebo

(65, 66). In the APOLLO-B study (NCT03997383), compared with placebo patisiran significantly improved the functional capacity, measured with 6-min walk test (6MWT), and quality of life (QoL) of patients with ATTR-CM at 12 months with no additional safety signals (reported at XVIII International Symposium on Amyloidosis, 4–8 September 2022, Heidelberg, Germany). However, there were no statistically significant benefits observed in composite secondary endpoints, including all-cause mortality. Although patisiran has fewer concerns about AE than inotersen, it needs to be administered by a trained healthcare professional and patients are exposed for long periods to corticosteroids and antihistamines to limit infusion reactions (67).

Since a normal physiological function of TTR is transporting vitamin A, reduction of TTR, due to either inotersen or patisiran, results in severe vitamin A depletion and requires daily supplementation to maintain normal levels (53, 56, 61, 62). Both drugs show improvement among patients with low to moderate disease burden. Recent data suggest patisiran also may have benefits among patients with ATTR-CM.

Vutrisiran is a second-generation siRNA formulation of patisiran and can block the expression of both ATTRwt and ATTRv genes (68). It has been approved for treatment of polyneuropathy. Compared with placebo, vutrisiran was shown to reduce serum levels of NT-proBNP, improve some echocardiographic parameters, and improve scintigraphy tracer uptake in ATTRv patients with polyneuropathy. It is currently under investigation for treatment of patients with ATTR-CM (NCT04153149).

Eplontersen is a ligand conjugated ASO with the same primary sequence as inotersen (69). Conjugation with ligand facilitates targeted uptake of the drug by hepatocytes, which has the potential for greater efficacy and lower toxicity than the unconjugated drug, inotersen (69). Eplontersen is reported to significantly lower TTR levels from baseline and be well tolerated. It is under investigation for treatment of both ATTR-CM and ATTR-PN. In the NEURO-TTRansform trial (NCT04136184), compared with placebo eplontersen slowed the progression of neuropathic disease and improved QoL among patients with hereditary ATTR-PN (reported at XVIII International Symposium on Amyloidosis). No specific safety concerns were reported.

Stabilizers

Another approach to treating ATTR amyloidosis is to stabilize the TTR tetramer protein complex, thereby preventing its dissociation into amyloidogenic TTR monomers and oligomers (Figure 1B) (70). Native TTR tetramer stabilization requires both thyroxine-binding pockets of the TTR tetramer to be occupied, thus requiring high concentrations of TTR stabilizers to prevent its dissociation (66).

Diflunisal, an oral non-steroidal anti-inflammatory drug (NSAID), stabilizes TTR tetramers (52). However, long-term use may be associated with increased fluid retention leading to heart failure, gastritis, peptic ulcer disease, and worsening of renal dysfunction (71). Therefore, diflunisal should be used with caution in older adults and in patients with severe congestive heart disease. Moreover, renal insufficiency limits its use in patients with ATTR-CM or ATTR-PN with cardiac or renal impairment requiring careful patient selection and management of drug-associated AEs (52).

Tafamidis is an orally bioavailable agent, and the only one approved to treat ATTR-CM (72). It occupies the thyroxine-binding sites in wild-type and several variants of TTR with high affinity and selectivity, thereby preventing their dissociation (73). Compared with placebo, treatment with tafamidis was associated with TTR stabilization in almost all patients, significantly lower all-cause mortality, lower rate of cardiovascular-related hospitalizations, and less decrease in 6MWT functional capacity indicating that tafamidis stabilized disease, delayed disease progression, and slowed decline in patient QoL (72, 74). However, a pre-specified subgroup of patients with New York Heart Association (NYHA) class III heart failure, representative of patients with advanced disease, had a higher rate of cardiovascular-related hospitalizations compared with placebo, i.e., an inverse relationship between effectiveness and NYHA class (72). However, improved survival was observed at 5-year follow-up among patients in NYHA class III compared with those who received placebo initially (reported at European Society of Cardiology Heart Failure Congress, 26–29 May 2022, Barcelona, Spain). Patients in NYHA class IV, representative of very advanced disease, were excluded from this trial. In all clinical studies, tafamidis demonstrated an appropriate benefit-to-risk ratio.

Acoramidis (AG10/ALXN2060) is a new TTR stabilizer under investigation for treatment of ATTR amyloidosis (75). Acoramidis binds TTR with greater selectivity than either tafamidis or diflunisal and increases serum levels of TTR tetramers and is well tolerated (75–77). In the ongoing open-label extension of the phase 2 trial (NCT03536767), serum TTR levels were below the lower limit of normal in 40.4% of patients and there was a median decrease from baseline of 479 pg/mL in serum NT-proBNP levels (78). Two randomized, double-blind, placebo-controlled, phase 3 studies (NCT03860935 and NCT04622046) are in progress to determine the efficacy and safety of acoramidis in patients with ATTR-CM.

Another stabilizer under investigation for treatment of ATTR amyloidosis is tolcapone (79). Like acoramidis, tolcapone binds TTR with greater selectivity than either tafamidis or diflunisal and increases serum levels of TTR tetramers (79, 80). However, tolcapone has a boxed warning for potentially fatal acute fulminant liver failure and is not suggested for patients with ATTR-CM (77).

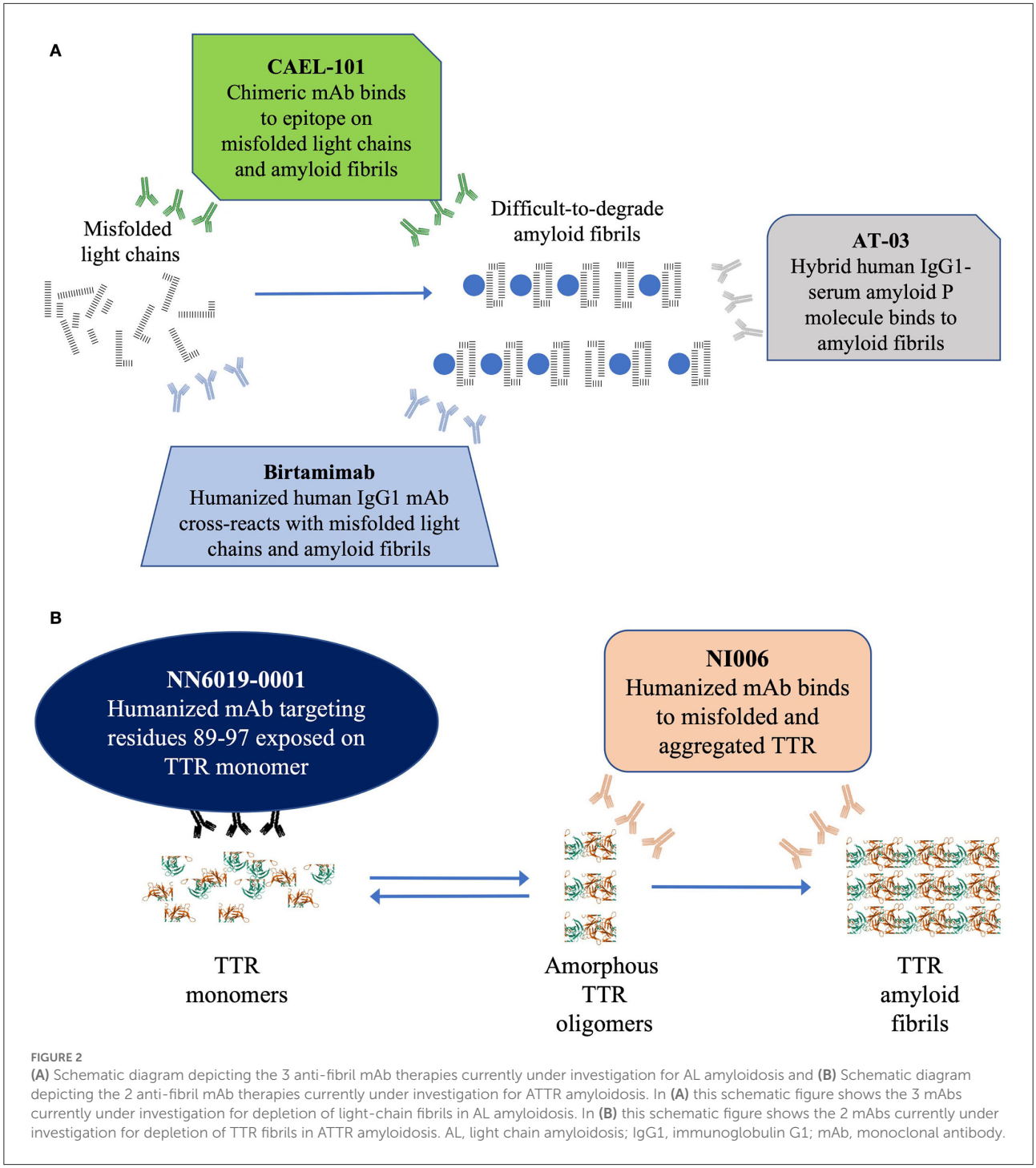

FIGURE 2
(A) Schematic diagram depicting the 3 anti-fibril mAb therapies currently under investigation for AL amyloidosis and **(B)** Schematic diagram depicting the 2 anti-fibril mAb therapies currently under investigation for ATTR amyloidosis. In **(A)** this schematic figure shows the 3 mAbs currently under investigation for depletion of light-chain fibrils in AL amyloidosis. In **(B)** this schematic figure shows the 2 mAbs currently under investigation for depletion of TTR fibrils in ATTR amyloidosis. AL, light chain amyloidosis; IgG1, immunoglobulin G1; mAb, monoclonal antibody.

Future anti-fibril therapies

Despite advances in treatment options, there are as yet no approved treatments for removal of amyloid fibrils already deposited in the organs, especially the heart (61, 81). These fibrils can continue to cause progressive damage to the organs resulting in death. The current hypothesis is that removal or depletion of these amyloid fibrils will decrease organ damage and restore function, particularly that of the heart resulting in improved survival (Figures 2A,B).

AL amyloidosis

There currently are 3 mAbs, birtamimab, CAEL-101, and AT-03 (Figure 2A), under investigation as anti-fibril agents (82–84). It is hoped that these antibodies provide direct proof of concept by depleting the deposits of light chain amyloid fibrils from organs improving their function.

Birtamimab, a fully humanized mAb developed to recognize a cryptic epitope on serum amyloid A protein, cross reacts with light chain amyloid fibrils and activates macrophage-mediated degradation and clearance of the fibrils (83). In a phase 1/2 trial, birtamimab was well tolerated at all doses administered up to 24 mg/kg (85). In the phase 2b PRONTO trial (NCT02632786), birtamimab failed to improve cardiac response, 6MWT, and NT-proBNP levels in previously treated patients with AL amyloidosis (86). Furthermore, a futility analysis of the phase 3 VITAL (NCT02312206) trial showed that birtamimab did not reduce all-cause mortality in newly diagnosed patients resulting in termination of the trial (87). However, a *post-hoc* analyses showed promising results among patients in Mayo 2012 Stage IV (85, 87, 88). A double-blind, placebo-controlled, phase 3 trial (AFFIRM-AL; NCT04973137) is currently recruiting patients to confirm these results (89).

CAEL-101, a chimeric mAb developed to recognize a cryptic epitope on immunoglobulin light chains, binds to misfolded free light chains and amyloid fibrils deposited in organs (90–92). In phase 1 trials, CAEL-101 demonstrated reductions in biomarkers of cardiomyopathy and nephropathy (93–95). The ongoing phase 2 trial demonstrated that CAEL-101 was well tolerated when administered with anti-PCD therapy that included daratumumab or as monotherapy after cessation of anti-PCD therapy. There are 2 concurrent randomized, double-blind, placebo-controlled, phase 3 trials actively recruiting patients with advanced cardiac disease 2015 European Modification of Mayo 2004 Stages IIIA (NCT04512235) and IIIB (NCT04504825) (96).

AT-03, a hybrid human mAb against serum amyloid P protein, binds all types of amyloid fibrils (84). AT-03 has recently completed a phase 1 biodistribution study (NCT05201911), the results of which have not yet been reported.

ATTR amyloidosis

There currently are 2 mAbs under investigation to deplete or remove TTR amyloid fibrils, NI006 and NN6019-0001 (formerly known as PRX004; Figure 2B) (73, 97). It is hoped that these antibodies will provide direct proof of concept by depleting TTR amyloid fibril deposits from organs and improving their function.

NI006 is a humanized mAb that selectively binds to a cryptic epitope that is exposed in misfolded TTR oligomers and aggregated TTR fibrils (97). In preclinical studies, NI006 bound with high affinity to both ATTRwt and ATTRv amyloid fibrils and facilitated their elimination *via* activation of phagocytic cells. A phase 1 study (NCT04360434) is in progress to determine the dosage and safety of NI006 in patients with ATTR-CM.

NN6019-0001 binds to a cryptic epitope that is exposed when TTR tetramers dissociate into monomers (73). NN6019-0001 binds and neutralizes the various prefibrillar species of TTR and prevents the formation of new amyloid fibrils (73). In phase 1 trials, NN6019-0001 was well tolerated at all doses and showed improvement in both global longitudinal strain and neurologic symptoms (reported at XVIII International Symposium on Amyloidosis, 4–8 September 2022, Heidelberg, Germany). A placebo-controlled, phase 2 study (NCT05442047) is currently recruiting patients to determine the efficacy and safety of NN6019-0001 at 10 and 60 mg/kg.

Conclusions

Although there have been considerable advances in the treatment of CA, there are still opportunities for improvement. Overall CR among patients with AL amyloidosis still hovers around 50% and does not always translate into organ response. Despite advances, disease continues to progress for many patients. Current therapies to treat both AL and ATTR amyloidosis are focused on eliminating or stabilizing the source of the amyloidogenic protein. However, there are no approved therapies that deplete or eliminate already deposited amyloid fibrils, which can continue to cause progressive organ damage, especially the heart, causing early death. There are promising studies focusing on amyloid fibril depletion in both forms of CA that are expected to add to the armamentarium available and to elucidate whether removal of preexisting fibrils will improve survival and QoL of patients.

Author contributions

CCQ was invited to contribute. All authors provided critical input into the concept and content of this review article. All authors approved the submission of the final article.

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Conflict of interest

CCQ, JC, DS, and MM are employed by Alexion, AstraZeneca Rare Disease. MF: consultant (Akcea, Alexion, Alnylam, Eidos, Ionis, Intellia, Pfizer, Prothena, and Sanofi); advisory board (Akcea, Alnylam, Eidos, Intellia, Pfizer, and Prothena). TD: consultant (Alnylam, GlaxoSmithKline, Pfizer, and Prothena); honoraria (Alnylam, Pfizer, and Prothena; research grants (GlaxoSmithKline and Pfizer); clinical trial support (Alnylam, Ionis, and Pfizer). PG-P: consultant (Alexion, Alnylam, AstraZeneca, Attralus, Bridgebio, Intellia, Ionis, Neurimmune, NovoNordisk, and Pfizer); speakers bureau (Alnylam, Bridgebio, Ionis, and Pfizer); grant support to his institution (Alnylam and Pfizer). MM: advisory board or DSMB (Alnylam, Eidos, Intellia, Ionis, NovoNordisk); research grants (Eidos, Janssen, Pfizer); clinical trial support (Alnylam, Attralus, Eidos, Ionis, Pfizer). GP: advisory board (Alexion, Argobio, Janssen, Protego); honoraria (Alexion, Argobio, The Binding Site Group, Janssen, Pfizer, Protego, Prothena, Sebia, and Siemens); research funding (Gate Bioscience, The Binding Site Group).

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Prevalence of anxiety and depression symptoms in a sample of outpatients with ATTR cardiac amyloidosis

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Patients with ATTR cardiac amyloidosis (ATTR-CA) face rare disease that could negatively influence psychological well-being with consequences on the course of the disease and quality of life. However, to date, no study analyzed the prevalence of anxiety and depression in patients with ATTR-CA and which clinical and sociodemographic characteristics are linked with these psychopathological conditions. A total of 109 consecutive patients (83% males) aged 62–90years with ATTR-CA were recruited. In order to better understand the prevalence of anxiety and depression in ATTR-CA, a control group composed by 33 individuals equaling gender, education, and age were recruited. The level of anxiety and depression was measured using the Italian version of the Hospital Anxiety and Depression Scale (HADS). Sociodemographic and clinic characteristics were registered. Almost half of patients (49%) reported a clinical level of depression or anxiety, or both. ATTR-CA patients reported higher levels of anxiety and depression than control group. Results showed that older patients with ATTR-CA, especially females, with more advanced disease could be more at risk to develop an anxious disorder. Furthermore, being a woman, and presenting with a greater severity of symptoms, would appear to be a risk factor for developing a depressive disorder. Overall, these results highlighted the high presence of anxiety and depression in ATTR-CA patients, suggesting to physicians to pay attention to the psychological well-being of ATTR-CA patients. In fact, a psychological support for patients with high level of psychopathological disease could reduce disease burden and improve quality of life in ATTR-CA population.

KEYWORDS

cardiac amyloidosis, psychological well-being, anxiety, depression, clinical characteristics, sociodemographic characteristics

1. Introduction

Several works have shown an increased prevalence of depression and anxiety in adults with different types of cardiovascular diseases (CVD) compared to people free from these conditions (Ormel et al., 2007; Knapp et al., 2020).

Despite the wide literature on the role of anxiety and depression on CVD, little research has investigated anxiety and depression in Transthyretin Cardiac Amyloidosis (ATTR-CA). ATTR-CA is a myocardial disease characterized by a pathological process of TTR derived amyloid deposition in the extracellular space, resulting in a progressive deterioration of cardiac function (Agha et al., 2018).

Although previous studies suggest that ATTR-CA patients reported anxious and depressive symptoms and that, in some cases, they are clinical (Stewart et al., 2018), no study has been conducted to analyze the prevalence of anxiety and depression in ATTR-CA and if clinical and sociodemographic risk factors are associated with anxiety and depression in patients with ATTR-CA.

Previous studies conducted in AL amyloidosis, a disease with a different systemic involvement, age of onset, and therapeutic approach, showed that anxiety and depression were common, reported in approximately 30% of subjects (Smorti et al., 2012, 2014, 2016; Lo et al., 2015; Shu et al., 2016; Lopes et al., 2018). Moreover, Smorti et al. found that time since the onset of cardiac symptoms was a positive predictor of anxiety in AL patients, whereas only the severity of cardiac symptoms was for depression (Smorti et al., 2012).

Our study aim is to fill the knowledge gap in ATTR-CA comprehensively analyzing the socio-demographic and clinical risk factors that may be associated with the presence of clinically significant levels of anxious and depressive symptoms in ATTR-CA patients.

2. Materials and methods

2.1. Participants and procedures

A total of 109 consecutive patients (90 males and 19 females) aged 62–90 years (M = 79.07; DS = 6.19) with ATTR-CA followed at the Tuscan Regional Amyloidosis Center in Florence, Italy, were recruited for the present study between September 2021 and June 2022. About type of ATTR-CA, 88.1% of patients were diagnosed with ATTR wild type (ATTRwt) and 11.9% with ATTR variant (ATTRv) with prevalent cardiac phenotype. All patients enrolled had a definite diagnosis and at the time of communication of the diagnosis, or of at the first evaluation in your center of a previously diagnosed patient in case of diagnosis made at another center, the same protocol is applied for all patients of our center. Each patient receives accurate counseling on the characteristics of the disease, prognosis, therapeutic possibilities, and any repercussions on family members in the event of a diagnosis of ATTR-CAv. For that reason, all patients have similar background in terms of knowledge about their disease. Moreover, with all patients, at the time of communication of the diagnosis, are discussed the pharmacological options with market access or involvement in clinical trials. However, it should be considered that to date in Italy, the only disease modified drug for ATTR-CA is Tafamidis that was refundable since the end of January 2022, therefore, patients enrolled in the study were not in treatment.

Inclusion criteria were: diagnosis of ATTR-CA according to standard international criteria (Garcia-Pavia et al., 2021), absence of cognitive impairment, and able to understand Italian language. Exclusion criteria was the presence of actual diagnosis of anxiety or depression with ongoing psychopharmacologic treatment. Only patients corresponding to the inclusion criteria were invited to participate. All contacted patients agreed to participate. Data collection was conducted by a trained psychologist who administered the questionnaire during a routine cardiological assessment. A small group of individuals without cardiac pathology balanced for gender and age (23 males; $M_{age} = 76.85$; SD = 5.97) were recruited from local community centers in order to compare the level of anxiety and depression between them and our ATTR-CA patients. The study was approved by the local Ethical Committee (CEAVC; protocol number 19476_OSS/2021). Participation to the study was voluntary and written informed consent was obtained from all patients before data collection by a study responsible who informed them about aims and procedure of the study.

2.2. Instruments and detected variables

2.2.1. Socio-demographic characteristics

All participants completed a questionnaire to collect socio-demographic data, such as age, gender, educational level, marital status, have children or not, working activity, and living condition.

2.2.2. Clinical characteristics

Only for ATTR-CA patients, Physicians recorded the following clinical data during cardiological assessment: Body Mass index (BMI), type of ATTR, months since communication of diagnosis, symptom severity according to NYHA class, the level of NT-proBNP, the glomerular filtration rate (GFR), interventricular septum (IVS), and Left Ventricular End Diastolic Diameter (LVEDD); LV posterior Wall (LVPW), Left Atrium Diastolic Diameter (LADD), Left Ventricular Ejection Fraction (LVEF),E/e', and National Amyloid Center (NAC) score as previously described (Gillmore et al., 2018; Cappelli et al., 2020), and medical treatment.

2.2.3. Psychopathological characteristics

The Italian version of the Hospital Anxiety and Depression Scale (HADS; Iani et al., 2014) was employed to assess the level of anxious and depressive symptoms. The HADS is a self-reported questionnaire composed by 14 items (seven assessing anxiety and seven assessing depression) rated on a four-point Likert scale from 0 to 3. Scores range from 0 to 21 for each subscale, with a higher score indicating a higher level of psychological symptomatology. In the present study, the HADS Cronbach's alpha was 0.86 and 0.81 for the anxiety and depression subscales, respectively.

2.3. Data analyses

The prevalence of anxiety and depression symptoms were estimated using the HADS cut-off points of 8. Based on score 8 on anxiety or depression HADS subscales participants with a clinical level of psychopathological symptomatology were identified. Comparison between ATTR-CA patients and control group was performed. Differences in presence of clinical level of psychopathological symptomatology according to socio-demographic and clinical variables were performed using chi-square analysis and Student's *t*-test, depending on the dichotomous or continuous nature of variables. Two regression analyses were performed to verify how the different significant variables were associated with anxiety and depression. All comparisons were calculated in IBM SPSS Statistics for Macintosh, Version 23.0 (IBM, Armonk, NY, United States), and $p < 0.05$ was considered significant.

3. Results

Socio-demographic and clinical characteristics of patients and control group are reported in Supplementary Table 1. As expected, most patients were male with an average age of almost 80 years, they were married, lived with their partner, and had at least one child. All patients were retired. Moreover, most of them had wild type ATTR-CA and they had been diagnosed for an average of 2 years before recruitment. At study, evaluation 19% of patients were in NYHA class I, 66% in NYHA class II, and 15% in NYHA class III. Echocardiographic evaluation performed at the moment of recruitment showed a slight reduction in left ventricular systolic function, and impaired left ventricular relaxation measured by the E/e' ratio (Supplementary Table 1). No significant differences emerged between ATTR-CA patients and control group on the socio-demographical variables.

Overall, 53 patients (48.6%) reported a clinical level of depression or anxiety, or both (Figure 1).

Taking in consideration anxiety and depression separately (and not comorbid), 36 patients (33%) meet criteria for clinical level of anxiety and 46 (42.2%) for depression. Among participant of control group, 5 of theme (15.2%) reported significant level of anxious and 7 (21.2%) of depressive symptoms. Moreover, ATTR-CA patients reported higher levels of anxiety and depression than control group.

Referring to anxious symptomatology, no significant differences emerged between anxious and not anxious patients on most socio-demographical and clinical characteristics excepted for gender, females reported more likely a clinical presence of anxiety than males (69 vs. 31%, $p = 0.011$); age, with patients with a clinical level of anxiety presented an older age ($p = 0.047$); and renal function impairment, with patients with a clinical level of anxiety showing a lower GFR ($p = 0.021$). Similar results are highlighted referring to depressive symptomatology. According to clinical depression levels, patients differed on gender, females reported more likely a clinical presence of depression than males (74 vs. 36%, $p = 0.042$); NYHA class, where patients in class III reported more likely the presence of clinical level of depression ($p = 0.015$), and NAC score, with a lower prevalence of patient with clinical level of depression in class 1 ($p = 0.05$). All these results are reported in Supplementary Table 2.

Finally, results of regression analysis conducted using anxiety as dependent variables and age, gender and GFR level as predictors, showed that only gender was significantly associated to the level of anxiety. The regression analysis conducted using depression as dependent variables and gender, NYHA class, and NAC score and predictors showed that female gender and higher NYHA class were significantly associated to the level of depression (see Supplementary Table 3).

4. Discussion

ATTR cardiac amyloidosis is a rare disease associated to a high mortality rate (Rapezzi et al., 2013; González-López et al., 2017), and the incidence of this disease is uniformly considered to increase. This is presumably due to better knowledge of the disease itself and the availability of imaging tests that can reveal its presence (Zampieri et al., 2021). Moreover, as many chronic diseases, with poor outcome, it can easily be accompanied by tiredness, pain, discomfort, anxious, and depressive feelings (Damy et al., 2022). An evaluation of the psychological burden should be considered mandatory due to the influences that a clinically significant psychopathological condition can have on the patient's well-being and on the course of his/her illness (Gathright et al., 2017). Several studies showed that ATTR-CA is linked to high levels of impairment in different domains, including physical health, quality of life, and reduced productivity (Stewart et al., 2018; Aimo et al., 2021). It is possible to hypothesize that the high levels of anxiety and depression can significantly and negatively influence all these aspects. Despite that, to our knowledge, no studies have investigated the prevalence of anxiety and depression in ATTR-CA patients, exploring which variables, both socio-demographic and clinical, may be most

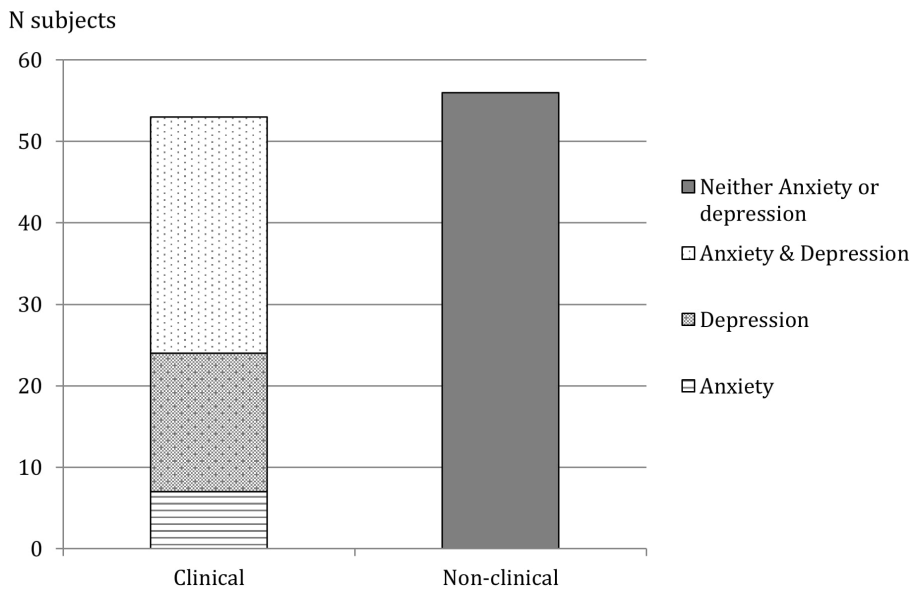

FIGURE 1
Clinical and non-clinical subjects according to HADS. Prevalence of anxiety and depression and both anxiety and depression are reported.

associated with these conditions. This knowledge could instead represent a useful tool for physicians who work with these patients. Knowing which aspects are most associated with psychopathological traits, such as anxiety and depression, could allow physicians to identify possible risk situations more quickly and promptly arrange a specific support. At this regard, the purpose of this study is therefore aimed at filling this aspect. Firstly, our results confirmed that depression and anxiety are prevalent among ATTR-CA patients, with almost half of patients (49%) reported a clinical level of depressive or anxious symptoms, or both. ATTR-v, given its nature as an inherited disease, may be associated with the development of feelings of guilt related to the possibility of having passed the genetic mutation to offspring (Stewart et al., 2018). Nevertheless, this study found that anxiety and depressive symptoms were not more prevalent in the subgroup of ATTR-v patients than those with ATTR-wt. However, considering that most patients had some form of ATTR-wt, caution is needed in interpreting these results and further investigation would be needed.

Comparing the prevalence of anxious and depressive symptoms with those reported in the general population aged 65–80 years, the prevalence of anxious symptoms among ATTR-CA is 3-fold higher (33 vs. 10% in the general population), and that of depressive symptoms almost 4-fold higher (42 vs. 9%) than general population (Djukanovic et al., 2017). Also in the present study, the levels of anxious and depressive symptoms in ATTR-CA patients were higher than in control group. Nevertheless, the prevalence of symptoms of anxiety and depression was comparable to that found in other cardiomyopathy (Singh et al., 2021). Moreover, it should be noted that our study was conducted during the COVID-19 pandemic, when the levels of symptoms of anxiety and depression increased in the general Italian population (and in the elderly) compared to the pre-COVID period (Fiorenzato et al., 2021).

This high prevalence highlights the need to pay particular attention to the anxiety and depression levels of ATTR-CA patients. Referring to anxiety, our results showed that older patients, especially females, with more advanced disease (suggested by lower GFR levels often linked to a cardiorenal syndrome) could be more at risk to develop an anxious disorder. Being a woman is also a risk factor for depressive disorder, along with greater symptom severity, as measured by the NYHA class scale and again the disease severity stage according to the NAC score. Although these data are significant results, it should be noted that the group is strongly unbalanced with respect to gender. This reflects the gender distribution within the ATTR-CA population, which predominantly affects the male population (Grogan et al., 2016). However, the low number of women in the sample must be cautious in the possibility of generalizing these results. Moreover, considering all significant variables, referring to anxiety symptoms, only gender was a significant predictor, while for depression symptoms both gender and NYHA class are predictors.

Unfortunately, all the other variables taken into consideration, both socio-demographic and clinic, did not show any significant association with the presence of clinical level of anxious and depressive symptoms. This does not allow us to identify a specific socio-demographic profile of a patient at risk of psychopathology, with particular characteristics, beyond gender and the above mentioned few other clinical indicators. These results are however very important, because they underline the high presence of clinical symptoms of anxiety and depression in the population of ATTR-CA patients, suggesting to pay attention, in general, to all patients followed for this disease.

In other words, our data seem suggest the relevance in amyloid referral centers a specific psychological assessment performed by trained health care staff should be provided to entire patient population. In fact, identify those subjects with psychopathological difficulties should be allow to refer them for further evaluation and take in charge by psychological staff. At our center, for example, for patients who scored clinical symptoms of anxiety and depression, our psychologist conducted a return to each patient of the questionnaire result and offered the opportunity to have a psychological support interview at the center itself. A psychological support could be able to reduce disease burden and improve quality of life and adherence to new disease modifying drugs that are changing the scenario of ATTR-CA.

4.1. Limitations

There are some study limitations. Firstly, the sample is small, it is possible that our results could be susceptible to type II error. However, ATTR-CA is a rare chronic disease and this study had a monocentric nature. This is undoubtedly another limitation. Due this monocentric nature this data should be confirmed in larger and multicentric, international population to avoid referral center bias. Nevertheless, given the lack of studies on this topic, the present study represents a starting point contributing to the literature on the prevalence of anxious and depressive symptoms in ATTR-CA population.

Another limitation is linked to the lack of other information which could influence the presence of anxious and depressive symptoms, such as the presence or number of hospitalizations, the socio-economic status, or a baseline psychological status before the diagnosis. Moreover, in the present study the presence of anxiety and depression was assessed by a self-report questionnaire. There is no doubt that a clinical evaluation carried out by a specialist would be more precise and reliable. However, our study was not intended to make a psychopathological diagnosis according to the DSM-5 criteria (American Psychiatric Association, 2014), but only to detect depressive or anxious symptoms above the cut off in our sample of patients with ATTR-CA, in order to highlight whether psychological distress was particularly presented in this clinical population. Moreover, the HADS is a valid measure for detecting the presence of anxious and depressive symptoms, widely used in hospitals and adapted to various medical diseases including cardiomyopathies. Furthermore, compared to other self-report assessment tools, the items have been created in a way to avoid ambiguous somatic symptoms that can be associated with various medical conditions, such as dizziness and lethargy (Zigmond & Snaith, 1983).

In conclusion, ATTR-CA is a debilitating disease not only physically but could also constitute a risk factor with respect to psychological well-being. In fact, despite the limitations of the present study, the results suggest the need to pay attention to the presence of anxiety and depression in this population. There is a need for increased awareness among the medical community about the prevalence of psychological disorders to provide a psychological assessment as soon as patients are referred to specialized centers for a diagnosis. In fact, since it is currently not possible to provide a specific profile of ATTR-CA patients with a higher risk of suffering from anxious or depressive symptoms, a specific psychological evaluation performed by healthcare professionals should be carried out in the amyloid reference centers trained on the entire patient population.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comitato Etico Area Vasta Centro (CEAVC). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MS contributed to the literature review, drafting, and revising the article and approval of the final version. LP also contributed to the literature review and drafting, design, and revision of the article. FS contributed to the literature review and drafting of the article. AA, MZ, and CM contributed in acquisition of data. AT contributed in data analysis. FP and MA contributed in data interpretation. CDM, IO, and FC conceived the study and reviewed and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2022.1066224/full#supplementary-material

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Cardiac transplantation in transthyretin amyloid cardiomyopathy: Outcomes from three decades of tertiary center experience

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Aims: Transthyretin cardiac amyloidosis (ATTR-CM) is a progressive and fatal cardiomyopathy. Treatment options in patients with advanced ATTR-CM are limited to cardiac transplantation (CT). Despite case series demonstrating comparable outcomes with CT between patients with ATTR-CM and non-amyloid cardiomyopathies, ATTR-CM is considered to be a contraindication to CT in some centers, partly due to a perceived risk of amyloid recurrence in the allograft. We report long-term outcomes of CT in ATTR-CM at two tertiary centers.

Materials and methods and Results: We retrospectively evaluated ATTR-CM patients across two tertiary centers who underwent transplantation between 1990 and 2020. Pre-transplantation characteristics were determined and outcomes were compared with a cohort of non-transplanted ATTR-CM patients. Fourteen (12 male, 2 female) patients with ATTR-CM underwent CT including 11 with wild-type ATTR-CM and 3 with variant ATTR-CM (ATTRv). Median age at CT was 62 years and median follow up post-CT was 66 months. One, three, and five-year survival was 100, 92, and 90%, respectively and the longest surviving patient was Censored > 19 years post CT. No patients had recurrence of amyloid in the cardiac allograft. Four patients died, including one with ATTRv-CM from complications of leptomeningeal amyloidosis. Survival among the cohort of patients who underwent CT was significantly prolonged compared to UK patients with ATTR-CM generally ($p < 0.001$) including those

diagnosed under age 65 years (p = 0.008) or with early stage cardiomyopathy ($p < 0.001$).

Conclusion: CT is well-tolerated, restores functional capacity and improves prognosis in ATTR-CM. The risk of amyloid recurrence in the cardiac allograft appears to be low.

KEYWORDS

amyloid, transplant, heart failure, TTR–transthyretin, outcome

Introduction

Systemic amyloidosis is characterized by extracellular tissue deposition of misfolded fibrillary protein. Amyloid is identified *ex vivo* by apple green birefringence when a tissue biopsy is stained with Congo red dye and viewed under cross-polarized light (1). A variety of normally soluble proteins, known as "fibril precursor proteins," have been identified which can misfold and self-assemble with an abnormal cross beta-sheet conformation resulting in fibrils that are proteolysis resistant (2). These different "amyloidogenic" proteins form the basis for the classification of amyloidosis. Clinical disease occurs when amyloid deposits disrupt tissue structure and function. Whilst amyloid deposition can occur in almost any organ of the body, cardiac amyloidosis is the established leading cause of mortality in systemic amyloidosis (3).

Transthyretin amyloid cardiomyopathy (ATTR-CM) is the most commonly diagnosed type of cardiac amyloidosis and may be either acquired (ATTRwt-CM) or associated with inheritance of a TTR variant (ATTRv-CM) (2). Historically, treatment for this inexorably progressive and ultimately fatal cardiomyopathy was supportive with meticulous fluid balance and diuretic therapy. However there have been landmark developments in disease-modifying therapy for patients with ATTR amyloidosis. Examples include TTR specific RNA interference or antisense oligonucleotide therapies such as patisiran and inotersen, TTR stabilizers such as tafamidis and acoramidis (4–6), and the CRISPR/Cas9 based gene-editing therapy NTLA-2001 (7). Whilst these agents show promise, to date they appear to slow or potentially halt disease progression rather than being curative. Treatments that bring about an overt clinical improvement and genuinely improve quality of life in patients with advanced ATTR-CM remain elusive.

Cardiac transplantation (CT) for patients with ATTRv-CM was first reported in 2003 (8). However, this was in the context of combined hepatic and CT, the dual objectives being to replace failing cardiac function and remove hepatic production of circulating variant, amyloidogenic TTR which is entirely liver-derived. Hepatic transplantation was mostly undertaken in young patients with hereditary ATTR amyloid polyneuropathy (ATTRv-PN) who typically carried the p.(Val50Met) TTR variant (9). In patients with ATTRv-PN deemed suitable for liver transplantation who had concurrent amyloid cardiomyopathy, a combined hepatic and cardiac transplant would sometimes be offered (10). However, the need for hepatic transplantation in patients with a predominant neuropathic ATTR amyloidosis has largely been superseded by the availability of aforementioned patisiran and inotersen, both of which have been shown to slow or halt disease progression. Consequently, younger patients with advanced ATTR-CM including ATTRwt-CM are increasingly considered for isolated CT (11). There is a paucity of published data on long term outcomes in patients who receive CT for ATTR-CM (11, 12). Allograft amyloid recurrence and accumulation in extra-cardiac organs have remained key concerns in patients with AL amyloidosis receiving CT (either isolated, or combined) (13), and similar concerns regarding potential amyloid recurrence exist in ATTR-CM. Whilst patisiran and inotersen are effective at slowing ATTR amyloid production, they remain licensed only for patients with ATTRv-PN. The risk of cardiac allograft amyloid recurrence is therefore potentially greater in patients, such as those with ATTRwt-CM, who do not routinely receive TTR suppressing agents. We present multicentre outcomes with CT in 14 patients with ATTR-CM evaluated at the UK National Amyloidosis Center and the Department of Cardiothoracic Science, Azienda Sanitaria Universitaria Integrata di Udine, Udine, Italy.

Materials and methods

Patients

All patients with ATTR-CM who attended the aforementioned tertiary centers and underwent CT between November 1991 and March 2020 were retrospectively identified from the institutions' respective databases. Patients who underwent combined liver and heart transplant were excluded. Censor date was 27th July 2022. Patients were considered for referral to a tertiary transplantation center on a case by case basis; typically patients were under aged 65 years with advanced cardiac failure attributable to ATTR-CM and had little or no significant comorbidity.

Patients were managed in accordance with the Declaration of Helsinki, the Declaration of Istanbul and the International Society for Heart and Lung Transplantation Statement on Transplant Ethics. Approval for retrospective analysis and publication of their anonymized data was obtained from the Royal Free London NHS Foundation Trust Ethics Committee (ref 06/Q0501/41).

Abbreviations: TTR, transthyretin; ATTR, transthyretin amyloid; ATTR-CM, transthyretin amyloid cardiomyopathy; CT, cardiac transplantation; ATTRv, variant/hereditary transthyretin amyloid; ATTRwt, wild type transthyretin amyloid; ATTR-PN, transthyretin amyloid polyneuropathy; Tc-DPD, Tc-3,3-Diphosphono-1-2-Propanodicarboxylic Acid; CMR, cardiac magnetic resonance; SSFP, steady state free precession; LGE, late gadolinium enhancement; MAG-IR, magnitude inversion recovery; PSIR, phase sensitive inversion recovery; MOLLI, modified look-locker inversion; ECV, extracellular volume; LVEF, left ventricular ejection fraction; IVSd, interventricular septal thickness in diastole; eGFR, estimated glomerular filtration rate; ISHLT, international society for heart and lung transplantation; LVH, left ventricular hypertrophy; CMV, cytomegalovirus.

Diagnosis

In each case, the diagnosis of ATTR-CM was established according to either validated non-biopsy criteria (14) and/or histological confirmation of cardiac ATTR amyloid deposits with imaging evidence of amyloid cardiomyopathy by echocardiogram and/or CMR. In brief, non-biopsy diagnostic criteria for ATTR-CM consist of echocardiographic and/or CMR imaging evidence suggestive of amyloid cardiomyopathy in together with a Perugini Grade 2 or 3 Tc-DPD scan and absence of a monoclonal gammopathy. Baseline/pre-transplant characteristics were established with biochemical tests which included full blood count, renal function, liver function tests, serum NT-proBNP and high sensitivity troponin T. Serum free light chains (retrospectively from a stored serum sample in patients transplanted before 2000), immunofixation and urine immunofixation were performed in all cases. NAC ATTR Stage was calculated on the basis of NT-proBNP concentration and MDRD eGFR, as previously published (15). Patients were followed routinely at NAC following CT with a full clinical evaluation, biochemical testing and echocardiography; selected cases underwent serial CMR and ^{99m}Tc-3,3-Diphosphono-1-2-Propanodicarboxylic Acid (^{99m}Tc-DPD) scintigraphy.

Bone scintigraphy

Patients underwent radionuclide scintigraphy following an intravenous injection of approximately 700 MBq of ^{99m}Tc-DPD. Whole body planar and single photon emission computed tomography with a low-dose, non-contrast CT scan (SPECT-CT) images of the heart were acquired 3 h post-injection using low energy, high resolution collimators. Cardiac uptake on all ^{99m}Tc-DPD scans was categorized according to the Perugini grading system (16).

Echocardiography

Image acquisition and analysis was performed by independent, experienced and appropriately accredited echocardiographers in accordance with the latest European Association of Cardiovascular Imaging guidance (17).

Cardiac magnetic resonance

Cardiac magnetic resonance (CMR) was performed, after 2005, on a 1.5T scanner (Magnetom Aera, Siemens Healthcare, Erlangen, Germany). Localizers and cine imaging with steady state free precession sequences (SSFP) was performed. The contrast agent used was 0.1 mmol./kg of Gadoterate meglumine. Late gadolinium enhancement (LGE) imaging was acquired with magnitude inversion recovery (MAG-IR) and phase-sensitive inversion recovery (PSIR) sequence reconstruction with SSFP read-outs. T1 mapping was performed with the modified look-locker inversion (MOLLI) recovery sequence. T1 mapping was repeated 15 min post-contrast to produce extracellular volume (ECV) maps. All CMRs were analysed by independent consultant cardiologists.

TABLE 1 Pre-transplant patient and disease characteristics among 14 patients with transthyretin amyloid cardiomyopathy (ATTR-CM) who underwent cardiac transplantation.

Patient	Sex	TTR variant*	Age at diagnosis	Time from diagnosis to CT (months)	NYHA class	NAC ATTR disease stage**	Serum NT-proBNP (ng/L)	LVEF (%)	IVSD (mm)	eGFR (ml/min)	Serum high sensitivity troponin T (ng/L)
1	M	WT	65	11.3	4	2	8778	36	22	64	40
2	M	p.(Val142Ile)	60	1.7	3	2	4609	38	20	56	Unknown
3	M	WT	56	21.1	3	2	4347	35	18	48	37
4	F	p.(Gly73Ala)	43	19.7	3	2	5791	50	20	75	24
5	M	WT	62	48.2	2	1	1057	31	16	90	34
6	M	WT	58	44.4	3	1	3374	56	20	55	81
7	M	WT	57	22.4	2	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
8	M	WT	59	29.8	3	1	3029	48	14	70	28
9	M	WT	56	7.3	3	2	3434	27	18	90	20
10	F	p.(Ser43Asn)	54	8.7	3	2	5764	39	18	52	80
11	M	WT	61	28.4	3	1	5193	40	18	41	152
12	M	WT	63	20	3	1	2076	39	15	85	102
13	M	WT	63	23	3	2	1345	37	14	43	68
14	M	WT	65	56.4	3	2	5838	27	19	43	43

*WT, wild type TTR genotype **NAC ATTR disease stage is only validated for use at time of diagnosis. Therefore, disease stages at diagnosis, not immediately pre-transplant were used.

Histology, proteomics, and immunohistochemistry

All biopsy samples were formalin-fixed and paraffin-embedded. Samples were stained with Congo red by the method of Putchtler, Sweat, and Levine (18). Amyloid fibril type was determined by immunohistochemical staining of amyloid deposits with a range of monospecific antibodies (19). Where necessary, laser microdissection and subsequent proteomic analysis definitively confirmed fibril type (20).

Genotyping

DNA extracted from blood was amplified by polymerase chain reaction assays and the whole coding region of the *TTR* gene was sequenced to identify any mutations.

Results

Pre-transplant characteristics

A total of fourteen cardiac allograft recipients were identified. Patient and disease-related characteristics are shown in **Table 1**. Twelve of the fourteen recipients were male. Mean age at CT was 59 years, median time from diagnosis to CT was 22 months (IQR 13–29 months). Nine patients had histological evidence of ATTR amyloid deposition on endomyocardial biopsy (EMB) accompanying characteristic cardiac imaging; the remaining patients fulfilled validated non-biopsy diagnostic criteria for ATTR-CM.

Eleven patients had ATTRwt-CM and the remaining 3 had ATTRv-CM associated with the p.(Val142Ile), p.(Gly73Ala), and p.(Ser43Asn) variants respectively. One patient was NYHA functional class IV, eleven patients were NYHA functional class III and the remaining two cases were NYHA functional class II. Median (range) NT-proBNP concentration pre-transplant was 4,202 ng/L (1,057–8,778 ng/L), median (range) left ventricular ejection fraction (LVEF) was 39% (27–56%) and mean (IQR) interventricular septal thickness (IVSd) was 18 mm (14–22 mm). At diagnosis, 8 patients had NAC ATTR stage II disease, 5 had stage I disease and the NAC ATTR stage was not evaluated in the remaining patient. It is notable that patients with NAC ATTR Stage III disease were likely excluded from consideration of CT on the basis of an estimated glomerular filtration rate (eGFR) of < 45 ml/min (19).

Outcomes

Survival

Post-transplant characteristics are shown in **Table 2**. Patients were followed up for a median (range) of 66 months (21–233 months). At Censor, 10/14 (71%) patients were alive. Post-CT 1-year survival was 100%, 3-year survival was 92%, and 5-year survival was 90%. Overall estimated post-CT survival in ATTR-CM patients by Kaplan Meier analysis was 17.7 years (95% CI: 13–21 years). Kaplan-Meier survival curves comparing patients who were and were not transplanted, the latter group stratified by NAC ATTR disease stage or by age $\leq$ 65 years at diagnosis, are shown in **Figure 1**. Kaplan Meier analysis showed substantially prolonged survival from diagnosis in patients who underwent CT compared to their non-transplanted counterparts regardless of age ($p = 0.008$) or disease severity ($p < 0.001$) at the time of diagnosis.

There were no reported episodes of significant post-operative bleeding. Two patients (patient 5 and 13) were successfully treated with intravenous steroids for allograft rejection in the immediate post-operative period. A further patient (Patient 4) required intravenous steroids for grade IIIA allograft rejection 8 months post CT. Two patients were successfully treated in hospital for cytomegalovirus infection during the immediate post-operative period.

At the time of Censor, all but one surviving patient were NYHA functional class I. Patient 1 developed left ventricular hypertrophy 12 years following CT determined to be secondary to hypertensive heart disease. Notably, Tc-DPD scintigraphy in this patient 12 years post CT did not show evidence of cardiac allograft amyloid infiltration. The clinical course of non-surviving patients is available in further detail in **Supplementary material 1**.

Renal impairment

Post-CT renal impairment, including both acute kidney injury thought to be due to perioperative hypoperfusion, and progressive chronic kidney disease (CKD) was common and occurred in 8/14 CT recipients. Three patients required temporary post-operative hemodialysis with two recovering normal renal function, a further four were left with CKD. The final patient developed progressive CKD due to calcineurin inhibitor use 12 years after the CT which progressed to renal failure and hemodialysis dependence 19 years post-CT despite a switch from cyclosporin to sirolimus 5 years prior.

Recurrence of amyloid in the cardiac allograft

All patients were assessed regularly at their respective tertiary centers following CT. All patients were assessed for graft amyloid recurrence with Tc-DPD scintigraphy and/or endomyocardial biopsies in combination with CMR imaging. No patient developed recurrent amyloid in the cardiac allograft despite the fact that 12/14 patients did not receive disease-modifying therapy for amyloidosis. Two patients (patient 2 and patient 10), both of whom had ATTRv-CM were commenced on patisiran 210 months and 6 months post-CT respectively following development of mild ATTR-PN. It is notable that patient 2 demonstrated a progressive increase in soft tissue uptake on Tc-DPD scintigraphy post-CT, despite having an allograft EMB that was persistently free from amyloid, no cardiac uptake of Tc-DPD, and no evidence of cardiac amyloidosis on CMR over 17 years after CT (**Figure 2**).

Discussion

Here we report multicentre experience in UK and Italy of CT in ATTR-CM. To our knowledge, this is the longest period of follow-up following CT reported to date in ATTR-CM and is the only such study that is multicentre. Both short-term and long-term outcomes were excellent with survival rates comparable to patients undergoing heart transplantation for non-amyloid indications. One-year survival in transplanted ATTR-CM patients was 100%, 3-year survival 92%, and 5-year survival 90%. The International Society for Heart and

TABLE 2 Outcomes, functional, and disease characteristics in patients with transthyretin amyloid cardiomyopathy (ATTR-CM) who underwent cardiac transplantation.

Patient	LVEF (%)	NYHA functional class	Serum NT-proBNP (ng/L)	eGFR (ml/min)	Significant rejection*	Significant infection**	Renal impairment	Amyloid recurrence***	Outcome (Follow up post-CT, months)	Comments
1	74	II	4627	42	No	No	No	No	Alive (142 m)	Diagnosed with hypertensive heart disease at censor date.
2	53	I	525	86	No	No	Yes–temporary, required dialysis	No	Dead (212 m)	Developed ATTR-PN. Commenced patisiran 210 months post-CT.
3	75	I	182	36	No	No	Yes–post operative CKD	No	Alive (109 m)	
4	60	N/A	382	36	Yes (8 months post CT)	No	Yes–post operative CKD	No	Dead (27 m)	
5	65	I	296	55	Yes (post operative)	No	No	No	Alive (28 m)	
6	56	I	1016	50	No	No	No	No	Alive (57 m)	
7	73	N/A	414	< 15	No	No	Yes–ciclosporin related CKD	No	Dead (233 m)	
8	60	I	611	39	No	No	Yes–post operative CKD	No	Alive (60 m)	
9	59	N/A	905	41	No	No	Yes–required temporary haemodialysis. Subsequent CKD	No	Dead (74 m)	
10	65	I	973	29	No	No	Yes–post operative CKD	No	Alive (29 m)	Developed ATTR-PN. Commenced patisiran 6 months post-CT.
11	50	I	674	46	No	No	Yes–temporary, required dialysis	No	Alive (72 m)	
12	67	I	65	60	No	Yes–CMV****	Yes–temporary	No	Alive (117 m)	
13	63	I	30	37	Yes (post operative)	No	No	No	Alive (60 m)	
14	65	I	620	37	No	Yes–CMV	Yes–post operative CKD	No	Alive (54 m)	

*Significant rejection was defined as rejection requiring treatment as defined by International Society of Heart and Lung Transplantation (ISHLT) criteria.
**Infection was deemed significant if it necessitated hospitalization within 1 month of cardiac transplantation.
***In all cases, amyloid recurrence was assessed for with Tc-DPD scintigraphy and/or endomyocardial biopsy.
****CMV, cytomegalovirus. Data points are the most recently available for all patients. NYHA class is at the time of Censor.

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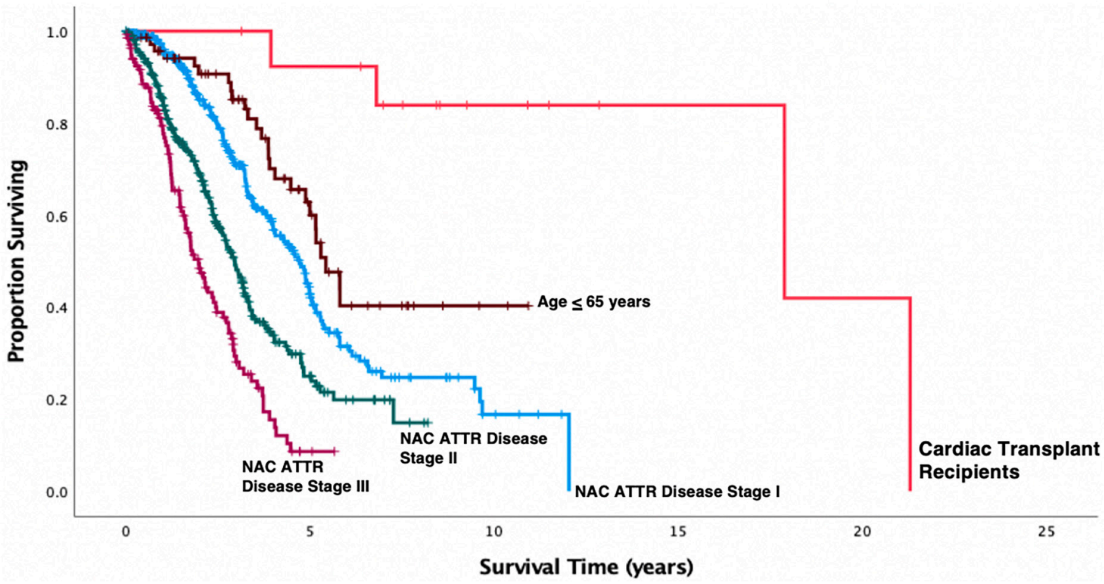

FIGURE 1
Kaplan-Meier survival curves in 814 patients with transthyretin amyloid cardiomyopathy (ATTR-CM) stratified by NAC disease stage and compared to the cohort who underwent cardiac transplantation. Cardiac transplantation in this small cohort of selected patients was associated with a substantial prolongation of life expectancy measured from date of first diagnosis compared to patients who did not undergo cardiac transplantation (Transplant vs. NAC ATTR stage I, $p < 0.001$; Transplant vs. NAC ATTR stage II, $p < 0.001$; Transplant vs. NAC ATTR stage III, $p < 0.001$) including the cohort of 71 NAC patients who were diagnosed with ATTR-CM under age 65 years ($p = 0.008$).

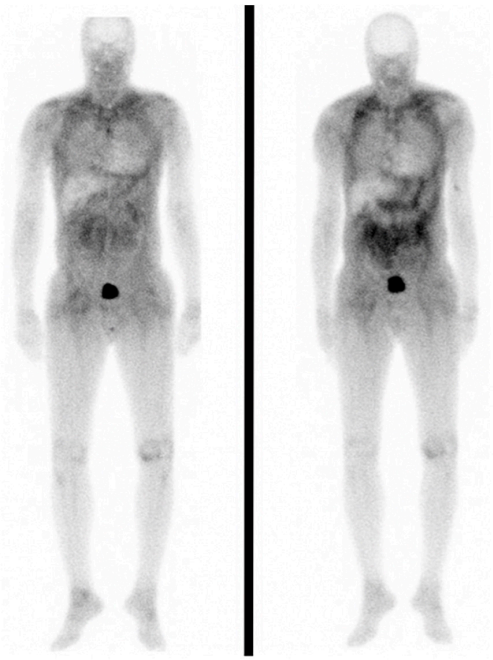

FIGURE 2
Anterior whole body planar ^{99m}Tc-DPD scintigraphy images in patient 2. Left and right panels were obtained seven and ten years post cardiac transplantation, respectively. Both scans are notable for the absence of cardiac uptake despite extensive soft tissue uptake which visibly increased across this 3 year interval.

Lung Transplantation (ISHLT) registry report a 1-year survival rate post-CT of 84% (21). Between 2012 and 2020, the UK National Health Service transplant registry reported CT 1- and 5-year survival rates of 84 and 70% respectively. Whilst our cohort is highly selected and relatively small, Kaplan Meier analysis indicates vastly improved survival compared to unselected ATTR-CM patients, including a UK cohort of 71 un-transplanted patients diagnosed under age 65 years.

Cardiac involvement is the most important determinant of mortality in systemic amyloidosis (3, 22). For example, patients with p.(V50M)-associated ATTR-CM accompanying ATTR-PN carry a prognosis of approximately 5 years, compared to up to ∼15 years in those with p.(V50M)-associated ATTR-PN alone (23). The role of "standard" heart failure therapies including ACE inhibitors, beta blockers and sacubitril/valsartan remains uncertain in ATTR-CM. Some authors argue these aggravate hemodynamic compromise and prohibit effective diuretic therapy (24). The role of SGLT2-inhibitors in ATTR-CM related heart failure is also unknown since ATTR-CM patients were excluded from relevant trials (25). Novel therapeutics such as patisiran, inotersen, and tafamidis, aimed specifically at modifying the inexorably progressive clinical course of ATTR amyloidosis are now in clinical use. However, whilst these agents may be effective at slowing or halting disease progression, there are no data to suggest that they are able to restore normal cardiac function in patients with established ATTR-CM (4–6). Therefore, CT remains the only therapeutic option that has the potential to restore normal cardiac function and quality of life in patients with advanced ATTR-CM.

The first case of CT in amyloid cardiomyopathy was reported in 1984 in a gentleman with cardiac AL amyloidosis (13, 26). Subsequent reports of CT in cardiac amyloidosis highlighted recurrence of amyloid in the cardiac allograft and poor outcomes in comparison to patients undergoing CT for non-amyloid indications, and resulted in amyloid cardiomyopathy being considered an absolute contraindication to CT in some centers (27). However, some case series of CT in amyloid cardiomyopathy consider the condition as a single entity with no differentiation between amyloid types (8,

11, 12), despite the fact that the natural history, organ tropism and prognosis of systemic AL amyloidosis indicate a more aggressive and multi-system disease phenotype than that of ATTR amyloidosis (15, 28). This stark difference in disease natural history coupled with the absence of detectable recurrence of ATTR amyloid in the cardiac allograft of any transplanted patient despite follow up of up to 19 years warrants consideration of the ATTR-CM indication for CT independently from that of cardiac AL amyloidosis. There is strong *in vivo* and *in vitro* evidence that presence of existing amyloid in a tissue promotes ongoing local amyloid deposition (29, 30); this phenomenon, known as "seeding," was evident in patient 2 who had ongoing accumulation of soft tissue amyloid following CT but did not develop amyloid in his cardiac allograft (confirmed by allograft biopsy 17 years post-CT) which was obviously amyloid naïve at the time of transplantation. One can therefore take encouragement from our cohort that ongoing, unaltered hepatic TTR synthesis does not lead to recurrent amyloid in the cardiac allograft in the short or medium term.

In patients with ATTRv amyloidosis, ATTR-PN accompanying ATTR-CM is a common and important clinical manifestation (2, 4, 23). Prior to the introduction of TTR suppressing and TTR stabilizing agents, liver transplantation to remove the circulating variant transthyretin, was the only available treatment for ATTR-PN. Whilst successful in patients with early-onset p.(V50M)-associated ATTRv amyloidosis who typically have ATTR-PN without ATTR-CM (31), liver transplantation in patients with established ATTRv-CM did not prevent ongoing deposition of wild-type ATTR amyloid deposits on top of the existing template of variant ATTR cardiac amyloid resulting in poor outcomes (32). As a result, combined liver and heart transplantation was performed in some patients with mixed ATTRv-CM and ATTRv-PN (33). However, with the advent of patisiran and inotersen, liver transplantation for ATTRv amyloidosis is now rarely undertaken. A small number of the > 130 known amyloidogenic TTR variants are particularly associated with leptomeningeal ATTR amyloid deposition including p.(Thr69Pro), p.(Leu32Pro), p.(Tyr134Cys), and p.(Gly73Ala) (30, 34–36). Leptomeningeal ATTR amyloid deposits are composed of TTR protein which is synthesized in the choroid plexus rather than liver-derived circulating TTR protein such that patisiran and inotersen are ineffective at preventing neurological disease progression which carries a poor prognosis. Patients being considered for CT with these particular disease-causing variants need careful consideration, highlighted by the poor outcome in patient 4 in our cohort. Whilst patisiran and inotersen specifically target the liver (30, 37), tafamidis has been shown to cross the blood brain barrier (37) and could conceivably stabilize TTR in the cerebrospinal fluid and thereby slow ongoing leptomeningeal ATTR amyloid deposition although this hypothesis remains speculative at the current time (6, 38).

Extracardiac ATTR amyloidosis is well-described in the literature (2). Whilst polyneuropathy, the hallmark phenotypic feature for which TTR gene silencers are licensed in ATTRv (4, 5), is unequivocally caused by amyloid deposits it remains uncertain to what extent amyloid deposits contribute to other common organ manifestations in patients with ATTR amyloidosis such as gastrointestinal disturbance, lumbar canal stenosis and joint pains despite their almost universal presence in the relevant tissue. A recent study reported development of extra-cardiac disease manifestations following CT in patients with ATTR-CM (39) which is entirely consistent with our findings in which two patients were diagnosed with amyloid polyneuropathy 210 and 6 months post CT. However, given the recent availability of TTR gene silencers that effectively halt the progression of ATTR-PN, we would argue that concerns surrounding the possible development of ATTR-PN post CT should not preclude suitable ATTR-CM patients from undergoing CT. With regular monitoring, symptoms of ATTR-PN can be detected at an early stage, and where indicated, TTR gene silencers can be introduced in a timely manner. The orthopedic symptoms which appear to be over-represented in patients with ATTR amyloidosis, such as lumbar canal stenosis and carpal tunnel syndrome, can be effectively alleviated by surgical or non-surgical intervention. Given that cardiac allograft function is typically preserved in ATTR-CM post-CT, such patients are usually able to tolerate general anesthesia from a cardiac perspective.

Our study has limitations. Pre-operative invasive physiological data is not readily available due to the fact transplantation was undertaken in institutions external to the individual amyloidosis centers. Whilst multicentre, the cohort is small and highly selected and we acknowledge the limitations of statistical analyses. Additionally, since disease-modifying therapies have only been available for the past 3–4 years, we are unable to report on outcomes with their long-term use following CT; however, the absence of amyloid recurrence within the cardiac allograft argues against the need for administration of such therapies to protect the cardiac allograft after CT in patients with ATTRwt-CM.

In conclusion, CT in selected patients with ATTR-CM is a robust intervention which restores quality of life and prolongs patient survival. The risk of cardiac allograft amyloid recurrence in the short and medium term appears to be negligible. Longer term follow-up studies will be required to determine whether administration of disease-modifying therapies post-CT provides any additional clinical benefit in patients with isolated ATTR-CM. The authors advise caution when considering the suitability of patients with ATTRv-CM for CT who carry TTR mutations which are known to be associated with important leptomeningeal amyloidosis.

Data availability statement

The anonymized raw data supporting the conclusions of this article can be made available by the authors upon reasonable request.

Author contributions

YR and AP conceived the study, carried out data collection, analysis, and manuscript preparation. CD carried out data collection and manuscript review. RP, AI, MR, AM, StL, LC, TR, SR, JaG, DR, IB, NK, DH, HL, AW, WM, SeL, CC, CW, LV, and AM-N contributed to data analysis and manuscript review and editing. MM, GS, UL, and PH contributed with data collection, manuscript preparation, and review. MF and JuG oversaw the study design, data collection, manuscript preparation, review, and final approval. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DH receives speaker fees from Akcea Therapeutics Ltd., and Alnylam UK. SeL receives support fees from Abiomed. MF has received consulting income from Intellia, Novo-Nordisk, Pfizer, Eidos, Prothena, Akcea, Alnylam, Caleum, Alexion, Jennsen and her institution has received clinical trial fundings from Pfizer, Eidos, and Alnylam. JuG receives consulting fees from Alnyman, Ionis, Eidos, Intellia, Pfizer, and ATTRalus.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.1075806/full#supplementary-material

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Stroke volume and myocardial contraction fraction in transthyretin amyloidosis cardiomyopathy: A systematic review

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Background: Cardiac amyloidosis (CA) is primarily a restrictive cardiomyopathy in which the impairment of diastolic function is dominant. Despite this, the left ventricular ejection fraction (LVEF) may be depressed in the late stage of the disease, but it poorly predicts prognosis in the earlier phases and does not represent well the pathophysiology of CA. Many echocardiographic parameters resulted important diagnostic and prognostic tools in patients with CA. Stroke volume (SV) and myocardial contraction fraction (MCF) may be obtained both with echocardiography and cardiac magnetic resonance (MRI). They reflect many factors intrinsically related to the pathophysiology of CA and are therefore potentially associated with symptoms and prognosis in CA.

Objectives: To collect and summarize the current evidence on SV and MCF and their clinical and prognostic role in transthyretin (TTR-CA).

Methods and results: We performed a systematic review following the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines. We searched the literature database for studies focusing on SV and MCF in patients with TTR-CA. We analysed the following databases: PUBMED, Cochrane Library, EMBASE, and Web of Science database. Fourteen studies were included in the review. Both SV and MCF have important prognostic implications and are related to mortality. Furthermore, SV is more related to symptoms than LVEF and predicts tolerability of beta-blocker therapy in TTR-CA. Finally, SV showed to be an excellent measure to suggest the presence of TTR-CA in patients with severe aortic stenosis.

Conclusion: Stroke volume and MCF are very informative parameters that should be routinely assessed during the standard echocardiographic examination of all patients with TTR-CA. They carry a prognostic role while being associated with patients' symptoms.

Systematic review registration: https://doi.org/10.17605/OSF.IO/ME7DS.

KEYWORDS

heart failure, amyloidosis, transthyretin, prognosis, stroke volume (SV), myocardial contraction fraction (MCF)

Introduction

Cardiac amyloidosis is caused by the progressive deposition of misfolded proteins, most commonly light chain (AL-CA) or transthyretin (TTR-CA) amyloid. This process disrupts the heart's structure and function, leading to heart failure (HF), reduced quality of life, and death.

Although been claimed to be a rare disease with an insidious presentation, the availability of new diagnostic tools (i.e., scintigraphy with bone tracer) and the increasing attention to the presence of echocardiographic "red flags" progressively increased the prevalence of the disease during the last decade (1, 2). AL-CA has an estimated annual incidence of 9.7–14.0 cases per million person-years in the United States, and autopsy studies revealed TTR-CA in 25% of subjects over 80–85 years old (3, 4).

The latest guidelines provide a classification of HF still based on LVEF, but this approach does not characterize the pathophysiology of restrictive cardiomyopathies (5). LVEF only describes the change in volumes during the cardiac cycle and is not a precise reflection of the antegrade flow developed during systole. In CA, amyloid deposition in the myocardium causes thickening of the ventricular wall and increased myocardial mass, which results in decreased compliance, diastolic dysfunction and raised filling pressures. Only in the late phases of the disease, with a massive expansion of extracellular volume (ECV), LVEF will decrease. Indeed, the disease progression is accompanied by a progressive impairment of systolic left ventricular function and a decrease in left ventricular diastolic volume, leading to a decline in stroke volume (SV) not necessarily associated with a decreased of LVEF. Recent studies have shown the predictive value of staging system based on biomarkers and several echocardiographic measurements of central cardiac function, but only few studies focused on SV (6–11).

The SV is a measure of ventricular performance that integrates many factors affecting the ventricle (preload, afterload, contractility, geometry), and that is also representative of the shortening and thickening of the myocardium (**Figure 1**); indeed, this parameter changes in the earlier stages of the disease (12). Moreover, a newer quantitative SV-derived marker of myocardial function, the myocardial contraction fraction (MCF), has been proposed by King et al. (13); MCF, defined as the ratio between the SV and the myocardial volume (MCF = SV/MV), is a more sophisticated volumetric measure of myocardial shortening which differentiates myocardial performance in similar degrees of hypertrophy.

This report aims to perform a systematic review, analysing the role of the SV and MCF in diagnosing, prognostic stratification, and managing of patients with TTR-CA.

Methods

We developed a systematic review following the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) amendment to the Quality of Reporting of Meta-analyses (QUOROM) statement. The protocol registration application for this study was performed in Open Science Framework (OSF) with the following doi: 10.17605/OSF.IO/ME7D. Two expert cardiologists (M.S., A.C.) independently and systematically searched PUBMED, Cochrane Library, EMBASE, and Web of Science database. The terms searched were: (amyloid*) AND [(transthyretin) OR (TTR)] AND [(echo) OR (stroke) OR (SV) OR (SVi) OR (stroke index) OR (cardiac output)]. The research was carried out in April 2022. Only original articles published in peer-reviewed journals were selected. The shortlisted studies were retrieved as full articles and appraised independently by two unblinded reviewers (A.C. and M.S.), with divergences solved after consensus, according to the following inclusion criteria: (i) English language; (ii) reporting data on echocardiographic-derived or CMR-derived SV/SVi and/or MCF; (iii) involving patients with TTR-related cardiac amyloidosis (wtTTR-CA, vTTR-CA), (iv) data published in peer-reviewed journal. SV is defined as the volume of blood pumped out of the left ventricle during each systolic cardiac contraction. It can be calculated by a doppler-derived method (representing specifically the antegrade SV) or as the difference between end-diastolic volume (EDV) and end-systolic volume (ESV). MCF is defined as the ratio of SV to myocardial volume (MV). Myocardial volume is generally calculated as the LV mass divided by the mean density of the myocardium (1.04 g/ml).

Exclusion criteria for this study were: (i) duplicate reports, (ii) gray literature; (iii) only abstract or posters; (iv) review or case report/series; (v) editorials. Outcomes of interest were diagnostic, prognostic and clinically meaningful findings correlated to SV/SVi and MCF in patients with TTR-CA. In particular, the aim of this systematic review is to describe available evidence relating SV/SVi and MCF to (i) symptoms, (ii) differential diagnosis and (iii) prognosis.

Results

Results of the search strategy

Overall, 643 citations were obtained. After the first screening, 600 records were excluded because they were out of the field of interest; the remaining 43 records were further examined. Of these, 16 were excluded with reasons (15 = duplicates, 1 = only abstract). Finally, of the 27 studies examined as full-text, 13 were excluded because they did not report any outcome of interest (**Figure 2**). Fourteen studies were finally included in the review (12, 14–26). Three studies provided data on the relation between symptom and SV (18, 19, 26). Three focused on the prognostic role of SV (14, 15, 20) and three on the prognostic role of MCF (12, 16, 17, 21). Only one study addressed the implication of the use of neuro-hormonal antagonists (i.e., beta-blockers) in CA patients, according to SV (24). Two study showed the diagnostic usefulness of SV in patients with aortic stenosis (AS) and CA (22, 23).

One compared right heart catheterization-derived (RHC) SV with doppler-derived SV in patients with CA (25).

Five studies out of 11 used the pulsed wave doppler measurement of LVOT velocities and LVOT diameter measurements (20, 23, 25, 26). Six studies calculated SV by linear left ventricular (LV) dimension measured by M-mode. One study used a bioimpedentiometry technique (18). One study used a doppler-derived SV for MCF calculation while six used SV calculated as difference between EDV and ESV (23). **Table 1** summarizes the main findings of each study and the methods applied for SV and MCF calculation.

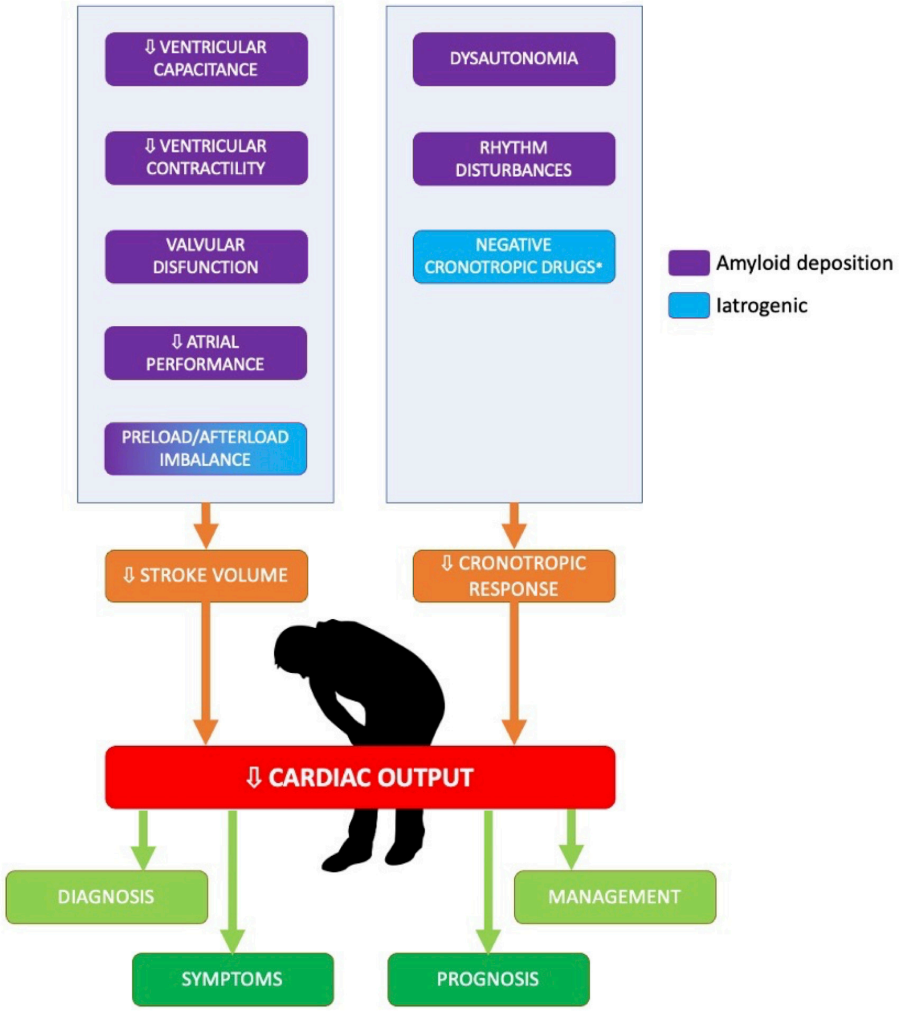

FIGURE 1
Determinants of low stroke volume and low cardiac output in patients with cardiac amyloidosis. The figure summarized the main determinants of low cardiac output in patients with cardiac amyloidosis.

Discussion

Stroke volume and myocardial contraction fraction assessment

Although there are no specific guidelines for SV assessment in patients with HF, echocardiographic recommendation for aortic stenosis grading suggest to deriving SV by the pulsed-wave doppler measurement of LVOT velocities and LVOT diameter measurements (27). This method was applied in 5 out of 11 studies (20, 23, 25, 26), one study used a bioimpedentiometry technique (18), and the remaining six studies calculated SV by the linear left ventricular (LV) dimensions measured by M-mode echocardiography (12, 14, 15, 21, 22, 24).

While the doppler-derived estimates are more representative of the real antegrade flow through the aortic valve during the cardiac cycle, others estimate based solely on the difference between EDV and ESV are representative of both antegrade and retrograde SV, and therefore are more influenced by the presence of significant mitral regurgitation. Notably, Granstam et al. (25) found RHC SV to be comparable to doppler-derived SV assessment in patients with CA: cardiac output (CO) and cardiac index (CI) assessed by RHC were both reduced in patients with amyloidosis [4.3 (3.3–6.7) L/min and 2.2 (1.0–3.8) L/min/m^2], and the calculated flows comparable to those obtained with echocardiography. At the same time, SV was similarly slightly reduced in both catheterization and echocardiography estimates [66 (51–89) and 65 (25–125) mL, respectively].

Interestingly, only one study out of seven examining MCF, used a doppler-derived SV for its calculation (12). The remaining six studies computed SV as the difference between EDV and ESV.

Stroke volume and symptoms

Figure 3 shows the mean baseline SVi (or SV if SVi was not reported) and LVEF values extrapolated from the selected studies population. While mean LVEF was generally preserved or slightly reduced, mean SV was significantly lower than typical reference values in most of the study populations. Starting from the assumption that the baseline low SV and its reduced reserve during exercise are among the main determinants of the reduced exercise tolerance of patients with CA, Clemmensen et al. (26) tried to evaluate the link between impaired exercise capacity and hemodynamic alterations during functional stress in patients with CA. Patients

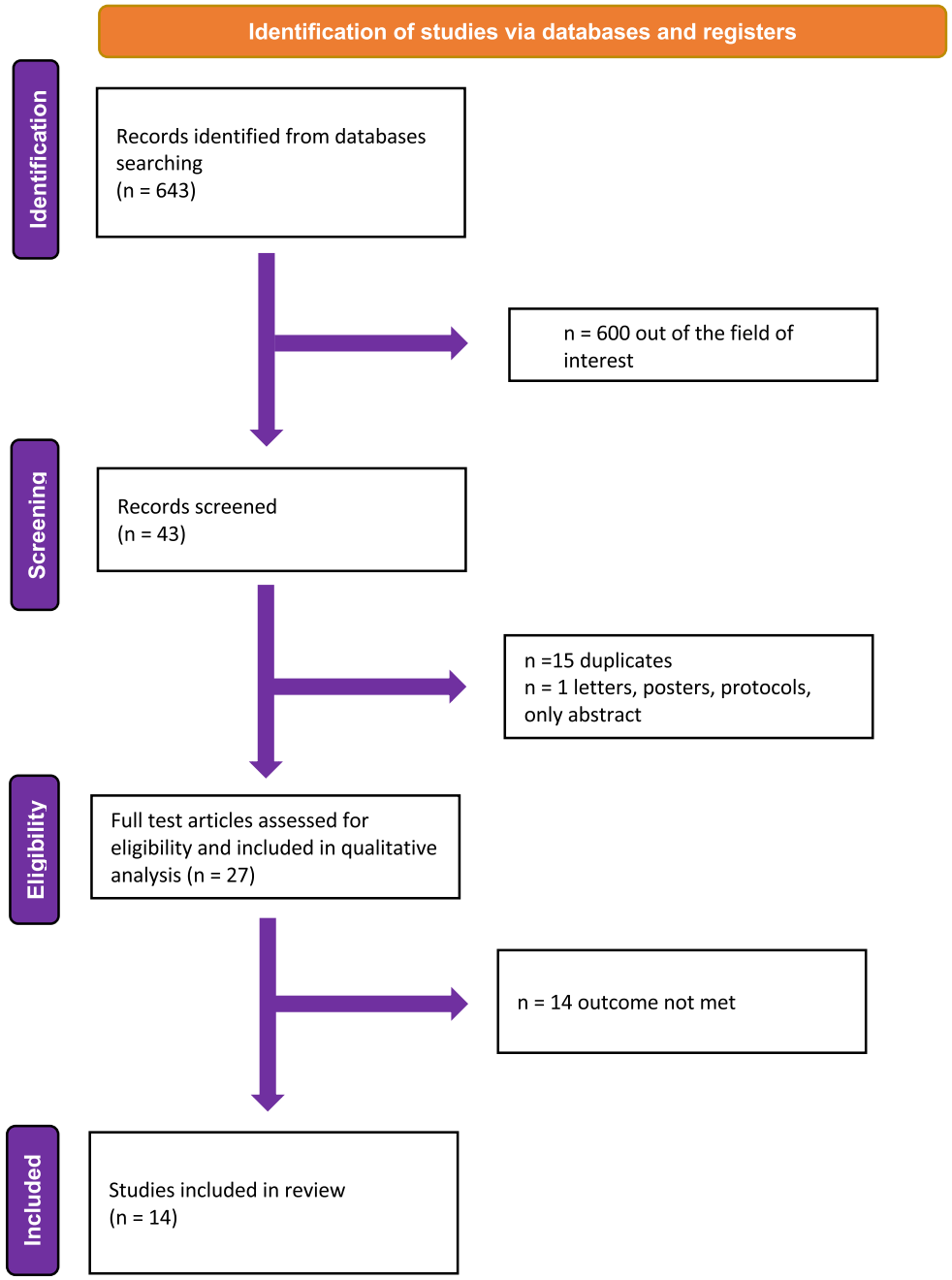

FIGURE 2
Study flow diagram.

with CA usually develop symptoms with physical activity because of rising filling pressures, which are necessary to maintain adequate SV. The authors studied 44 subjects, 24 with confirmed CA and 20 without CA (control group). The first group comprised wtTTR-CA (n = 10), vATTR-CA (n = 5) and AL-CA (n = 9) patients. CA patients had reduced CI ($P < 0.01$) due to severely reduced SVi. They also presented lower VO^2 max (normalized per body weight) than controls (15 ± 6 vs. 33 ± 7 mL/min/kg; $P < 0.001$) and had a severely reduced inotropic myocardial reserve.

Starting from the previous finding of a lower rate of oxygen consumption at peak exercise (peak VO^2) in wtTTR-CA, vTTR-CA, and AL-CA, Monfort et al. (18) performed exercise testing with oxygen consumption measurement and SV measurement by bioimpedentiometry in African-American patients with vATTR-CA. At peak exercise, CI increased by approximately 2-fold compared to a 3-fold increase in age-matched controls. All patients were not receiving beta-blocking therapy, and the two groups reached similar peak HR. Furthermore, HR recovery expressed as a percent decrease in peak HR at 1 and 3 min post-exercise was blunted in vATTR-CA patients compared with the control group.

Finally, in the study by Arenja et al. (19), a significant correlation was found between MCF and NYHA class, and therefore with symptoms. MCF was significantly reduced according to the increase in NYHA class in a cohort of CA patients (19). The same correlation was not present between LVEF and NYHA classes.

TABLE 1 Summary results of the selected studies.

References	Parameters	N°	Summary of the study finding	Overview of univariate/multivariate regression
Castano et al. (22)	SV*, MCF*	151 -84% no CA -16% TTR-CA	Significant univariate predictors of ATTR-CA included SVI <35 ml/m^2 and a decreased MCF, but in multivariable logistic regression only average mitral annular S' remained significantly associated with ATTR-CA.	**Predictors of ATTR-CA (univariate analysis).** SVi < 35 ml/m^2: OR 4.53, 95% CI 1.68–12.21; $P = 0.003$. MCF: OR for 1% unit decrease 1.10, 95% CI 1.05–1.15; $P < 0.001$.
Tendler et al. (16)	MCF*	66 -27% wtTTR-CA -21% vTTR-CA -52% AL-CA	There was no significant difference in LVEF between patients who survived the study period and those who died, while there was a significant difference in MCF. At the univariate analysis MCF, as a continuous parameter, was significantly associated with death while LVEF was not, and at the multivariate analysis, an MCF <30 was an independent risk predictor of death, driven by a higher risk in AL-CA subjects than ATTR-CA.	**Predictors of death (univariate analysis).** MCF: HR for each 1% increase 0.972, 95% CI 0.947–0.998; $P = 0.035$. **Predictors of death (multivariate analysis).** MCF < 30%: HR 2.841, 95% CI 1.214–6.648; $P = 0.016$.
Aimo et al. (24)	SV*	99 -64% wtTTR-CA -3% vTTR-CA -33% AL-CA	AL amyloidosis, reduced function of left heart (lower SV and FE) and right heart function (TAPSE) were predictors of adverse events during beta-blocker therapy and were associated with HF hospitalization; lower systolic blood pressure predicted need for dose reduction.	**Predictors of cardiovascular events or need for dose reduction during BB therapy (univariate analysis).** SV: negatively associated with events ($P = 0.036$). **Predictors of HF hospitalization in patients started on BB (univariate analysis).** SV: negatively associated with events ($P = 0.027$). CO: negatively associated with events ($P = 0.017$).
Monfort et al. (18)	SV	33 -45% no CA -55% vTTR-CA	At CPET, ATTRv-CA patients had reduced changes (relative to increase in VO^2) in CI and SV compared with controls (suggesting a poor inotropic myocardial reserve).	**Peak SV index,% change from rest.** vTTR-CA vs. Controls: 18.0% ± 6.5 vs. 30.5% ± 5.9 $P < 0.0001$. **Peak cardiac index,% change from rest.** vTTR-CA vs. Controls: 92% ± 31 vs. 144% ± 22; $P < 0.0001$.
Ruberg et al. (20)	SV	29 -62% wtTTR-CA -38% vTTR-CA	Statistically significant univariate predictors of mortality for the entire cohort at baseline were disease duration, HR > 70, baseline SV, LVEF < 50%, presence of V122L mutation.	**Predictor of death (univariate analysis).** SV: HR for 1 ml increase 0.96, 95% CI 0.92–1.00; $P = 0.05$.
Bhuiyan et al. (14)	SV*	29 -62% wtTTR-CA -38% vTTR-CA	At multivariable survival analysis, baseline LVEF <50% was associated with increased mortality. Declines in LVEF were lower than decrease in SV; declines in LVEF were strongly correlated with declines in SV, but not with declines in end-diastolic volume.	**Correlation analysis.** SV correlation with LVEF: r = 0.769, $P = 0.0093$. EDV correlation with LVEF: r = –0.306, $P = 0.389$.
Siepen et al. (21)	SV*, MCF*	191 -100% wtTTR-CA	LVEF, SVi, and MCF weren't predictors of mortality.	**Predictor of death (univariate analysis).** SVi (ml/m^2) c-statistics = 0.429. MCF (%) c-statistics = 0.383.
Arenja et al. (19)	MCF*	330 -30% control -24% wtTTR-CA -8% vTTR-CA -24% AL-CA -18% HCM -12% IHD	In HF, MCF discriminates CA from other forms of LVH (better than LVEF) and comparable to LVMI in discriminating LVH from controls. Cut-off value for MCF <50% and for LVEF <60% could best identify patients with a high probability for CA.	**Discrimination between HCM and CA.** Diagnostic performance of MCF for discriminating AL from HCM: AUC 0.84, $P < 0.0001$).
Rubin et al. (17)	MCF*	530 -30% wtTTR-CA -70% vTTR-CA	Most of the patients who died during follow-up had a lower value of MCF and a lower mean MCF at baseline compared to survivors. LVEF was lower at baseline in those who died, but still in the normal range in both cohorts. At univariate analysis, MCF <25% had a greater predictive value for mortality than EF <50%. At multivariate analysis MCF <25% was independently associated with a greater risk of death.	**Predictor of death (univariate analysis).** MCF < 25%: HR 8.46, 95% CI 4.8–14.9, $P < 0.0001$. **Predictor of death (multivariate analysis).** MCF < 25%: HR 5.37, 95% CI 1.82–15.9, $P = 0.0024$.
Nitsche et al. (23)	SV, MCF	191 -8% wtTTR-CA -1% AL-CA -91% no CA	The usefulness of SVi for the detection of CA-AS was tested; while GLS did not reliably differentiate AS from CA-AS, SVi showed good discriminative power by ROC analysis (0.77, 95% CI 0.69–0.86; $P < 0.002$), comparable to extracellular volume by CMR. SVi was also associated with CA at univariate logistic regression analysis and at multivariate analysis.	**Prediction of cardiac amyloidosis (univariate analysis).** OR for SVi increase 0.21, 95% CI 0.08–0.56; $P = 0.002$. **Prediction of cardiac amyloidosis (multivariate analysis).** OR for SVi increase 0.30, 95% CI 0.10–0.87; $P = 0.027$.

(Continued)

TABLE 1 (Continued)

References	Parameters	N°	Summary of the study finding	Overview of univariate/multivariate regression
Clemmensen et al. (26)	SV	44 -23 wtTTR-CA -11% vTTR-CA -20% AL-CA -45% no CA	CA patients had reduced CI ($P < 0.01$) as a result of severely reduced SVi. They also presented lower VO^2 than controls (15 ± 6 vs. 33 ± 7 mL/min/kg bwt; $P < 0.0001$). Furthermore, CA patients had a severely reduced inotropic myocardial reserve. Only small exercise-induced increases in left ventricular stroke work index (LVSWI) and preload-adjusted left ventricular stroke work (LV-PASW) were seen in CA patients. The poor LVSWI and LV-PASW reserve was mainly attributable to only a small increase in SV during exercise.	**Increase in SVi during exercise (controls vs CA patients).** ΔSVi: 4 mL/m^2 (range: −1 to 8) vs. 14 mL/m^2 (range: 5– 25); $P < 0.0001$. **Increase in CI during exercise (controls vs CA patients).** ΔCI: 2 ± 2 vs. 7 ± 2 L/min; $P < 0.0001$.
Granstam et al. (25)	SV	14 -36% wtTTR-CA -64% AL-CA	Assessment of echocardiographic-derived SV is feasible and comparable to RHC-derived SV in patients with CA.	**SVi estimation comparison (echo Doppler-derived vs right heart catheterization).** SV was similarly slightly reduced in both catheterization (66 mL, IQR 51–89) and echocardiographic assessment (65 mL, IQR 25–125).
Chacko et al. (15)	Svi*, MCF*	1,240 -62% wtTTR-CA -38% vTTR-CA	SVi, right atrial area index, LS and severe AS were independently associated with patient survival in the overall population; E/e' was associated with survival if severe AS patients were excluded. LS, SVi, and severe AS remained independently associated with survival also after adjustment for NYHA class and for NAC staging system (eGFR and NT-proBNP).	**Risk of death (univariate analysis).** SV: HR for 1 ml increase 0.95 (95% CI 0.93–0.96).
Knight et al. (12)	SV*, MCF*	322 -35% wtTTR-CA -23% vTTR-CA -41% AL-CA	At the univariable analysis, SVi and MCF were predictive of mortality. At multivariable Cox model analysis adjusted for age and sex, SVi remained independently predictive of mortality while in a multivariable model, the only parameter that remains independently predictive of mortality was TAPSE.	**Risk of death (univariate analysis).** SVi: HR for 5 ml decrement 1.40, 95% CI 1.24–1.57; $P < 0.001$. MCF: HR for 10% decrement 1.55, 95% CI 1.32–1.81. **Risk of death (multivariate analysis).** SVi: HR for each 5 ml/m^2 decrement 1.24; 95% CI 1.04–1.48, $P = 0.019$. MCF: HR for each 10% decrement 1.25; 95% CI 1.00–1.57, $P = 0.053$.

SV* and MCF*, in these studies the estimation of stroke volume and MCF was from linear left ventricular (LV) dimensions measured by M-mode echocardiography; AL-CA, light chain cardiac amyloidosis, AS, aortic stenosis; ATTR-CA, transtiretin cardiac amyloidosis; CI, cardiac index; CO, cardiac output; CPET, cardio-pulmonary exercise test; EDV, end-diastolic volume; eGFR, estimated glomerular filtration rate; ESV, end-systolic volume; HCM, hypertrophic cardiomyopathy; HR, heart rate; IHD, ischemic heart disease; LS, longitudinal strain; LV-PASW, left ventricular pressure-adjusted stroke work; LVEF, left ventricular ejection fraction; LVOT, left ventricular outflow tract; LVSWI, left ventricular stroke work index; MCF, myocardial contraction fraction; MV, myocardial volume; NT-proBNP, N-terminal pro-brain natriuretic peptide; RHC, right heart catheterization; SV, stroke volume; TAPSE, transannular plane excursion; vTTR, variant TTR; wtTTR, wild type TTR.

To summarize these findings, patients with amyloidosis presents with low SV and low CI despite usually normal LVEF. They also have low SV and CI reserve. These parameters are linked to reduce peak exercise VO^2 and exercise tolerance. Consistently, also the MCF is significantly reduced according to the increase in NYHA class.

Prognostic stratification

Stroke volume

Recent studies have shown the predictive value of several echocardiographic features, such as LVEF, average strain rate, E/e', TAPSE, and SVi (10, 11). In 2011, Bhuiyan et al. (14) wanted to evaluate the end-diastolic pressure-volume relation and other pressure-volume indices in patients with TTR-CA to determine how these indices change over time and whether abnormal pressure-volume relations and indices of pump function were associated with reduced survival. They studied 29 patients with TTR-CA (both wtTTR-CA and vTTR-CA forms) over 18 months, and found that, at multivariable survival analysis, initial LVEF <50% was associated with increased mortality (HR 6.6, 95% CI 1.1–40.3). They also found that declines in LVEF were of a lower magnitude than the decrease in SV because of concomitant reductions in EDV over time. In fact, declines in LVEF were strongly correlated with declines in SV (r = 0.769, $P = 0.0093$), but not with declines in EDV (r = –0.306, $P = 0.389$). This shows that SVi changes in the earlier stages disease, and it might be an early predictor of a decrement in LVEF and, consequently, of the patient's outcome.

One year later, Ruberg et al. (20) tried to find clinical, echocardiographic, or biochemical baseline parameters that could predict the course of the disease, examining 29 patients with TTR-CA (11 vTTR-CA and 18 wtTTR-CA). They showed for the first time that SV could be a useful tool for the prognostic stratification of CA. Indeed, they found SV to be a predictor of death at univariate analysis (HR 0.96 for each ml increase, 95% CI 0.92–1.00, *P*-value 0.05). In 2020, Chacko et al. (15) studied a larger sample of cases with more than 1,000 patients with TTR-CA from the National Amyloidosis Center (NAC) of London (62% of patients had wtATTR-CA, 25% had V122l-associated vTTR-CA, 10% had T60A-associated vTTR-CA, and 3% had non-V122I non-T60A-associated vATTR-CA). In this study SVi, right atrial area index, longitudinal strain and severe aortic stenosis (AS) were independently associated with patient survival in the overall population after adjustment for NYHA class and a validated staging system (including eGFR and NT-proBNP), highlighting their independent prognostic role for survival prediction. Interestingly, this study also showed different degrees of disease severity across the different genotypes: compared to wtTTR-CA, patients with V122I mutation had similar increases in LV wall thickness but significantly lower indices of LV function (including SVi, LVEF, and MCF). Notably, in V122I patients, SVi resulted as a weaker predictor of mortality than wtTTR-CA. One possible explanation could be that in the study, SVi was calculated as the difference of VTD and VTS indexed to BSA and not with a PW-doppler approach; therefore, a possible overestimation of SVi could

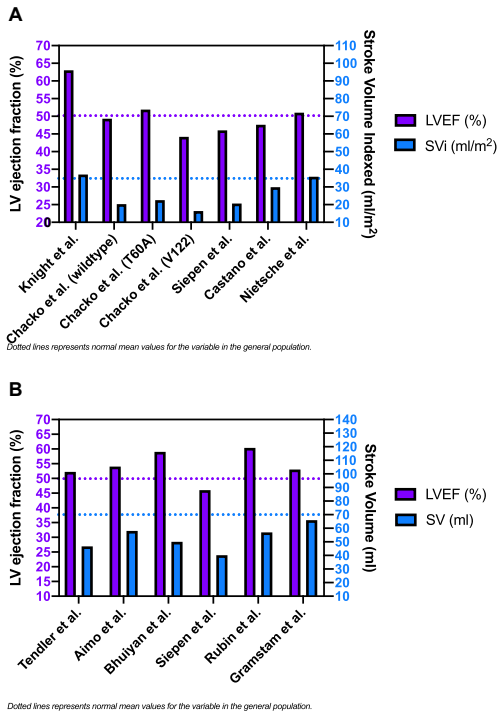

FIGURE 3
Baseline left ventricular ejection fraction, stroke volume index [panel **(A)**] and stroke volume [panel **(B)**] in patients with cardiac amyloidosis.

have happened in those with moderate-to-severe mitral regurgitation, negatively affecting the predictive value of this parameter in vTTR-CA. Significant MR was indeed more frequent in patients V122I vTTR-CA versus wtTTR-CA patients (40.7 vs. 27.1%).

Interestingly, following this publication from the NAC of London, Rosenblum et al. (28) performed an analysis of the echocardiographic-derived pressure-volume loops of the same cohort. All patients with ATTR-CA demonstrated impaired diastolic properties with leftward shifted end-diastolic pressure relationship (EDPVR), especially for those with V122I variant, which presented the lowest chamber function and stroke volume (28).

Myocardial contraction fraction

Myocardial contraction fraction might give important prognostic information for CA patients. The progressive amyloid deposition in the myocardium causes an increase in left ventricular MV and a decline in SV with a deterioration of the ventricular function and, therefore, a decline in MCF (17). The first to study the predictive power of MCF in cardiac amyloidosis were Tendler et al. (16) in 2014. They studied a small population of 66 patients with AL-CA and TTR-CA, hypothesizing that MCF would be superior to LVEF in predicting survival among patients with CA. Interestingly they did not find a significant difference in LVEF between patients who survived the study period and those who died, while they found a significant difference in MCF. At the univariate analysis, MCF, as a continuous parameter, was significantly associated with death while LVEF was not, and at the multivariate analysis, an MCF <30% was an independent risk predictor of mortality, driven by a higher risk in AL subjects than ATTR amyloidosis. MCF did not differ between patients with AL and ATTR amyloid, even though subjects with TTR-CA had a larger increase in MV than subjects with AL, corroborating the hypothesis of a direct detrimental effect of light chains on myocardial function. The direct effects of light chains on cardiac performance have already been demonstrated before, but this data highlights the MCF power to measure myocardial contractility and the consequences of amyloid infiltration on myocardial performance, regardless of the different mechanisms by which this occurs. After this study, the interest in MCF grew, as it seemed to be a revolutionary parameter capable of prognostically stratifying the patient with CA more subtly and completely. It was, therefore, unexpected when Siepen et al. (21), in 2017, published their study with the intent to analyse clinical predictors of mortality in 191 patients with TTR-CA and showed that both SV and MCF were not significantly correlated with survival. It is essential to notice that Siepen's study population was bigger than the Tendler's, but with a limited number of fatal events and little statistical power. Furthermore, this study did not use the doppler-derived method for SV calculation.

Two other studies analysing this parameter were published in less than a year to clarify its role. Knight et al. (12) studied 322 patients and analysed 11 commonly measured (at CMR and echocardiography) structural and functional cardiac parameters, which were categorized into three groups, according to their likelihood of being abnormal across the degree of myocardial infiltration (low burden/intermediate/high burden variables) (12). Cardiac amyloidosis burden was quantified using CMR-derived extracellular volume. In the univariate analysis, the SVi, and MCF were predictive of mortality. In multivariate regression SVi was an independent predictor of mortality (HR for each 5 ml/m^2 decrement 1.24; 95% CI 1.04–1.48, $P = 0.019$), and in the model including MCF, this last one did not reach statistical significance for a few points (HR for each 10% decrement 1.25; 95% CI 1.00–1.57, $P = 0.053$).

Finally, Rubin et al. (17) published a study with the same Tendler's hypothesis (that MCF could be a better predictor of survival than LVEF) but with a larger population counting 530 patients, all presenting TTR-CA. They found that most of the patients who died during follow-up had a lower value of MCF and a lower mean MCF at baseline versus those who did not. The LVEF was lower at baseline in those who died but still in the normal range in both cohorts. In multivariate analysis, MCF <25% was independently associated with a greater risk of death. Therefore, the prognostic role of this parameter seems to have been confirmed. Still, it is crucial to notice that, in all these studies, MCF has been calculated using LV mass and volumes not directly measured and consequently subjected to error. It is undoubtedly attractive that MCF, even if measured with the simplest method, can predict adverse outcomes, but studies analysing actual volumetric chamber data are lacking.

Stroke volume in patients treated with beta-blockers

Except for tafamidis, which is currently the only disease modifying treatment available for cardiac amyloidosis, most of the medical management of CA patients is based on treatment of its complications (e.g., hemodynamic deterioration, arrhythmias, and systemic embolism). On this regard, the systematic use of neurohormonal antagonist in the setting of CA is still debated. Specifically, beta-blockers are perceived to be poorly tolerated or contraindicated in the setting of CA because of the fear of hypotension, conduction disturbances or impossibility of

adequately increasing CO, especially because of the typical restrictive pathophysiology observed in these patients. In the observational study of Aimo et al. (24) patients started on a beta-blocker (56%) did not show a higher frequency of hypotension ($p = 0.97$), fatigue ($p = 0.83$), syncope ($p = 0.13$), symptomatic bradycardia ($p = 0.65$), need for pacemaker implantation ($p = 0.51$), or HF hospitalization ($p = 0.59$) compared to the others. On the other hand, in this study, SV ($p = 0.027$) ad CO ($p = 0.017$) resulted predictors of HF, while CO was predictive of syncope in patients treated with beta-blockers (24). These findings show that in CA patients treated with beta-blockers, SV and especially CO are related to symptoms, and the use of rate-limiting drugs should be carefully evaluated on a tailored base.

Diagnostic role of SV and MCF in patients with coexistent AS or unexplained LV hypertrophy

Stroke index and MCF might be useful tools to raise the diagnostic suspicion of CA also in patients with hypertrophy. It is estimated that almost 15% of the AS population and 30% of the subset with "low-flow low-gradient" pattern may have CA (29). In these patients, significant myocardial thickening is naturally attributed to long-standing pressure overload and is recognized as a potential sign of a storage disease. Coexisting CA and AS has been associated with worse outcomes (22, 23). Castano et al. (22) used 99mTc-PYP scintigraphy to examine 151 elderly patients with severe symptomatic AS undergoing TAVR, and they found a prevalence of TTR-CA of 16%, and a greater percentage of this group had low-flow low-gradient AS. In this study, Castano proposed an evaluation model consisting of echocardiographic parameters comprising s', SVi, and MCF to select patients with TTR-CA and, consequently, refer for a 99mTc-PYP amyloid scan before TAVR. Using logistic regression models, significant univariate predictors of TTR-CA included a SVi <35 ml/m^2 (OR 4.53, 95% CI 1.68–12.21; $P = 0.003$) and a decreased MCF (OR for 1% unit decrease 1.10, 95% CI 1.05–1.15; $P < 0.001$). Nitsche et al. studied 191 consecutive patients with AS scheduled for TAVR. The 81.7% of this population underwent complete standardized assessment (echocardiography, ECG, CMR, 99mTc-PYP, serum and urine free light chain measurement, biopsy in AL) (23). The authors tested SVi for the detection of CA. While longitudinal strain did not reliably differentiate AS from CA-AS, SVi showed good discriminative power by ROC analysis, comparable to extracellular volume by CMR. SVi was also associated with CA by univariate logistic regression analysis (OR 0.21, 95% CI 0.08–0.56; $P = 0.002$) and by multivariate analysis (OR 0.30, 95% CI 0.10–0.87; $P = 0.027$).

In 2017, Arenja et al. (19) studied with CMR 230 patients with left ventricular hypertrophy (LVH), including 132 patients with a confirmed diagnosis of CA [AL-CA ($n = 80$), vTTR-CA ($n = 27$), wtTTR-CA ($n = 25$)], 60 with hypertrophic cardiomyopathy and 38 with hypertensive heart disease (HHD). The mean value of MCF was reduced in all groups (HCM, 80.0 ± 20.3%; TTR-CA, 74.9 ± 32.2%; HHD 92.6 ± 20%; with $P < 0.05$ for all), and the lowest MCF value was in patients with AL-CA (50.5 ± 20%, $P < 0.05$ vs. all other groups).

Myocardial contraction fraction outperformed LVEF and left ventricular mass index (LVMI) in discriminating between different etiologies of LVH and between AL-CA and other forms of LVH (AUC = 0.84, $P < 0.001$). Moreover, cut-off values for MCF $< 50\%$ and LVEF $< 60\%$ allowed for identifying patients with a high probability of CA. This higher ability of MCF to discriminate AL-CA from other forms of LVH can be explained by a higher grade of LV geometric deformation or a greater level of contractility dysfunction in AL-CA, with an increase in LV mass and a decrease in end-diastolic LV volume that appears more pronounced than in other forms of LVH.

Conclusion

The findings of this systematic review highlight the role of SV and MCF in the diagnosis and prognostic stratification of patients with CA. Being the results of the several factors, SV and MCF should be considered very informative parameters to be routinely assessed during a standard echocardiographic examination of all patients with TTR-CA. They carry both a diagnostic and a prognostic role while being associated with patients' symptoms. With the advance and availability of disease-modifying treatment for TTR-CA, they may also emerge as possible parameters to evaluate disease progression and response to treatments. This should be confirmed in further exploratory studies. It is essential to notice that discrepancies between some trials may be partly explained by the different methods used to estimate SV, which was not performed by a doppler-derived technique in most studies. Finally, data correlating SV and MCF with heart failure hospitalization are lacking and should be investigated further.

Data availability statement

The original contributions presented in this study are included in this article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SM and CA conceived and design, systematic literature research, and critical writing and revising the intellectual content. SF, MD, FG, DB, PR, TE, PG, and CG revised the intellectual content. RC conceived and design. All authors contributed to the article and final approval of the version to be published.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewers DT and GS declared a past co-authorship with the authors PR and RC to the handling editor.

Publisher's note

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Screening approaches to cardiac amyloidosis in different clinical settings: Current practice and future perspectives

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Cardiac amyloidosis is a serious and progressive infiltrative disease caused by the deposition of amyloid fibrils in the heart. In the last years, a significant increase in the diagnosis rate has been observed owing to a greater awareness of its broad clinical presentation. Cardiac amyloidosis is frequently associated to specific clinical and instrumental features, so called "red flags", and it appears to occur more commonly in particular clinical settings such as multidistrict orthopedic conditions, aortic valve stenosis, heart failure with preserved or mildly reduced ejection fraction, arrhythmias, plasma cell disorders. Multimodality approach and new developed techniques such PET fluorine tracers or artificial intelligence may contribute to strike up extensive screening programs for an early recognition of the disease.

KEYWORDS

cardiac amyloidosis, heart failure, ATTR, AL, screening, aortic stenosis, nuclear medicine

1. Introduction

Cardiac amyloidosis (CA) is an infiltrative cardiomyopathy characterized by extracellular deposition of an amorphous substance called amyloid, whose formation is secondary to misfolding of different precursors proteins. The most frequent forms of CA are light-chain (AL) and transthyretin-related (ATTR) amyloidosis that can be classified in hereditary (ATTRv) or wild-type (ATTRwt) depending on whether a mutation of TTR gene has been identified (1). Although CA has been historically considered a rare condition, recent advances on medical education and global awareness have conducted to an exponential increase of prevalence of the disease (2). ATTR and AL amyloidosis may

Abbreviations
AI, artificial intelligence; AL, light chain amyloidosis; AS, aortic stenosis; ATTR, transthyretin-related amyloidosis; ATTRv, variant transthyretin-related amyloidosis; ATTRwt, wild-type transthyretin-related amyloidosis; CA, cardiac amyloidosis; CMR, cardiac magnetic resonance; CT, computed tomography; CTR, carpal tunnel release; CTS, carpal tunnel syndrome; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; LV, left ventricle; PET, positron emission tomography; SUV, standardized uptake values.

share some clinical aspects although organ tropism is generally different (Figure 1). CA may simulate heterogenous cardiac conditions, therefore specific clinical and instrumental features, so called "red flags" (listed in Table 1), have been identified over the years to help physicians to reach a definite diagnosis. The aim of this review was to describe the most common clinical settings in which diagnosis of CA may arise and to examine little-explored fields of interest about CA screening.

TABLE 1 Main red flags related to amyloidosis.

Extracardiac	Cardiac
Polyneuropathy	Low QRS voltage compared to LV thickness
Macroglossia	Pseudo-infarction ECG pattern
Bilateral carpal tunnel syndrome	AV conduction disease
Lumbar spinal stenosis	Granular sparkling
Renal insufficiency, proteinuria	Pericardial effusion
Vitreous deposits	Reduced longitudinal strain with apical sparing
Family history	Increased ECV, elevated T1 values

2. Common clinical settings leading to CA diagnosis

2.1. CA and orthopedic conditions

It is common knowledge that in patients with ATTR-CA the deposition of amyloid can be observed in soft tissue structures (i.e., ligaments) in addition to myocardial tissue, leading to carpal tunnel syndrome, lumbar spinal stenosis (3, 4), or biceps tendon rupture (5). Among these conditions, carpal tunnel syndrome (CTS) is the most frequent in ATTR-CA population (6). The underlying pathophysiology is still unclear. It has been suggested that repeated mechanical stimuli on ligaments and myocardium could favor amyloid deposition, but there is no such scientific evidence. An Italian study found that CTS history was present in 14% of ATTRv and 25% of ATTRwt patients, significantly higher compared to general population (4.1%). CTS was often bilateral, and it usually occured 5 to 9 years prior to CA diagnosis. In AL patients, no signs of increased incidence of CTS were observed (7). In the last years, researchers have been developing many strategies for earlier disease detection, including carpal tunnel release (CTR) specimens' analysis. For instance, in a cohort of 98 adults undergoing CTR, 10 patients were found to have a positive tenosynovial biopsy for amyloid presence (7 ATTR, 2 AL and 1 untyped) after Congo red staining. Only two patients had cardiac involvement (1 AL amyloidosis and 1 ATTR) at the time of screening for CA, for an overall yield of 2%. To note, only those with a positive tenosynovial biopsy underwent a screening for CA, and this could explain the low prevalence of cardiac involvement in this population (8). In 2019, Fosbøl et al. attempted to esteem the risk of future amyloidosis associated with CTS using a Danish nationwide registry of patients who underwent CTR surgery and comparing them to a sex- and age-matched cohort from the general population. They found that the absolute incidence of future diagnosed amyloidosis among CTS patients is low, but significantly higher compared to general population (cumulative incidence of 0.10% by 10 years in the CTS group vs. 0.006% among control subjects). Also, CTS population showed a higher risk of heart failure (HF), with a hazard of 1.5 times that of control subjects and a cumulative incidence of 4% at 10 years. However, in this study the association of CTS with heart failure did not negatively affect the early mortality when compared to a HF population without CTS (9). These results have been recently confirmed in a Danish study (10) which evaluated the association of previous CTS surgery and cardiovascular outcomes in patients who underwent permanent pacemaker implantation. In fact, authors found that previous CTS surgery was associated with increased risk of new-onset HF and increased risk of diagnosed amyloidosis after pacemaker implantation. Also, no association between previous CTS surgery and increased

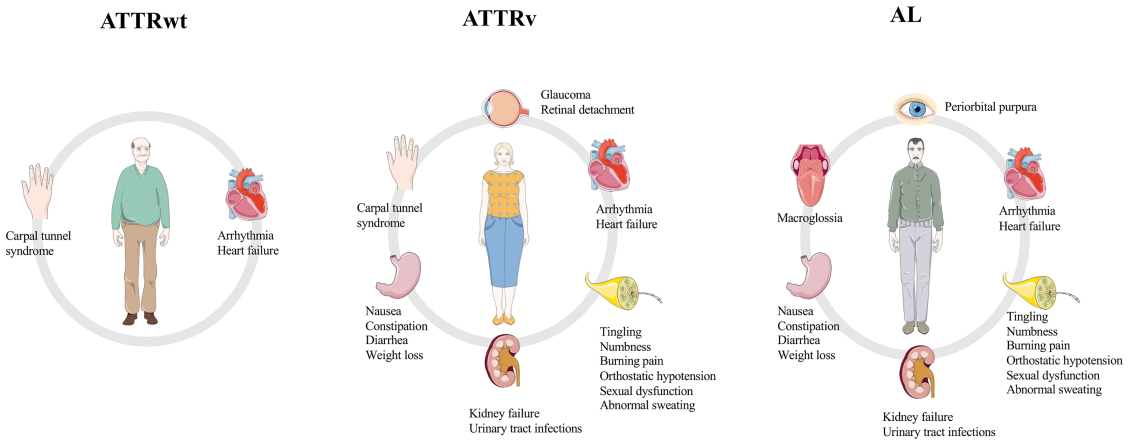

FIGURE 1
Major manifestations of the three most common subtypes of amyloidosis. ATTRwt, wild-type transthyretin-related amyloidosis; ATTRv, variant transthyretin-related amyloidosis; AL, light-chain amyloidosis. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

mortality could be established. Lately, Westin et al. published the results of the CACTUS (Cardiac Amyloidosis Carpal TUnnel Syndrome) study, a trial designed to determine the prevalence of undiagnosed CA in patients 5–15 years after bilateral CTR (11). They identified 250 subjects (50% females) with a median age of 70 years old with prior bilateral CTR and evaluated them for CA by performing echocardiography, bone scintigraphy, and monoclonal protein studies. CA was diagnosed in nearly 5% of patients. All cases were wild-type transthyretin amyloidosis (ATTRwt). As underlined by the authors, when focusing on men ≥70 years, prevalence of undiagnosed CA rises to 21.5%, therefore playing a potential role for systematic screening in this population. Notably, most of ATTRwt diagnosis (10 of 12 patients) had very early-stage disease. Globally, orthopedic conditions are very common in patients affected by CA (predominantly ATTR). Indeed, Rubin and colleagues have found that 25.9% and 18.8% of patients with ATTRwt-CA and ATTRv-CA, respectively, underwent hip or knee arthroplasty. Compared to the general population, surgical procedures were significantly more common among patients with ATTR-CA (hip arthroplasty: RR: 5.61, 95% CI: 2.25–4.64; knee arthroplasty: RR: 3.32, 95% CI: 2.25–4.64). As for other musculoskeletal disorders, arthroplasty occurred significantly before ATTR-CA diagnosis, and it was not significantly more common in AL patients compared to general population (12). Consequently, a history of a multidistrict orthopedic surgery should be definitely considered a red flag for suspicion of ATTR amyloidosis.

2.2. CA and aortic stenosis

It is well known that aortic stenosis (AS) and ATTRwt share the same epidemiological background as their prevalence is particularly high in male octogenarians, but whether this represents a simple epidemiological association, or a causal relationship is still an unanswered question. As previously summarized by Rapezzi et al. (13), there are three main hypotheses that may explain the association between those two conditions: (1) an age-dependent incidence of both diseases, thus involving the same subgroups of the population; (2) amyloid deposition involves the aortic valve, promoting or accelerating valvular degeneration; (3) AS itself can possibly trigger or cause transthyretin cardiac amyloidosis by increasing left ventricular (LV) wall shear stress. The question is relevant as both diseases carry a high burden of morbidity and mortality and the effect of their coexistence on patients' prognosis has not fully clarified, although recent data suggest that treatment options of AS improve survival even in patients affected by CA and consequently they should not be withheld (14). Moreover, along with population ageing, the absolute number of people affected is considerable and represents a public health issue in most developed countries.

ATTRwt's prevalence among aortic stenosis cohorts is variable between 4% and 16%, mainly influenced by the mean age of the studies' population and by the diagnostic criteria or imaging exams used for diagnosis (15). In a very early study, Treibel and colleagues (16) prospectively enrolled 146 patients with severe AS requiring surgical valve replacement and performed intraoperative myocardial biopsies, finding a prevalence of 6% of occult transthyretin CA. In another small single-centre prospective study, Longhi et al. (17) performed a 99mTc- 3,3-diphosphono-1,2-propanodicarboxylic acid (DPD) scintigraphy to detect occult ATTR-CA based on echocardiographic features. They applied an "echocardiographic red flags" approach to 43 elderly patients with AS referred for aortic valve replacement (surgical or transcatheter), including one or more of these features: increased thickness of atrioventricular valves, interatrial septum or right ventricular free wall, pericardial effusion, and myocardial granular sparkling. 5 of the 43 patients enrolled had at least one red flag at echocardiogram and all of them showed high cardiac uptake at 99mTc-DPD scintigraphy (prevalence = 11%). Similar percentages are reported in more recent clinical studies in which the systematic search of CA associated with AS was made by using multimodal imaging techniques (14, 18). To note, they all confirmed that bone scintigraphy shows a better sensitivity to detect ATTR-CA than cardiac magnetic resonance (CMR), even in presence of valvular abnormalities. Recently, computed tomography (CT) scan has been addressed as an additional emerging tool for identifying CA in patients with AS. In a single tertiary referral center study, an additional post-contrast acquisition for extracellular volume (ECV) evaluation was added to routine CT scan evaluation for transcatheter aortic valve replacement planning. All patients underwent bone scintigraphy as well. CT-measured ECV showed a good correlation with Perugini score, suggesting that this technique can reliably detect AS-CA and quantify the degree of infiltration (19).

Recently, researchers' efforts have been focused on identifying clinical, biochemical, electrocardiographic, and echocardiographic features that could help clinicians to raise the suspicion of underlying CA in patients affected by AS. Although some instrumental characteristics can be shared by both conditions (particularly LV hypertrophy), the identification of multiple distinctive features (listed in **Table 2**) should promptly trigger a diagnostic work-up for CA. A scoring system called RAISE was

TABLE 2 Characteristic parameters of cardiac amyloidosis in aortic stenosis.

Clinical	Male sex
	Age >80 years
	Carpal tunnel syndrome (or multidistrict orthopedic surgery)
ECG	Atrial fibrillation
	Atrio-ventricular and intra-ventricular conduction abnormalities
	Discrepancy between left ventricular mass and voltages
	Q waves (pseudo-infarction pattern)
Echocardiography	Severe left ventricular hypertrophy
	Low flow-low gradient aortic stenosis
	Right ventricle hypertrophy (>5 mm)
	Marked diastolic dysfunction ($E/e' > 15$)
	Severe atrial enlargement
	Very reduced S′ wave at mitral annulus Tissue Doppler
	Reduced global longitudinal strain with "apical sparing" pattern

developed in a multicenter international study enrolling patients referred for transcatheter aortic valve replacement (14), including five domains: LV remodeling (LV hypertrophy and/or diastolic dysfunction, 1 point), older age (>85 years old, 1 point), cardiac injury (serum high sensitivity troponin I >20 ng/L, 1 point), systemic involvement (carpel tunnel syndrome, 3 points), and electrical remodeling (right bundle branch block, 2 points or low QRS voltage, 1 point). Authors suggest that scores of ≥2 points would promote further investigation by bone scintigraphy and light-chain assessment thanks to a high sensitivity (93.6%), but scores ≥3 points shows a better specificity (84% vs. 52%).

2.3. CA and bone scintigraphy for non-cardiac reasons

Studies investigating prevalence of cardiac uptake in individuals undergoing bone scintigraphy for oncologic or rheumatologic reasons provide the most reliable information about the prevalence of CA in the general population. Recently, Aimo et al. performed a systematic meta-analysis of published studies ($n = 5$) and they found a prevalence of CA among patients undergoing bone scintigraphy for non-cardiac reasons of 1%, with a higher likelihood in men and increasing prevalence with age (20). The largest study in this setting was published in 2014 by Longhi and colleagues (21). Authors retrospectively analyzed 12,400 99mTc-DPD bone scans made for oncologic (95%) or rheumatologic (5%) indications in people aged >65 years: unexpected myocardial tracer uptake was present in 0.36% ($n = 45$) of patients, reaching 1.4% among male octogenarians. Of them, 14 patients with a median age of 82 years underwent a comprehensive cardiological evaluation, including ECG, echocardiogram and endomyocardial biopsy in selected cases. Five patients eventually received a diagnosis of ATTRwt, and 1 patient was found to have ATTRv. Despite the small number of definite diagnoses of ATTR-CA, this study highlighted for the first time the possibility of pre-clinical identification of patients affected by ATTR-CA in an unselected population. A subsequent study investigated the prevalence of myocardial tracer uptake in bone scans in an older cohort, finding a higher percentage of unexpected positive scans (3.88% of males over 75 years old) (22). Interestingly, cardiac uptake was associated with a higher risk of HF hospitalization during follow-up (OR: 2.60, 95% CI: 1.09–5.74, $p = 0.022$).

A recent Australian study found a prevalence of 0.43% of cardiac uptake in a heterogeneous cohort of over 3,000 patients who underwent 99mTc-hydroxy-methyl-diphosphonate (HMDP) bone scans for non-cardiologic reasons (23). Data confirmed that prevalence increases with age, and it is higher in males than females (1.44% vs. 0.17% in subjects >65 years old). Notably, nearly 29% of patients (998/3472) were <65 years old but none of them had cardiac uptake, corroborating the idea that positive bone scans in younger individuals are quite rare.

In this study, a positive correlation between positive scans (visual score ≥2) and septal wall thickness measured by echocardiography was observed, as well as between LV mass and the amount of HMDP uptake (measured by heart to whole body ratio); conversely, the degree of tracer uptake correlated negatively with LVEF, suggesting that higher HMDP uptake is associated with more advanced disease. However, in this population positive HMDP scans were surrogate marker of ATTR-CA, as AL amyloidosis was not systematically excluded, representing a possible confounding variable.

2.4. CA and heart failure

Nowadays, heart failure (HF) represents a considerable public health issue as it affects approximately 5% of the general population aged ≥60 years and HF with preserved ejection fraction (HFpEF) accounts for near the half of HF diagnosis in US (24). To date, few treatments have demonstrated to reduce cardiovascular events or mortality in HFpEF overall population. For such reason, it is crucial to exclude a possible specific cause of HFpEF, especially if disease-modifying therapies are available.

HFpEF strongly correlates with CA, since its pathophysiology is characterized by increase of myocardial stiffness causing an impaired LV relaxation, leading to high LV end-diastolic pressure and impaired ventricular filling (25). Since Mohammed et al. (26) have found that the age- and sex-adjusted prevalence of wild-type TTR amyloid was higher in HFpEF patients than in control subjects (odds ratio: 3.8, 95% confidence interval: 1.5 to 11.3; p 0.03) on histological screening in LV autopsy specimens, CA has been found to be a common etiology in such patients within several subsequent clinical studies. It should be noted that small quantities of transthyretin amyloid are commonly seen in the autoptic analysis of hearts of elderly patients, but their contribution to the burden of HFpEF is unclear. Indeed, whether the neurohumoral dysregulation and metabolic imbalance seen in HFpEF population can cause a destabilization of wild-type transthyretin molecules or on the other side the cytotoxic and profibrotic effect of the primary deposition of amyloid fibrils can cause the diastolic dysfunction underlying the disease is still an open question. In 2015, Gonzalez-Lopez and colleagues (27) prospectively screened a population of 120 patients aged >60 years old admitted to the hospital for HFpEF by performing 99mTc-DPD scintigraphy. They found an intense cardiac uptake (grade 2 or 3) in approximately 13% of cases; of these, no one was found to carry a genetic mutation of transthyretin gene. Notably, only 4 patients underwent to endomyocardial biopsy confirmation and light-chain amyloidosis (AL) was not systematically excluded. Afterwards, a multimodal cardiovascular imaging approach was tested to phenotype a population of patients over 65 years old with HFpEF in 2016 (28). In this study, 49 patients admitted to the hospital for signs and symptoms of HF and a LV ejection fraction >45% with no evidence of coronary artery disease underwent a complete evaluation with all clinical available cardiovascular imaging modalities, including echocardiography, cardiac magnetic resonance and 99mTc-DPD scintigraphy. The authors found a prevalence of 18% in ATTR-CA, similarly to Gonzalez-Lopez's group findings, 12% of AL cardiomyopathy and 6% of

hypertrophic cardiomyopathy. In this paper, a combination of CMR and 99mTc-DPD scintigraphy, in addition to biochemistry, clinical and echocardiographic evaluation, appeared to expand clinicians' possibility to characterize this population, contributing to the current idea that HFpEF is a complex and heterogeneous group in etiology and pathophysiology.

More recently, a Swedish study investigated the prevalence of ATTR-CA in a heart failure population with myocardial hypertrophy (29). Indeed, the investigators performed a 99mTc-scintigraphy in an unselected cohort of elderly patients (median age 84 years) with heart failure, irrespective of ejection fraction, and increased wall thickness (defined as interventricular septum >14 mm). Fourteen of the 86 (20%) investigated patients had a cardiac uptake of grade 2 or 3 at bone scintigraphy, while 5 patients had an uptake of grade 1. Only one patient was found to carry a mutation in TTR-gene. All patients were evaluated with blood and urine samples to exclude AL amyloidosis. Although a different patient recruitment protocol, these data appear to be in line with the previous ones. In fact, this study has remarkably focused on patients with myocardial hypertrophy, suggesting that the disease could not be confined only to HFpEF, as more than half of the cohort had an ejection fraction <50%.

2.5. CA and plasma cell disorders

The incidence of AL amyloidosis is ≈0.8%/100.000 population and symptomatic cardiac involvement seems to be present in 30% to 50% of cases (30). Most patients do not present multiple myeloma and in most cases the underlying plasma cell dyscrasia would be classified as monoclonal gammopathy of undetermined significance. AL amyloidosis may indeed complicate approximately 10%–15% of cases of plasma cell disorders (31). Cardiac amyloidosis is one the major parameter to impact on survival in AL amyloidosis (32), therefore an early recognition of signs of cardiac disease must represent an essential goal in order to start as soon as possible a specific therapy.

Nowadays, screening a plasma cell dyscrasia by assessing serum and urine protein electrophoresis with immunofixation and serum free light chains represents an imperative part of the diagnostic work-up in patients with suspected CA (33). Conversely, a systematic consensus on how and when to screen CA in plasma cell disorders is lacking. Patients with AL amyloidosis without cardiac involvement at baseline should be followed at least once a year in order to rule out the onset of CA, especially in those patients without a good hematologic response to treatment (34). Also, patients with a monoclonal gammopathy without AL amyloidosis should undergo to periodical cardiologic assessments, although the epidemiologic burden of the disease makes it challenging. Therefore, some authors suggest to screen pre-symptomatic amyloid organ involvement simply by assessing laboratory biomarkers (e.g., NT-proBNP, albuminuria, alkalin phosphatase) (35). Cardiac biomarkers (NT-proBNP and troponin) play an essential role on the diagnosis and the estimation of prognosis in AL-CA (32, 36), even in the absence of overt cardiac involvement defined by standard criteria. Recently, Sherpley et al. conducted a prospective observational study on Mayo stage I patients (N-terminal pro b-type natriuretic peptide <332 ng/L, high sensitivity cardiac troponin <55 ng/L) without cardiac involvement on echocardiogram. All patients also underwent to CMR, which documented signs of CA in 28% of patients. On multivariate analysis, N-terminal pro b-type natriuretic peptide >152 ng/L and cardiac involvement on CMR were prognostic. Interestingly, not all patients with elevated NT-proBNP had abnormal CMR and vice versa. Authors suggest that NT-proBNP may be detecting cardiac damage by light chain proteotoxicity before structural amyloid deposition (potentially detected by CMR), while some patients may have non-proteotoxic light chains where the structural changes are already evident on CMR. In conclusion, this study underlines the importance of combining standard cardiologic evaluation to NT-proBNP and CMR, which both play a complementary and crucial role on defining cardiac involvement (37).

2.6. CA and LV hypertrophy

LV hypertrophy is certainly one of the most recognized features of CA, although its presence is shared by many other conditions such as hypertrophic cardiomyopathy, Anderson-Fabry disease and hypertensive heart disease. Many strategies have been developed to detect CA in this context. For instance, an Italian study prospectively enrolled 343 patients aged ≥40 years referred to a Tertiary Centre with a previous echocardiographic diagnosis of hypertrophic cardiomyopathy (38). All patients underwent to a next-generation sequence genetic testing, of which 11 (3.2%) resulted positive for a TTR gene mutation and 1 for ApoAI mutation. Among remaining mutation-negative patients, authors investigated further with laboratory analyses, bone scintigraphy, and fat abdominal biopsy those who presented at least one "red flag" for CA, including pericardial effusion, symmetric LV hypertrophy or granular sparkling texture of the myocardium, thus increasing the number of total diagnoses of CA to 32 patients (9% of study population). Remarkably, prevalence of CA increased with age, ranging from 1% at ages 40–49 years to 26% above 80 years. The role of echocardiographic "red flags" has been widely investigated as a preferable approach for the diagnosis of CA. In 2020 Boldrini et al. (39) have proposed a multiparametric echocardiographic score with a very good diagnostic accuracy (area under the curve 0.87, 95% CI: 0.85–0.90) including relative wall thickness, E/e', TAPSE, longitudinal strain and "apical sparing" (defined as relative apical longitudinal strain >1.0, using the equation average apical LS/average basal LS + mid-LS) (40), within a sub-cohort of patients with LV hypertrophy and suspicion of CA. For the record, apical sparing More recently, the investigators of the AC-TIVE Study (41) aimed to assess the prevalence of CA in a much more unselected population. In fact, in the Phase 1 they evaluated more than 5,000 consecutive patients aged ≥55 years undergoing routine echocardiography to investigate the usefulness of echocardiographic "red flags" in detecting AC among general population by looking for hypertrophic and non-dilated left

ventricles with preserved ejection fraction in the first place ("CA compatibles"). Of the initial study population, 1,169 (22%) exams were CA-compatibles, and among these, 381 exams (33% of CA compatibles—7% of the total) were "CA suggestive", based on the presence of well-known clinical or echocardiographic "red flags". Notably, in this context thickening of the interatrial septum was the most frequent echocardiographic feature, followed by pericardial effusion, restrictive LV filling pattern, granular sparkling appearance of the myocardium, thickened atrio-ventricular valves and apical sparing pattern upon speckle-tracking analysis. In Phase 2 of the AC-TIVE study (4) they investigated the prevalence of CA among patients with echocardiographic red flags (the aforementioned "CA suggestive" population) by following the conventional diagnostic flow-chart for CA. Among patients who completed the diagnostic work-up ($n = 217$), 62 received a final diagnosis of CA with an estimated prevalence of nearly 29%. In this phase, statistical analyses showed that apical sparing alone or a combination of at least two red flags, had a diagnostic accuracy >70%, providing a guide for physicians in proceeding with further tests for AC when performing echocardiography for any reason. CMR may play a supplementary role in the instrumental evaluation of LV hypertrophy. In fact, in addition to the morphological and functional assessment of myocardial walls, CMR offers tissue characterization by measuring native T1 and native T2 (possibly with corresponding quantitative values in T1 mapping and T2 mapping) and extra-cellular volume (ECV). Those three measures have been proven in many studies to add both a diagnostic and prognostic value, even in distinguishing AL and ATTR amyloidosis (42, 43).

Figure 2 summarizes our proposed algorithm for the screening of CA in different clinical scenarios.

3. Future perspectives

As previously explained, the epidemiology of CA is vertiginously evolving, as if a "Pandora's box" was just opened by international scientific community. **Figure 3** recapitulates the prevalence of this condition fitting to the different clinical settings we investigated in this review. Yet, various clinical and experimental fields may still be explored to better define the most appropriate approaches to screen and then manage the disease.

For instance, atrial and ventricular arrhythmias and conduction disturbances are frequently found in patients affected by CA, nonetheless a systematic screening in this setting—still lacking—could offer further insights on the matter. Above all, atrial fibrillation and atrio-ventricular delays appear to be the most common (44, 45). Nevertheless, non-sustained ventricular arrhythmias are rather frequent and carry a debatable prognostic role (46), probably not higher as usually thought, as showed in a multicenter retrospective Italian cohort of 181 patients affected by CA (51 AL and 130 ATTR), in which the presence of non-sustained ventricular arrhythmias was related to the severity of disease but not with mortality in the total population and in each amyloidosis subtype (47). The underlying pathophysiology

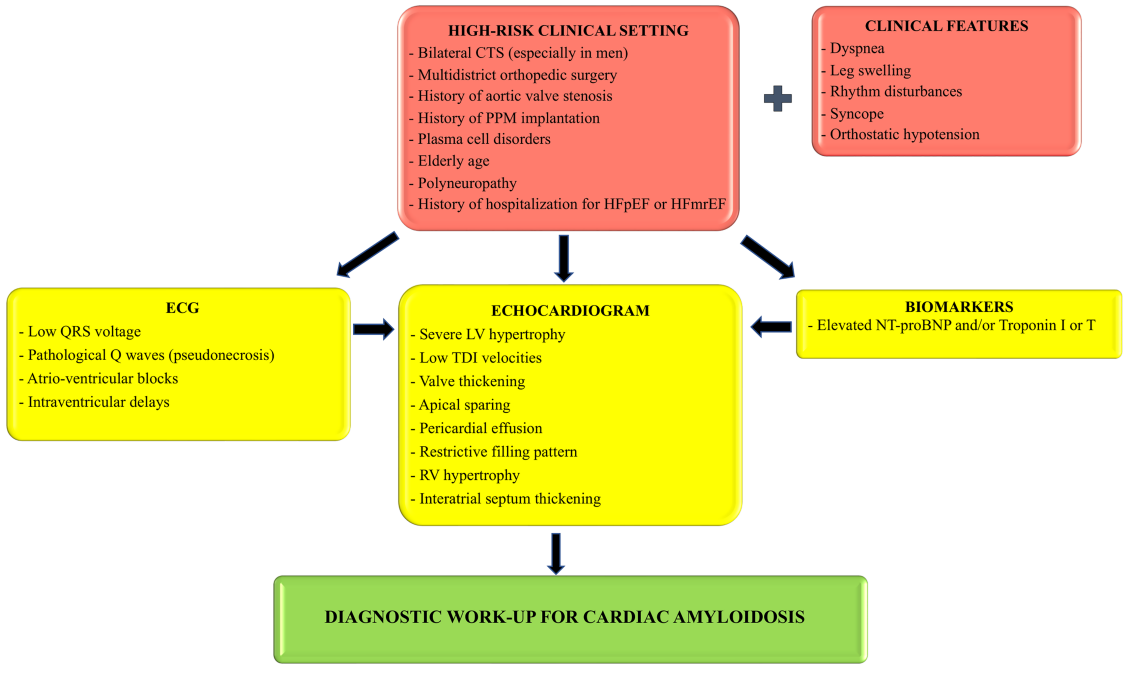

FIGURE 2
Proposed screening algorithm for patients belonging to high-risk clinical settings. ECG, echocardiogram and cardiac biomarkers can all be used as a first step methodic in the suspicion of cardiac amyloidosis, although echocardiogram is imperative before starting a diagnostic work-up. CTS, carpal tunnel syndrome; PPM, permanent pacemaker; HFpEF, heart failure with preserved ejection fraction; HFmrEF, heart failure with mildly reduced ejection fraction; LV, left ventricular; TDI, tissue Doppler imaging; RV, right ventricular.

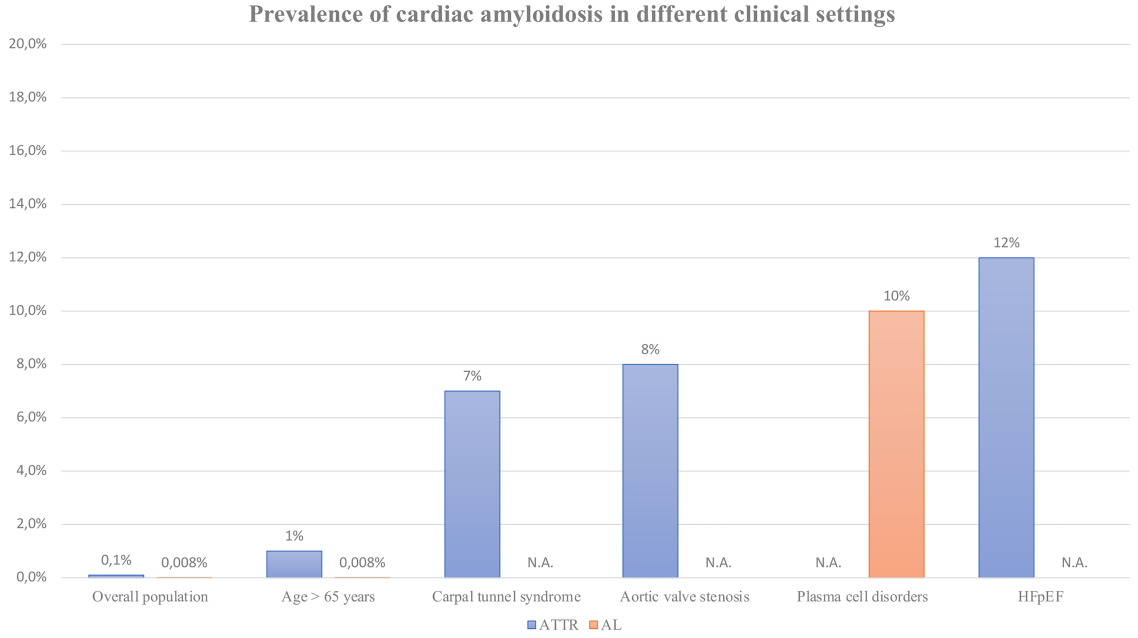

FIGURE 3
Prevalence of cardiac amyloidosis in different clinical settings. Data represent a rough estimation according to the main published evidence in literature. Not applicable (N.A.) was used in case of missing solid data. ATTR, transthyretin-related amyloidosis; AL, light-chain amyloidosis; HFpEF, heart failure with preserved ejection fraction.

is not completely understood as there is no convincing histological evidence of amyloid involvement of the conduction system tissue (48). Most likely, the pathogenesis of arrhythmias in CA is multifactorial (49). Amyloid deposition has been shown to cause wall thickening and disarray of myocardial fibers, which *per se* can disrupt the transmission of electrical impulses along conduction fibers. Also, there is an emerging hypothesis for a cytotoxic role of transthyretin molecule involving the dysregulation of intracellular calcium signaling causing action potential prolongation, in addition to oxidative stress and apoptosis (50). Furthermore, amyloid deposition from transthyretin monomers is thought to be neurotoxic in ATTRv patients and can cause the drive loss of sympathetic nerve fibers, which may contribute to arrhythmogenesis (51). Interestingly, electrophysiologic studies have shown not only prolonged AH intervals in CA patients, but also abnormal HV intervals, even in presence of a relatively normal QRS duration, reflecting an extended conduction system disease which determines an equal delay in both ventricles. This suggests that a narrow QRS does not exclude an intranodal conduction disease in this subset of patients and therefore should be considered in the diagnostic work-up of syncope (52). As result, permanent pacemaker implantation is often required. About 13% of patients affected by ATTRwt have a history of pacemaker implantation prior to diagnosis (53). In a recent prospective study conducted by Porcari et al. on a cohort of patients affected by ATTR-CA or AL-CA, 8.9% of patients underwent to pacemaker implantation during a median follow-up of 33 months, with an overall median time to PPM implantation at 18 months. An history of atrial fibrillation, a longer PR interval and QRS >120 ms on baseline ECG appeared to be independent risk factors for pacemaker implantation (54). In accordance with those data, a systematic search for CA (especially in presence of other "red flags") among patients with implanted devices could represent an intriguing field of research.

Among the instrumental methods to screen and diagnose CA, positron emission tomography (PET) has been gaining share, although its use on clinical practice is still hypothetical. In a screening setting, PET tracers may have a superior role both on detection of amyloid deposits and differentiation between ATTR and AL. For instance, 18F-Fluorbetaben was first tested by Law et al. in a small group of patients and showed higher standardized uptake values (SUV) in cardiac amyloidosis (both AL and ATTR forms) than hypertensive heart disease (55). A recent study by Genovesi et al. reported that 18F-Fluorbetaben kinetic of myocardial retention in AL-CA was much longer than ATTR-CA and in control subjects (56). 18F-Flutemetamol has only been tested in a small study which reported myocardial uptake exclusively in amyloid cardiomyopathy and not in the control group. Moreover, TBR (target-to-background ratio, the ratio between myocardial SUV and blood-pool SUV) was particularly high in the single patient with AL-CA enrolled in the study (57). An ongoing multicenter phase 2 study is evaluating the repeatability of organ-specific quantitation of 124I AT-01 tracer using PET/CT in ATTR and AL amyloidosis (NCT05235269). This radiotracer represents a novel pan-amyloid binding imaging agent. Preliminary data suggest that it may be useful to identify pre-symptomatic stage of CA (for instance in ATTRv carriers) and to detect amyloid deposits within the whole body, potentially allowing to replace tissue

biopsy for the assessment of organ involvement, especially in AL amyloidosis.

Lastly, artificial intelligence (AI) represents a widespread technique whose application in cardiology has been promisingly tested and approved in several contexts (58). AI describes a computational program that can perform tasks that are normally characteristic of human intelligence. In medicine, this typically involves data, health records or information extracted from images, used to predict a likely diagnosis, identify a new disease, or select a best choice of treatment. Grogan et al. collected 12-lead ECG data from 2,541 patients with AL-CA or ATTR-CA referred to Mayo Clinic between 2000 and 2019, matched for age and sex with 2,454 controls. A subgroup of 2,997 cases and controls were used to train a deep neural network to predict the presence of CA. 426 (84%) of the patients with CA were detected by the model, predicting the presence of CA more than 6 months before the clinical diagnosis in 59% of cases (59). A first pragmatic trial will be soon conducted at Mayo Clinic (NCT05557162) to prospectively evaluate the use of the AI-ECG dashboard in everyday practice. The principle aim of the study will be to prove if an alert system AI-ECG and enhanced algorithms enable earlier diagnosis of CA on compared to standard practice arm. Further clinical trials need to be performed, hopefully by combining multi-modality imaging, with the ultimate goal of making diagnosis of CA ever more extensive and accessible.

4. Conclusions

CA is a threatening disease which may be hiding in multiple conditions such as carpal tunnel syndrome, aortic stenosis, heart failure, plasma cell disorders. In those scenarios, detecting the disease may be difficult because some features overlap each other, therefore the application of a multimodality imaging approach along with the awareness and recognition of specific characteristics, so-called "red flags", are fundamental. Although the cost-effectiveness of a screening strategy in CA has not been scientifically proven yet, this extensive and meticulous approach can be beneficial for various potential reasons: - intercepting a pre-symptomatic phase in which "disease-modifying" therapies may act the most; - recognizing an underlying neoplastic process such as AL amyloidosis; - identifying hereditary CA from probands to siblings; - better understanding (and maybe predicting) the cardiac disturbances of recruited patients; - tailoring supportive cardiac and non-cardiac therapy within the peculiar CA pathophysiology and natural history. On this basis, the efforts of scientific community must continue along the direction of increasing knowledge of the disease.

Author contributions

AGC and AA participated in the writing of the manuscript. AG and GS provided images. GS, AP, MS, PM, AG, RD, VP, OL, PG, PC, CG, SL, NG and EB critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

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Risk stratification in transthyretin-related cardiac amyloidosis

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Transthyretin related cardiac amyloidosis (TTR-CA) is an infiltrative cardiomyopathy that cause heart failure with preserved ejection fraction, mainly in aging people. Due to the introduction of a non invasive diagnostic algorithm, this disease, previously considered to be rare, is increasingly recognized. The natural history of TTR-CA includes two different stages: a presymptomatic and a symptomatic stage. Due to the availability of new disease-modifying therapies, the need to reach a diagnosis in the first stage has become impelling. While in variant TTR-CA an early identification of the disease may be obtained with a genetic screening in proband's relatives, in the wild-type form it represents a challenging issue. Once the diagnosis has been made, in order to identifying patients with a higher risk of cardiovascular events and death it is necessary to focus on risk stratification. Two prognostic scores have been proposed both based on biomarkers and laboratory findings. However, a multiparametric approach combining information from electrocardiogram, echocardiogram, cardiopulmonary exercise test and cardiac magnetic resonance may be warranted for a more comprehensive risk prediction. In this review, we aim at evaluating a step by step risk stratification, providing a clinical diagnostic and prognostic approach for the management of patients with TTR-CA.

KEYWORDS

risk stratification, cardiac amyloidosis, natural history, transthyretin, heart failure, arrhythmias

Introduction

Transthyretin related cardiac amyloidosis (TTR-CA) is an infiltrative cardiomyopathy caused by extracellular deposition of transthyretin (TTR)-derived insoluble amyloid fibrils in the myocardium. TTR-CA is generally considered to be rare but in the last 20 years, due to advanced technology and improvement of diagnostic tools, it has been increasingly recognized (1). Two distinct types of the TTR protein (variant, vTTR, or wild type, wtTTR) become unstable, and misfolding forms aggregate resulting in amyloid fibrils.

The true prevalence of both forms of TTR-CA is hard to define, since the familial form present a highly uneven geographical distribution and the senile form is often underdiagnosed (2). The development of effective and specific drugs for TTR-CA marked the beginning of a new era for this disease once deemed incurable (3, 4).

Clinical course of TTR-CA is characterized by two different stages (**Figure 1**): a *pre-symptomatic stage*, when fibrils progressively infiltrate the heart, causing initial and subclinical structural and functional alterations; in this stage, patients are usually asymptomatic and often elude the diagnosis; in a subsequent time, the *symptomatic stage*,

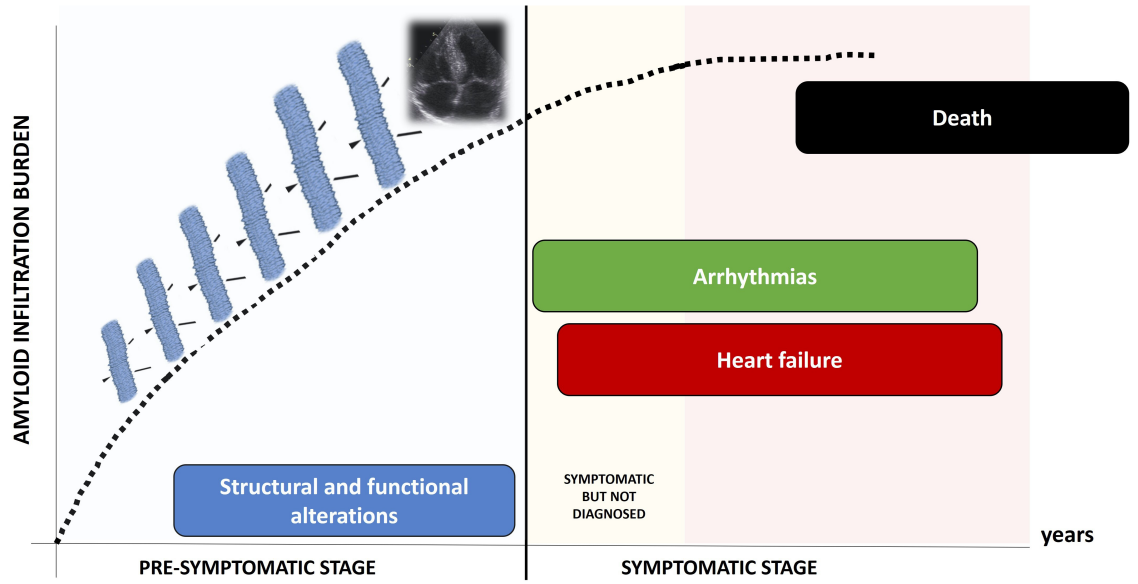

FIGURE 1
Natural history of TTR-CA: the pre-symptomatic stage and the symptomatic stage.

the disease clinically manifests: patients suffer from progressive heart failure (HF), arrhythmias and conduction system disease, undergo clinical evaluation and are eventually diagnosed. Death occurs in a median time of 3–4 years (1).

To improve care and risk stratification of TTR-CA patients, the identification of clinical and instrumental features associated with both the development and the progression of the disease is of paramount importance. Although the number of diagnoses of TTR-CA has increased markedly during the last 20 years (5), the main challenge in the management of this disease still remains its early recognition. Indeed, most of patients achieve a diagnosis when they have already reached hard endpoints, as arrhythmias or HF hospitalizations. Moreover, the specific TTR-CA therapies result more effective in patients with mild symptoms, further emphasizing the need to identify TTR-CA patients before clinical conditions worsen and HF develops and progresses.

Pre-symptomatic stage

Before the occurrence of clinical manifestations, clinical efforts need to be focused on recognizing those patients who can develop the disease and need to be screened.

In vTTR, more than 140 different mutations of the TTR gene have been described and specific variants often correlate with different clinical manifestations, ranging from a prevalent cardiac phenotype to mixed and prevalent neurological ones (6). The various mutations are associated with specific phenotypes and some of them are endemic in specific geographical areas (7). For example, the variant Val30Met has different manifestations and penetrance according to the geographical location, ranging from a fast-progressing disease dominated by neuropathy with early onset and high penetrance (Portuguese form), to a slowly progressing disease with late onset and low penetrance (Swedish form) (8, 9). Typical "cardiogenic" variants, like Val122Ile in North America and Ile68Leu in Italy, are characterized by clinical manifestations very similar to those of wtTTR, including a high prevalence of carpal tunnel syndrome (CTS), with a comparable age of onset (seventh-eight decade of life) (10, 11). These cardiac forms share with the wtTTR also the male prevalence, in contrast to mixed forms like those caused by Phe64Leu or Glu89Gln, in which the gender disparity is milder or absent (12, 13). In general, women who carry a pathogenic variant less likely have cardiac involvement and among asymptomatic carriers there is a relatively larger female presence (14), suggesting a lower penetrance in women. Furthermore, it has been reported that inheriting the pathogenetic TTR variant from the mother can cause an anticipation of disease onset and consequently a higher penetrance (15). In summary, carrying a pathogenic TTR mutation confers a variable risk of developing CA, which depends on the specific variant, the geographical area, gender and transmitting parent (father vs. mother). Genotype-positive phenotype-negative individuals should be periodically visited in order to detect the development of minor disease signs and in this way allow an early initiation of a specific therapy. It has been proposed that the clinical follow-up should start about 10 years before the predicted age of disease onset (PADO), estimated from the typical age of onset associated with the specific mutation, the age of onset of the affected relatives and the sex of the transmitting parent (16).

In wtTTR, extracardiac manifestations like CTS, which can represent an early sign of the systemic disease, are likely the only predictive factors of its development. CTS is a very frequent finding among TTR-CA patients, especially in the wild-type form, because of selective amyloid deposition in the transverse carpal ligament (17). This particular localisation may

be explained by the presence of repetitive mechanical stimuli in carpal tunnel area, as well as in the heart, that facilitate TTR amyloidogenesis through the activation of plasminogen (18). Compared to the general population, the prevalence of CTS in TTR-CA is higher, ranging from 15% to 60%, especially in men in the seventh and eight decades (19, 20). It is well known that the diagnosis of CTS is often followed by the development of CA with a characteristic latency of 5–10 years (21). This interval is the most likely explanation for the low incidence of CA (2%) found by Sperry et al. (22) in patients undergoing carpal tunnel surgical release, despite the fact that amyloid deposits have been found in 10% patients. On the other hand, this approach offers the opportunity of a very early screening of patients at risk to develop TTR-CA in the following years. Indeed, a history of CTS has been associated with a 12 times higher risk of amyloidosis as compared to matched control subject without CTS; an odd that raises to 30 times in the case of bilateral CTS (23). A recent study (24) has shown that the prevalence of wtTTR, 5–15 years after surgery for bilateral CTS, reached 8.8% in men, getting closer to the aforementioned prevalence of TTR deposits in the carpal tunnel ligament (22), and suggesting that amyloid deposition in this specific site could predict future development of CA. Post hoc subgroup analysis has highlighted a prevalence of 25.7% in men >70 years old, after excluding patients with BMI > 30 and occupational risk factors for CTS. Moreover, this screening approach has allowed an early diagnosis, considering that almost all the TTR-CA patients identified had low disease severity scores (24). The presence of left ventricular (LV) hypertrophy or other red flags, especially NT-proBNP and a relative apical sparring pattern, may allow to increase the sensitivity of the screening method (25, 26).

Finally, some echocardiographic features may raise the suspicion of TTR-CA in the context of the LV hypertrophy. In a multicentric study, evaluating more than 1,000 patients with increased heart wall thickness, in which amyloidosis was suspected, relative wall thickness, evidence of diastolic dysfunction (E/e'), TAPSE and strain variables assessing the relative apical sparing had best diagnostic accuracy to individuate those with amyloid infiltration (27). Moreover, a simple score, obtained by the product of relative wall thickness and E/e' ratio, has been demonstrated to possibly have a role as an initial screening tool for patients with suspected TTR-CA (28). Recently, Merlo et al. in a multicentric Italian study enrolling 5,315 unselected consecutive patients undergoing echocardiogram for reasons other than known or suspected cardiac amyloidosis (CA), showed that 1.2% of them reached a diagnosis of TTR-CA. Echocardiographic findings as non-dilated, hypertrophic hearts with LV ejection fraction >50% in combination with apical sparing or at least two red flags (i.e., restrictive filling pattern, granular sparkling, pericardial effusion, interatrial septum thickness >5 mm, atrio-ventricular valve thickness >5 mm) provide a diagnostic accuracy >70% (29). An ECG discordance with echocardiographic findings of hypertrophied not dilated LV or a slightly increase of cardiac biomarkers further increase the suspicion of TTR-CA (1, 8).

Symptomatic stage

Risk factors for heart failure and death

HF is the main complication of TTR-CA, both in terms of number of hospitalizations and of mortality. In recent years, TTR-CA has been increasingly recognized as a cause of HF hospitalizations, exceeding the rate of 65 cases per 100,000 people/year in several regions of the United States (30). Current pharmacological management of HF is limited in patients with TTR-CA: drugs as beta blockers and renin angiotensin system inhibitors may be poorly tolerated (31). Moreover, the reduced size of LV cavity and the frequent involvement of right ventricle may hamper the use of long-term ventricular assist devices. Finally, heart transplantation may be an effective option, but only in carefully selected patients (32).

For these reasons, risk stratification of TTR-CA patients is imperative in order to identify patients with at risk of a faster disease progression towards HF, as this subset requires earlier and more aggressive therapies, as well as closer monitoring. In this regard, a multimodal approach, which integrates clinical, biomarkers and instrumental indicators need to be used (**Figure 2**).

Risk scores

In 2016, Grogan et al. (33) proposed for wtTTR a three-group classification system (Mayo score) according to cut-offs of troponin T (50 ng/L) and NT-proBNP (3,000 ng/L). The 4-year survival was 57%, 42% and 18% for stage I (both values below the cut-off), stage II (one marker above the cut-off) and stage III (both markers above the cut-off), respectively. This staging system is not validated for vTTR. Moreover, the use of troponin T appeared to be overwhelmed by the current worldwide diffusion of newer high sensitivity troponin assays, which not only differ in sensitivity, but also give different numerical results as compared to older troponin assays. In 2018, Gillmore et al. (34) developed a three-stage grading system (NAC score) for vTTR and wtTTR amyloidosis using NT-proBNP (cut-off: 3,000 ng/L) and glomerular renal filtrate (cut-off: 45 ml/min/1.73 m^2) with median survival of 69, 47 and 24 months in stage I, II and III, respectively. Since the number of early diagnosis has recently increased, a very early stage (Ia) defined by low NT-proBNP concentration (<500 pg/ml, < 1,000 pg/ml with atrial fibrillation) and need for low doses of loop diuretics (<0.75 mg/kg) has been proposed (35) to better risk stratify CA patients. Stage Ia patients had a longer median survival compared with stage Ib (>100 months vs. 75 months), comparable to the age- and gender-matched general population. Despite this, a considerable cardiovascular morbidity still characterizes this stage, getting worse during the follow-up period, even in the patients with primary non-cardiovascular clinical presentation (36). Besides the aforementioned inter-assay variability of troponin, the accuracy of the "Mayo" staging system (33) is limited by the incompleteness of data regarding some variables and by the lack of external validation. Compared with NAC staging system, externally validated in an unrelated French cohort (34), it provides less effective prognostic discrimination, especially between stage I and stage II (35).

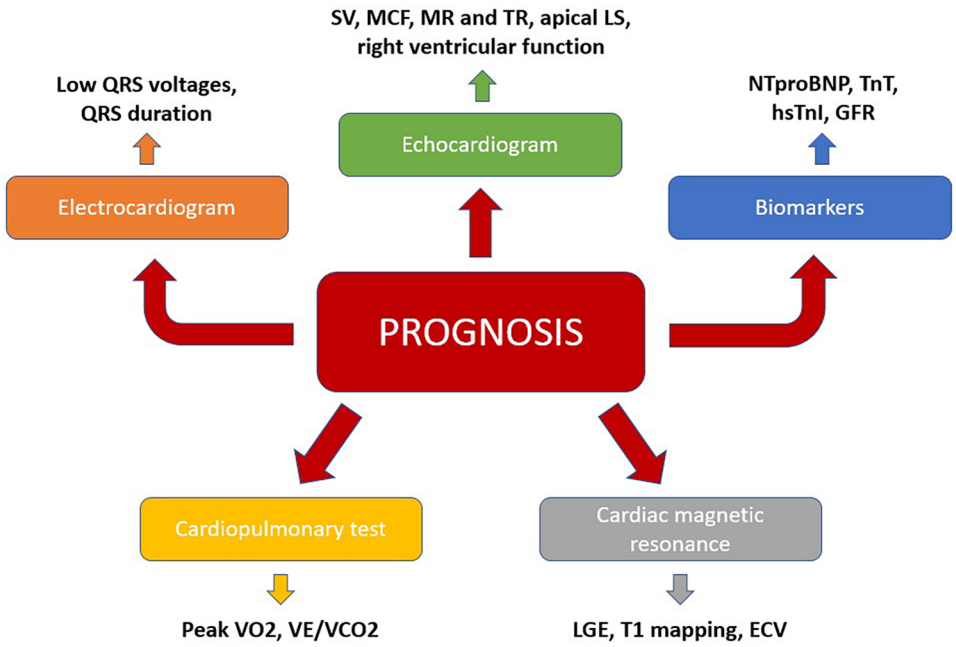

FIGURE 2
A multiparametric approach to predict prognosis in TTR-CA. ECV, extracellular volume; GLS, global longitudinal strain; GFR, glomerular filtration rate; LGE, late gadolinium enhancement; MR, mitral regurgitation; TR, tricuspid regurgitation; VCO_2, carbon dioxide output; VE, minute ventilation; VO_2, maximum oxygen consumption.

Alongside these two scores, other clinical markers have been proposed in recent years to improve their accuracy. In 2020, Cheng et al. (37) demonstrated that diuretic dose and NYHA functional class were strong independent predictors of all-cause mortality and the composite outcome of all-cause mortality or cardiac transplantation. This study, including both vTTR e wtTTRpatients, reported the incremental value of these parameters added to the Mayo and NAC staging systems. According to a recent study by the University of Heidelberg (38), the risk score proposed by Gillmore et al. for TTR-CA may not be sufficient to predict outcomes leading to advanced HF. In this study, a simple risk stratification score ("HeiRisk" score) including clinical parameters and biomarkers was generated to identify patients with end-stage cardiac light-chain (AL) or TTR amyloidosis in order to facilitate clinical decisions, such as listing for heart transplantation. This study showed that only cardiac biomarkers - hsTnT (cut off: 55 pg/ml) and NT-proBNP (cut off: 6,330 ng/L) - and QRS duration (cut off: 104 ms), but not haemodynamic measures, were significant predictors in TTR-CA.

However, all these scores are binary systems with few variables and this, whilst ensures simplicity of use, may be a limitation for an accurate prediction of outcomes, essential to evaluate the effects of new therapies; for this purpose a multiparametric approach is probably required.

Electrocardiogram

The most striking electrocardiographic abnormality in patients with CA is the reduction of QRS voltages, particularly in the limb leads, and the disproportion between QRS voltages and LV thickness at echocardiography (39). Low QRS voltage is defined as a QRS amplitude <5 mm (0.5 mV) in all peripheral leads. This finding, considered pathognomonic of the disease, is present in 60% of AL and only in 20% of TTR-CA, and may reflect the burden of amyloid infiltration in the heart. In a recent study by Cipriani et al. (40), low QRS voltages paired with an advanced disease stage independently predicted cardiovascular death. Together with the NAC staging, low QRS voltages provided incremental prognostic value in TTR-CA.

Echocardiogram

Cardiac amyloid deposition usually causes HF with preserved ejection fraction. Therefore, different echocardiographic tools have been proved useful to define the prognosis of TTR-CA patients, beyond LV ejection fraction. A decreased SV index, which represents a marker of advanced disease, has shown to independently predict prognosis, even after adjustment for NYHA class and NAC staging system (41). Chacko et al. have demonstrated a progressive worsening of structural and functional echocardiographic parameters over time, although only worsening in the degree of mitral and tricuspid regurgitation at 12- and 24-month assessments associated with a worse prognosis (42). Moreover, myocardial contraction fraction (MCF), which is the ratio of LV systolic output to LV myocardial volume, has shown promising result to predict outcomes in CA patients. In the THAOS registry (43) the median survival of patients with MCF < 25% was less than 3 years compared with 6.8 years of patients with MCF ≥ 25%.

In recent years, assessment of LV global longitudinal strain (GLS) has proved to be of great diagnostic and prognostic significance. In patients with TTR-CA, GLS is reduced showing the characteristic apical sparing pattern with a "cherry on top"

appearance at the bull's eye plot. Recently, a reduction in apical longitudinal strain (cut off: −14,5%) have shown to be an independent predictor of major cardiac adverse events (44). On the contrary, longitudinal strain of the basal and midcavity sections, where amyloid infiltration is more marked and early, has not been found to predict prognosis (44). These data suggest that a reduction of apical longitudinal strain, typical of the advanced stages of the disease with severe amyloid deposition, is uncommon and probably less helpful in the early course of CA.

Right ventricular dysfunction, assessed by TAPSE (cut off: 14 mm), has been associated with a higher rate of cardiovascular events (45, 46). A recent study (46) has also highlighted that right ventricular free wall strain (cut off: 16%) may have an independent prognostic role for all causes of death. A study by Bandera et al. (47) demonstrated that increased atrial stiffness, identified using echo speckle tracking and characterized by a reduction in the reservoir and contractile function of the atrium, remains independently associated with prognosis after adjusting for known predictors. Notably, the absence of atrial contraction,foundin 22% of patients in sinus rhythmis associated with a significantly poorer prognosis compared topatients who maintain an effective mechanical contraction,andsimilar tothose with atrial fibrillation (47).

Cardiopulmonary exercise test

The cardiopulmonary exercise test (CPET) is the gold standard test todetermine prognosis in chronic HF with reduced ejection fraction (48). CPET is performed to assess the cardiocirculatory exercise response, together with the ventilatory and peripheral muscular responses. All of these parameters can be altered in amyloidosis due to the restrictive cardiomyopathy, cardiac denervation and chronotropic insufficiency. The main CPET characteristics of CA patients include reduced peak VO_2, increased VE-VCO_2 slope and episodes of oscillatory ventilation (EOV) (49). Peak VO_2 and circulatory power has been found to be strongly and independently predictive of death or HF (50, 51). The combination of peak VO2 (cut off: 13 ml/min/kg) and NT-proBNP was the best predictor of all-cause mortality and the composite of mortality or HF-related hospitalization (45). Furthermore, the increase in VE/VCO_2 slope (cut off: 40), resulted from several factors like autonomic dysfunction, right ventricular dysfunction and the absence of tidal volume rise during exercise, was shown to be associated with clinical events in wtTTR (49, 52).

Cardiac magnetic resonance

Cardiac magnetic resonance (CMR)has the ability to provide unique information about myocardial tissue composition. Indeed, it can identify and quantify cardiac amyloid deposition, using late gadolinium enhancement (LGE) and T1 mapping with calculation of extracellular volume (ECV).

In CA, LGE shows a characteristic global subendocardial pattern, generally associated with abnormal myocardial and blood-pool gadolinium kinetics (53). Non-contrast T1-mapping has great diagnostic accuracy for CA, being more sensitive than LGE imaging for identifying early disease (54). Transmural LGE has been associated with higher mortality compared to subendocardial pattern, remaining an independent negative predictor of survival in multivariable Cox models, as well as NT-proBNP and stroke volume indexed (55). Both native T1 mapping and ECV correlate with mortality, but only ECV remains independently predictive of prognosis after adjustment for other prognostic factors, as evidence of its robustness as a marker of cardiac infiltration (56).

Risk factors for arrhythmias

Although the clinical course of TTR-CA is dominated by HF and its manifestations, arrhythmias and conduction system diseases are also very common (57). Sudden cardiac death has been reported to be one of the main causes of death (58), although often from pulseless electrical activity. Moreover, cardiac arrhythmias are associated with increased in-hospital mortality and acute HF exacerbations (59).

Atrial fibrillation is the most commonly observed heart rhythm disturbance in CA, especially in wtTTR, where it can be detected in up to 70% of patients (60, 61). The progressive diastolic dysfunction and the increase of filling pressures, together with the selective deposition of amyloid in the atria walls (62), lead to atrial structural and functional remodeling - also called atrial myopathy -, which accounts for the frequency of supraventricular arrhythmias. Age, HF, LV ejection fraction, left atrial size and right atrial pressure have shown to be independent predictors of developing atrial fibrillation (63). A history of atrial fibrillation is strongly associated with prevalent and incident HF (63); however, in contrast to other etiologies of HF, in TTR-CA atrial fibrillation doesn't seem to impact survival and all-cause mortality (60, 61, 63). Previous studies have emphasized the high prevalence of intracardiac thrombi in CA, in particular in patients with atrial fibrillation (64, 65). Restrictive filling pattern and low left atrial appendage emptying velocities at transesophageal echocardiogram have been shown to predict the presence of intracardiac thrombi (63). Furthermore, a significant proportion of arterial thromboembolic events occurred in patient in sinus rhythm or despite adequate anticoagulation therapy due to the amyloidosis related atrial myopathy, that causes a progressive decline of atrial function and, eventually, an electromechanical dissociation (66, 67). In view of this, in patients at high risk of thromboembolic events the execution of a transesophageal echocardiography should be considered before direct current cardioversion (68, 69).

High-grade atrioventricular (AV) blocks are present in 9.5% of TTR-CA patients at the time of diagnosis (70). Amyloid fibrils infiltrate the conduction system, making an increasing number of patients pacemaker (PMK)-dependent as the disease progresses (71). Several studies reported that device implantation is required in about 9%–11% of patients in the years following the diagnosis (70–72). PMK implantation impacts on outcomes, as right ventricular pacing may be associated with worsening HF symptoms, LV ejection fraction decline and mitral regurgitation severity (73). In a recent paper, it has been showed that history of atrial fibrillation, PR interval >200 ms and QRS duration predict future PMK implantation. The presence of these features should advice a close monitoring, while the absence of all these

risk factors allow to exclude with great accuracy the need of PMK in the first 6 months after diagnosis (72).

Ventricular tachyarrhythmias, although frequent, have not been thought to contribute significantly to overall mortality in CA, especially TTR-CA (74). On the other hand, previous studies (75, 76) have reported a high rate of appropriate and successful implantable cardioverter defibrillator (ICD) therapies, even if involving mostly AL patients. A recent retrospective study cohort of 130 TTR-CA patients (77) have documented a high rate of ventricular arrhythmias and appropriate ICD therapies, in particular in those patients with systolic dysfunction. The evidence of non-sustained ventricular tachycardia (NSVT) and a history of unexplained syncope has been proposed as criteria for ICD implantation (78). In contrast, in a recent meta-analysis, the predictive value of NSVT has been debated and it has been shown that a NYHA class III-IV is associated with lower rate of appropriate ICD therapies. The physiopathological explanation of this result is that the focal amyloid deposits and associated fibrosis in the early stage of the disease can act as arrhythmogenic foci (79).

However, no studies have demonstrated a survival benefit related to ICD implantation, highlighting the need to better select patients at risk of lethal arrhythmic events. Furthermore, in an elegant study (80) CA was associated with a mortality rate of 26.9% at 1 year after ICD implantation compared with 11.3% among a propensity-matched cohort of patients with other non-ischemic cardiomyopathies; in this context the Authors found 5 predictors of mortality: a history of syncope, NSVT, diabetes mellitus, cerebrovascular disease and renal dysfunction. Therefore, it is clear that the risk of lethal arrhythmias should be balanced with the risk of other competitive causes of mortality. In this regard, ICD implantation should probably be considered in patients with lesser cardiac involvement and in the early stages of the disease (81).

Therapeutic implication of disease staging

The main goal of the emerging disease-modifying therapies - TTR gene silencers and TTR stabilizers - is to prevent further generation or deposition of amyloid fibrils. For this reason, an early diagnosis and a prompt start of this specific treatment allows to obtain a significant benefit in terms of survival and quality of life. On the other hand, patients with delayed diagnosis and advanced disease are unlikely going to benefit from these therapies (82). This is especially true in older patients with higher risk of competitive non-cardiovascular causes of mortality. Moreover, in the ATTR-ACT study (3) patients with NYHA class III disease at baseline had higher rates of cardiovascular-related hospitalizations, suggesting an unfavourable cost-benefit ratio of Tafamidis in this subgroup of patients. Risk scores have not been systematically used as criteria for inclusion or exclusion of patients in trials, neither as endpoints to determine drug efficacy. Nevertheless, it is reasonable to think that the use of these scores would be informative of the potential benefit of the treatment or of its futility.

Conclusion

TTR-CA is increasingly recognized, particularly in older patients. The advent of new disease-modifiers therapies highlights the importance of reaching the diagnosis early, ideally in the pre-symptomatic stage. A multiparametric approach, including not only biomarker scores, but also clinic, electrocardiographic and imaging data, is suggested for a careful risk stratification of mortality and HF-related events, in order to tailor CA management and therapy and to improve outcomes. Identifying reliable predictors of arrhythmic events is still an unmet need and the role of ICD in CA remains unclear. The improvement of survival hopefully related to new therapies will likely change this scenario.

Author contributions

RS, EC, LM, GT and BM: drafted the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The handling editor [JG] declared a past co-authorship with the authors [GT, RS].

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Treating amyloid transthyretin cardiomyopathy: lessons learned from clinical trials

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An increasing awareness of the disease, new diagnostic tools and novel therapeutic opportunities have dramatically changed the management of patients with amyloid transthyretin cardiomyopathy (ATTR-CM). Supportive therapies have shown limited benefits, mostly related to diuretics for the relief from signs and symptoms of congestion in patients presenting heart failure (HF). On the other hand, huge advances in specific (disease-modifying) treatments occurred in the last years. Therapies targeting the amyloidogenic cascade include several pharmacological agents that inhibit hepatic synthesis of TTR, stabilize the tetramer, or disrupt fibrils. Tafamidis, a TTR stabilizer that demonstrated to prolong survival and improve quality of life in the ATTR-ACT trial, is currently the only approved drug for patients with ATTR-CM. The small interfering RNA (siRNA) patisiran and the antisense oligonucleotide (ASO) inotersen have been approved for the treatment of patients with hereditary ATTR polyneuropathy regardless of the presence of cardiac involvement, with patisiran also showing preliminary benefits on the cardiac phenotype. Ongoing phase III clinical trials are investigating another siRNA, vutrisiran, and a novel ASO formulation, eplontersen, in patients with ATTR-CM. CRISPR–Cas9 represents a promising strategy of genome editing to obtain a highly effective blockade of *TTR* gene expression.

KEYWORDS

transthyretin cardiac amyloidosis (ATTR-CA), treatment, tafamidis, siRNA, antisense oligonucleotide, gene editing, heart failure

1. Introduction

Amyloidosis is a rare disease caused by the deposition of misfolded fibrillar proteins causing morphological and functional changes in the infiltrated tissues (1). More than 40 different precursor proteins can undergo the substantial molecular transformation to form amyloid fibrils, but most cases of cardiomyopathy (CM) are due to the accumulation of transthyretin (ATTR) or immunoglobulin light chains (AL-CM) (2–4). AL-CM is traditionally considered the most common of these conditions. However, ATTR-CM is increasingly being diagnosed and is emerging as an under-recognized cause of heart failure (HF) in older adults (5, 6). It accounts for 12%–13% of HF with preserved ejection

fraction (HFpEF) cases, 5%–7% of patients with presumed hypertrophic cardiomyopathy and 8% of those with severe aortic stenosis (7–10).

Almost all serum TTR is synthesized and secreted in the liver; the choroid plexus and retinal pigment epithelium are other sites of production. Cardiac involvement in ATTR-CM manifests typically as left ventricular thickening and/or HFpEF with increased left ventricular wall thickness and diastolic dysfunction (11, 12). Based on the sequence of the *TTR* gene, ATTR-CM is classified as wild-type (ATTRwt-CM) (without mutation) or mutated/variant (ATTRv-CM) (with a mutation) (5) ATTRwt typically has a late symptom onset (>60 years of age), while symptom onset in patients with ATTRv may occur at younger ages. More than 150 mutations and deletions in the *TTR* gene have been identified (13). The mutations most commonly associated with cardiac involvement are Val122Ile, Thr60Ala, Leu111Met and Ile68Leu (14). Previous studies have shown a prevalence of ATTR-CM in male sex, particularly in ATTRwt. Historically, ATTR-CM phenotype was thought to be less severe in women compared to men, based on non-indexed echocardiographic parameters. A more recent analysis by Patel et al. showed that overall structural and functional phenotype was similar between sexes when indexed to body size, with the only significant differences pointing towards a mildly worse phenotype in females (15). The use of non-indexed parameters could have led not only to underestimate CA severity in female sex, but also to a later diagnosis (age at diagnosis was 3 years higher in females compared to males) and this can partially explain the under-representation of female sex in clinical trials (15, 16).

Recent advances have improved the treatment of ATTR-CM and several clinical trials are ongoing. The aim of our review is to summarize recent findings and future perspectives in the treatment of ATTR-CM.

2. Transplantation in ATTR amyloidosis

Until recent times, orthotopic liver transplantation or combined heart-liver transplantation were considered the only available disease-modifying treatments for vATTR amyloidosis (17). Importantly, tissue TTR deposition can progress even after liver transplantation, since TTR amyloid fibres promote subsequent deposition of circulating TTRwt (18). Liver transplantation is not an option for patients with ATTRwt-CM. A few studies conducted on patient with ATTRwt-CM showed excellent outcomes for heart transplantation in highly selected patients, with 92%–100% survival at 3 years and 90% at 5 years (19–21). Risk of allograft amyloid recurrence in short and medium terms appear to be negligible (19).

Due to limited organ availability, exclusion of older patients and of those with advanced systemic disease, the risks related to surgery and life-long immunosuppression, organ transplantation is not suitable for the vast majority of patients with this condition. With the improvement of target therapies in the amyloidogenic cascade, transplantation is not a therapeutic option anymore in many centres.

3. ATTR disease-modifying therapies

In the last decades research focused on disease-modifying drugs, that act through different mechanisms: (i) inhibition of amyloidogenic TTR synthesis, (ii) stabilization of the native TTR tetramer structure and (iii) removal of misfolded proteins (**Figures 1**, **2**) (13, 22, 23).

3.1. ATTR silencers

No ATTR-silencer is currently approved for the treatment of patients with ATTR-CM, whereas two gene silencers are currently approved for the treatment of patients with ATTRv polyneuropathy, either with or without cardiac involvement: patisiran and inotersen.

3.1.1. Small interfering RNAs

Patisiran is a small RNA-interfering (siRNA) molecule that inhibits hepatic synthesis of TTR by binding RNA silencing complexes (**Figure 1**). Phase I and II studies showed that patisiran is safe and effective in reducing serum TTR levels (24–26). The APOLLO trial, a randomized, placebo-controlled, phase 3 trial involved 225 patients with ATTRv-polyneuropathy, of whom 126 had concomitant cardiac involvement (27). Patients were randomly assigned in a 2:1 ratio to intravenous patisiran (0.3 mg per kilogram of body weight) or placebo every 3 weeks. Patisiran slowed polyneuropathy progression, assessed using the modified Neuropathy Impairment Score + 7 (mNIS + 7) as primary endpoint, and other measurements including the Norfolk Quality of Life-Diabetic Neuropathy (Norfolk QOL-DN) questionnaire (**Table 1**) (27). The effects of patisiran on cardiac structure and function were assessed in a pre-specified subpopulation of patients with evidence of cardiac amyloid involvement at baseline ($n = 126$). The pre-specified cardiac subpopulation comprised patients with a baseline left ventricular wall thickness ≥13 mm and no history of hypertension or aortic valve disease. Patisiran reduced mean left ventricular wall thickness (least-squares mean difference ± SEM: −0.9 ± 0.4 mm, $P = 0.017$), improved global longitudinal strain (−1.4 ± 0.6%, $P = 0.015$) and cardiac output (0.38 ± 0.19 L/min, $P = 0.044$), led to increased end-diastolic volume (8.3 ± 3.9 ml, $P = 0.036$) at month 18 and reduced N-terminal pro-B-type natriuretic peptide (NT-proBNP) levels at 9 and 18 months. Moreover, patients in the treatment group had a 46% reduction in the rate of hospitalizations due to cardiovascular (CV) causes and all-cause death compared with those receiving placebo (28). Fontana et al. compared 16 patients with ATTRv amyloidosis treated with patisiran (of whom 12 also received diflunisal) with 16 matched untreated patients with ATTRv amyloidosis. A total of 82% of treated patients showed >80% knockdown in circulating TTR. Patisiran therapy was associated with a reduction in extracellular

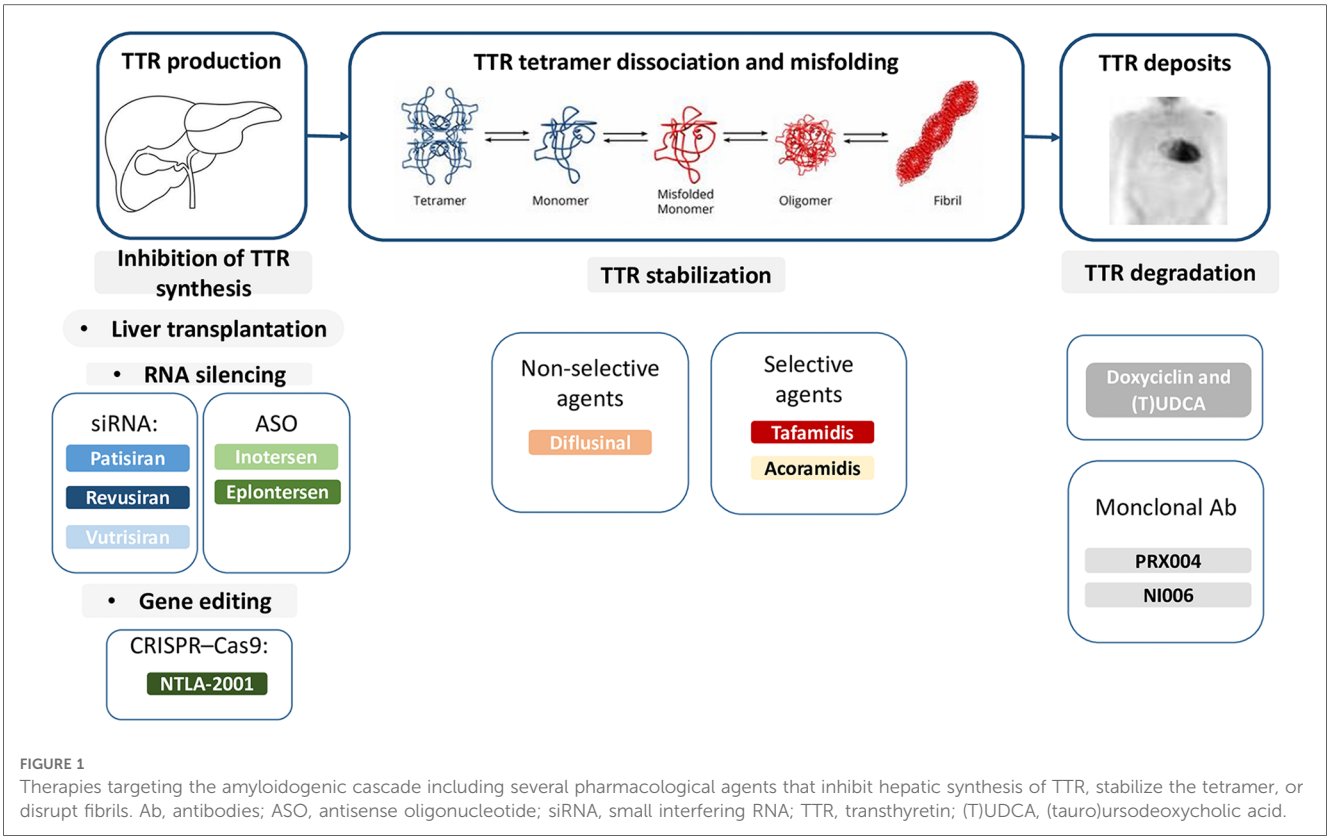

FIGURE 1
Therapies targeting the amyloidogenic cascade including several pharmacological agents that inhibit hepatic synthesis of TTR, stabilize the tetramer, or disrupt fibrils. Ab, antibodies; ASO, antisense oligonucleotide; siRNA, small interfering RNA; TTR, transthyretin; (T)UDCA, (tauro)ursodeoxycholic acid.

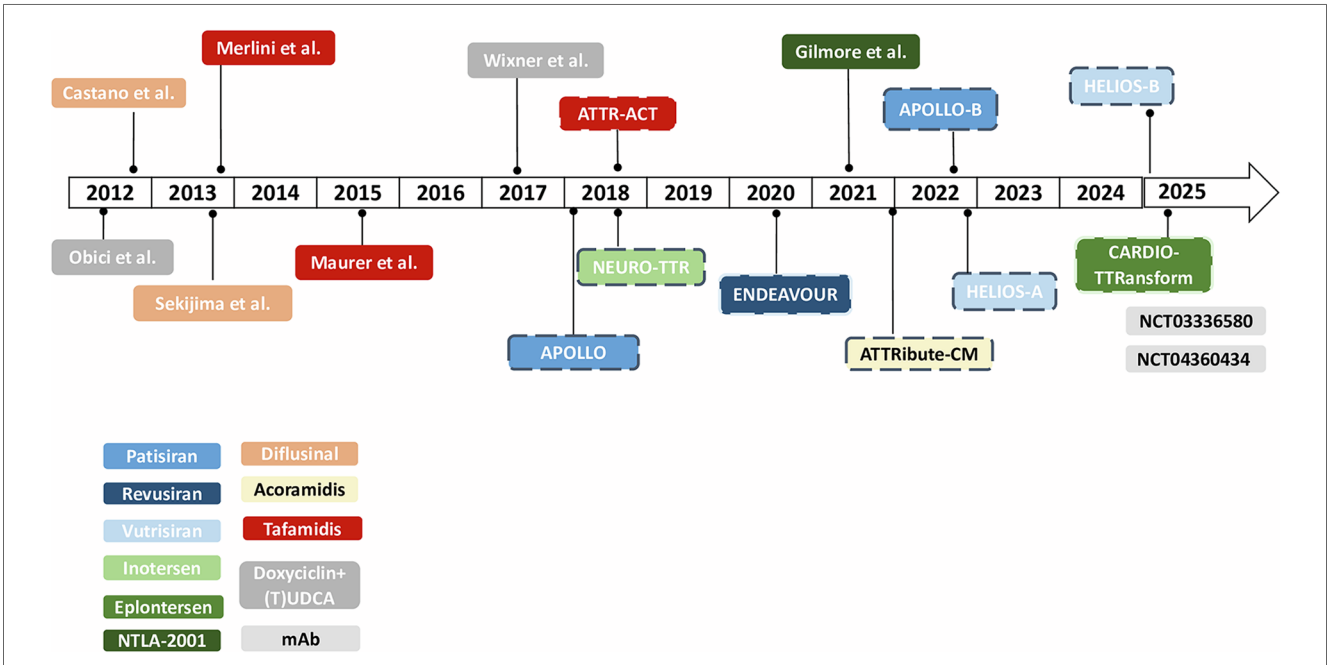

FIGURE 2
Timeline of clinical trials in ATTR-amyloidosis. *Dotted lines refer to phase III clinical trials. mAb, monoclonal antibodies; (T)UDCA, (tauro)ursodeoxycholic acid.

volume (ECV) {adjusted mean difference between groups: −6.2% [95% confidence interval (CI): −9.5% to −3.0%]; $P = 0.001$} although changes in ECV were highly heterogeneous, with only 6 patients (38%) experiencing an absolute ECV reduction greater than the 3% arbitrary threshold and 3 (19%) showing an increase (38). Patisiran was also associated with a decrease in NT-proBNP concentrations and an increase in 6-minute walking test (6MWT) distances after 12 months of therapy (39). Recently, the

TABLE 1 Therapies for amyloid transthyretin cardiomyopathy (ATTR-CM): evidence from clinical trials.

Drug	Study name (year)	Study design	Population	Outcomes
TTR silencers				
SiRNA				
Patisiran	APOLLO (2018) (28, 29)	Phase III, multicentre, randomized, double-blind, placebo- controlled trial; 2:1 randomization to IV patisiran (0.3 mg/kg) or placebo once every 3 weeks for 18 months	225 patients with ATTRv-PN (patisiran $n = 148$; placebo $n = 77$) 126 (56%) patients with concomitant cardiac involvement	Patisiran significantly improved neuropathy scores, QoL, walking parameters, nutritional status and activities of daily living compared to placebo. Patisiran reduced mean LV wall thickness, improved GLS and CO, led to increased LVEDV at month 18 and reduced NtproBNP levels at 9 and 18 months compared to placebo. Reduction of 46% in the rate of CV hospitalization and all-cause death.
	APOLLO-B (2022) (27)	Phase III, randomized, double-blind, placebo-controlled multicenter (patisiran vs placebo)	Patients with ATTRv- or ATTRwt-CM and history of HF; NT-proBNP ranging from 300 ng/L to 8,500 ng/L; 6MWD ≥150 m	Patisiran significantly improved 6MWT and QoL, assessed by KCCQ, at 12 months.
Revusiran	ENDEAVOUR (2020) (30)	Phase III, multicentre, randomized, double-Blind, placebo-controlled; 2:1 randomization to SC revusiran (500 mg) or placebo daily for 5 days, then weekly for 18 months	206 patients with ATTRv- CM (revusiran $n = 140$; placebo $n = 66$)	NA (trial stopped early due to increased mortality compared with placebo
Vutrisiran	HELIOS-A (2022) (31)	Phase III, multicentre, randomized, open-label; 3:1 randomization to SC vutrisiran (25 mg) once every 3 months or IV patisiran (0.3 mg/kg) once every 3 weeks, for 18 months	164 patients with ATTRv- PN (vutrisiran $n = 122$; patisiran $n = 42$)	Vutrisiran signficantly improved neuropathy scores and QoL at 9 months
	HELIOS-B	Phase III, multicentre, randomized, double-blind, placebo- controlled; SC vutrisiran (25 mg) or placebo once every 3 months	Patients with ATTRv- CM or ATTRwt- CM	Estimated study completion date 2025
ASO				
Inotersen	NEURO-TTR (2018) (29)	Phase III trial, randomized, double-blind, placebo controlled; 2:1 randomization to weekly subcutaneous injections of inotersen (300 mg) or placebo.	172 patients with stage 1–2 ATTRv-PN (inotersen $n = 112$; placebo $n = 60$) 108 (63%) patients had ATTRv-CM	Inotersen improved the course of neurologic disease and QoL. No differences in global longitudinal strain and other echocardiographic variables at 15 months Several adverse events, including severe events such as glomerulonephritis and thrombocytopenia, causing durg discontinuation.
Eplontersen	CARDIO-TTRansform	Phase III, multicentre, randomized, double-blind, placebo- controlled trial; randomization to SC injections of either eplontersen or placebo once every 4 weeks	Patients with ATTR-CM (estimated 1400 participants)	Estimated study completion date 2025
Gene editing—CRISPR-Cas9				
NTLA-2001	Gillmore et al. (2021) (32)	Phase I, multicentre, randomized, open- label, placebo- controlled; single IV dose of NTLA-2001: 0.1 mg/kg ($n = 3$) or 0.3 mg/kg ($n = 3$)	6 patients with ATTRv-PN	Interim results from the first two single- dose groups of the trial: administration of NTLA-2001 showed a 52% (47%–56%) in patient receiving 0.1 mg/kg and 87% (80%–96%) in patient receiving 0.3 mg/kg reduction in serum TTR protein concentration from baseline
TTR stabilizers				
Diflunisal	Castano et al. (2012) (33)	Single-arm, open-label study; oral diflunisal 250 mg b.i.d.	13 patients with ATTRv-CM or ATTRwt-CM	Administration was safe, even if not associated with significant change in cardiac structure, function or biomarkers
	Sekijima et al. (2013) (34)	Single-centre study; Diflunisal was administered orally at 500 mg/day	40 patients with ATTRv amyloidosis	Diflunisal was well tolerated and stabilized TTR tetramer
Tafamidis	ATTR-ACT (2018) (35)	Phase III, multicentre, double blind, placebo-controlled trial; 2:1:2 randomization to oral tafamidis 80 mg daily, tafamidis 20 mg daily, or placebo for 30 months	441 patients with ATTRv-CM or ATTRwt-CM (pooled tafamidis: n = 264; placebo: $n = 177$)	Tafamidis was associated with lower all-cause mortality than placebo (HR, 0.70; 95% CI, 0.51–0.96) 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, 0.56–0.81). Differences in overall survival emerged after approximately 18 months. Tafamidis showed also benefits on 6MWT and KCCQ-OS, already relevant at 6 months.

(*Continued*)

TABLE 1 (Continued)

Drug	Study name (year)	Study design	Population	Outcomes
Acoramidis (AG10)	ATTRibute-CM	Phase III, randomized, double-blind, placebo-controlled study; randomization acoramidis (AG10) 800 mg administered orally twice a day vs placebo	Patients with ATTRv-CM or ATTRwt-CM and history of HF	Acoramidis did not change 6MWD at month 12 compared to placebo. An improvement in KCCQ-OS and a decline in Nt-proBNP concentrations as well as in serum TTR concentrations were observed.
TTR disrupter				
Doxycicline and TUDCA	Obici et al. (2012) (36)	Phase II, open-label study with doxycycline (100 mg twice/day) and TUDCA (250 mg three times/day) administered continuously for 12 months	20 patients with TTR-amyloidosis (both FAP or CM)	Doxycicline and TUDCA stabilized the disease for at least 1 year. NIS-LL: stable; LV wall thickness: stable or reduced; NYHA class: no change; NT-proBNP: stable or increased; Improvement of SF-36 physical and mental scores
Doxycicline and UDCA	Wixner et al. (2017) (37)	Phase II study; Part 1: 12-month doxycycline 200 mg/day for 4 weeks and UDCA 750 mg/day with intermittent discontinuation for 2 weeks; Part 2 (6 months): no treatment.	28 patients with ATTR-CM	High discontinuation rate due to treatment failure, side effects and voluntary dropouts. NT-proBNP: no change at month 6; increase at month 12 No increase in LV septum thickness at month 12
Monoclonal antibodies (mAb)				
PRX004	NCT03336580	Phase I study	Subjects with ATTR amyloidosis	Ongoing
NI006	NCT04360434	Phase I study	Patients with hereditary or wild-type ATTR-CM	Ongoing

positive results of the APOLLO B trial have been announced. The APOLLO-B trial randomized patients with ATTRv- CM or ATTRwt- CM and a history of HF (but current clinically stable), NT- proBNP levels ranging from 300 ng/L to 8,500 ng/L and a 6MWD of ≥150 m to patisiran or placebo. Patisiran met the primary endpoint with a statistically significant improvement in 6MWT compared to placebo at 12 months (−8 m in Patisiran arm vs. −25 m in placebo group, with an overall improvement of 14.7 m with Patisiran compared to placebo). It is questionable whether this improvement might be considered significant in clinical practice. Patisiran also met the first secondary endpoint of improvement in quality of life (QoL) as assessed with Kansas City Cardiomyopathy Questionnaire (KCCQ) (40).

Revusiran is a siRNA that was investigated in the phase III ENDEAVOUR trial, enrolling patients with ATTRv-CM. The trial was stopped early due to the high rates of death (13% of patients receiving revusiran and 3% of those receiving placebo) recorded during a median follow-up of 7 months. The majority of patients enrolled in the trial died because of HF. A *post hoc* safety investigation of patients treated with revusiran found that a greater proportion of those who died had ≥75 years and more advanced HF, although a role of revusiran could not be excluded (41).

Vutrisiran is a second-generation siRNA targeting TTR mRNA64 similar to patisiran, with an enhanced stabilization chemistry that allows its subcutaneous administration every 3 months. An initial phase I study proved its safety and efficacy (42). The subsequent HELIOS-A trial included 164 patients with ATTRv-PN who received a 25 mg dose of vutrisiran every 3 months ($n = 122$) or active comparator patisiran ($n = 42$). At 9 months, vutrisiran met the primary endpoint of improvement in mNIS +7 score and of all secondary endpoints (31). Vutrisiran is being studied for the treatment of both ATTRv and ATTRwt-CM in the phase III, randomized, double- blind, placebo-controlled, multicentre HELIOS-B trial. The primary endpoint is a composite of all- cause mortality and recurrent CV events (CV hospitalizations and urgent HF visits) at 30–36 months (NCT04153149) (Table 1). Full results are expected in early 2024.

3.1.2. Antisense oligonucleotides

Antisense oligonucleotide (ASO) can silence target mRNA sequences by a variety of mechanisms, mostly involving degradation mediated by the endogenous ribonuclease RNase H1. Inotersen is a 2′-O-methoxyethyl–modified ASO, that binds the 3′ untranslated region of human TTR mRNA (both wildtype and variant) and inhibits the production of liver TTR-protein (Figure 1). It is administered weekly by subcutaneous injection. Inotersen was initially tested in a phase I, randomized, placebo-controlled, double-blind, dose-escalation study with patients receiving the highest dose regimen showing the greatest reduction in serum TTR levels up to 76% (43).

The NEURO-TTR was a randomized, double-blind, placebo controlled, phase 3 trial that randomized 172 patients in a 2:1 ratio to weekly subcutaneous injections of inotersen (300 mg) or placebo. Inotersen improved the course of neurologic disease and QoL (Table 1). However, several adverse events occurred in the treatment group (e.g., injection site reactions, nausea, headache, fatigue, fever and thrombocytopenia) causing drug discontinuation in 14% of the cases. Furthermore, serious adverse events as glomerulonephritis (3%) and thrombocytopenia (3%, with one consequent death) were registered. Thus, frequent platelet count and renal function monitoring is recommended during treatment (29). This important adverse effects might result in treatment cessation, and have significant implications on the use of this drug in both current clinical practice and in the setting of a clinical trial. In the subgroup of patients with cardiac

disease (as well as in the whole population) from the NEURO-TTR trial no significant differences were reported regarding echocardiographic parameters at the 15-month follow-up (29). A small single-centre, open-label study including patients affected by ATTRwt-CM and NYHA I-III showed, at a 2- and 3-years follow-up, a reduction in left ventricular (LV) mass measured by magnetic resonance imaging (MRI) and an increase in exercise tolerance as measured by 6MWT. The main adverse effect was inflammation and induration associated with subcutaneous injection; a few patients developed generalised "flu-like" symptoms 24–48 h after the injection, at first or second exposure and without further episodes. At 3 years, the average platelet count was decreased by 15% but none of the patients had bleeding episodes or severe thrombocytopenia (44). A further study is ongoing (NCT03702829).

Eplontersen is a novel antisense nucleotide that is administered subcutaneously every 4 weeks and with no serious adverse effects reported in a phase I study. NEURO-TTRansform is a phase III trial that randomized 140 patient with stage 1 or 2 hereditary transthyretin-mediated amyloid polyneuropathy (ATTRv-PN) in a 6:1 ratio to either eplontersen 45 mg once every 4 weeks or inotersen 300 mg once a week until the pre-specified week 35 interim efficacy analysis. Participants received daily supplemental doses of the recommended daily allowance of vitamin A. Participants included in the inotersen reference arm were crossed over to eplontersen at Week 37 after completing the Week 35 assessments. The final efficacy analysis at week 66 compared eplontersen with the historical placebo arm from NEURO-TTR trial (45). At 66 weeks, patients treated with eplontersen demonstrated a statistically significant and clinically meaningful change from baseline vs. placebo group on the co-primary endpoints of modified Neuropathy Impairment Score +7 (mNIS + 7) and Norfolk Quality of Life Questionnaire-Diabetic Neuropathy (Norfolk QoL-DN). The trial also met its third co-primary endpoint demonstrating a statistically significant reduction in serum TTR concentration in the eplontersen vs. placebo group. TTR reductions were consistent with those reported in the interim analysis at week 35. Eplontersen' efficacy in ATTR-CM is being studied by CARDIO-TTRansform study that will randomize 1,400 patients with ATTR-CM and NYHA class I-III to eplontersen (subcutaneous injection once every 4 weeks) or placebo. The primary endpoint is a composite of CV mortality or recurrent CV clinical events up to week 140. Secondary outcome will evaluate also functional capacity and QoL. (NCT04136171).

3.2. ATTR stabilizers

Tetramer stabilizers prevent monomer misfolding and deposition by binding to the T4-binding site on TTR (e.g., tafamidis and diflunisal) or by mimicking the super-stabilizing TTR variant T119M (e.g., acoramidis).

3.2.1. Non-selective agents: diflunisal

Diflunisal is a non-steroidal anti-inflammatory (NSAID) drug that can be classified as a non-selective ATTR stabilizer agent. In 2006, a phase 1 study showed that diflunisal (250 mg per os b.i.d.) complexes to the T4 binding site and stabilizes TTR tetramers, thus preventing amyloid fibril formation *in vitro*. In a single-arm, open-label study (mean follow-up 0.9 ± 0.3 years) involving 13 patients with confirmed ATTRwt- or v-CM, diflunisal was safe, but did not improve cardiac structure, function, or biomarkers (33). In a further study including 40 ATTRv amyloidosis patients, diflunisal stabilized TTR tetramer structure over a mean follow-up of 38.0 ± 31.2 months. As a NSAID, the main adverse effects include gastrointestinal bleeding and renal failure (34).

3.2.2. Selective stabilizers: tafamidis, acoramidis

Tafamidis is a small molecule that stabilizes TTR tetrameric structure by binding to the protein's thyroxine binding sites. In the early 2010s tafamidis (at a dosage of 20 mg) proved effective and safe for the treatment of patients with early-stage Val30Met transthyretin familial amyloidotic polyneuropathy (FAP) with a reduction in the neurological symptoms' progression (46). The results were then confirmed in a phase 2 open-label, single-treatment arm study enrolling patients with non-Val30Met TTR amyloidosis (47).

Tafamidis was firstly approved by the European Medicines Agency (EMA) for the treatment of TTR amyloidosis in adult patients with stage 1 symptomatic polyneuropathy but not by the US Food and Drug Administration (FDA).

The ATTR-ACT trial, published in September 2018, was a multicentre, international, double-blind, placebo-controlled, phase 3 trial that randomized 441 patients with ATTR-CM (both variant and wt) in a 2:1:2 ratio to tafamidis 80 mg, 20 mg or placebo. The primary outcome was a hierarchical composite of all-cause mortality, followed by frequency of cardiovascular-related hospitalizations. Over a 30-month follow-up the rates of the primary endpoint were lower among patients in the treatment arm compared to those who received placebo ($P < 0.001$). Tafamidis was associated with both lower all-cause mortality [hazard ratio, 0.70; 95% confidence interval (CI), 0.51–0.96] and a lower rate of CV-related hospitalizations than placebo, with a relative risk ratio of 0.68 (0.48 per year vs. 0.70 per year; 95% CI, 0.56–0.81) (48). Differences in overall survival emerged after approximately 18 months. Secondary endpoints included performance on 6MWT and KCCQ-Overall Summary (KCCQ-OS) score. The benefits of tafamidis on these secondary endpoints were already evident at 6 months (48). Long-term extension study with all patients subsequently switched to the highest dose supported tafamidis 80 mg (bioequivalent of tafamidis free acid 61 mg) as the optimal dose, considering the lack of adverse events and a significant reduction (30%) in the relative risk of death on a median follow-up of 51 months (30). Among patients enrolled in the ATTR-ACT trial, 335 were affected by ATTRwt-CM and 106 by ATTRv-CM. Patients with ATTRwt had milder disease and lower rate of disease progression over the study. However, tafamidis showed similar benefits on mortality and QoL in both subgroups (49). A *post hoc* analysis of ATTR-ACT reported a reduction in patients treated with tafamidis of both CV hospitalization (−32%) and mean length of

stay per CV-related hospitalization events. Taken together, tafamidis reduced by 2.62 days CV-related hospitalizations per patient per year and in a subgroups analysis of patients with NYHA functional class I or II, by 3.96 days (50). Earlier treatment initiation was associated with better outcomes. Indeed, an interim analysis of the long-term extension to the pivotal ATTR-ACT showed that patients initially treated with tafamidis in ATTR-ACT had substantially lower mortality than those first treated with placebo and then transitioned to tafamidis [79 (44.9%) deaths with continuous tafamidis and 111 (62.7%) with placebo to tafamidis, hazard ratio, 0.59 (95% CI, 0.44–0.79); $P < 0.001$] (51). The reduction in mortality was similar in patients with ATTRwt and patients with ATTRv (≈40% in each), whereas there was a greater reduction in patients with NYHA class I or II (44%) than NYHA class III (35%) in the continuous tafamidis group compared with the placebo to tafamidis group (51).

Based on the results of the ATTR-ACT trial and of these subgroup analyses showing greater benefits in patients with NYHA functional class I and II, the 2021 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure recommend tafamidis for the treatment of patients with ATTRv or ATTRwt cardiomyopathy, and NYHA class I or II to reduce symptoms, CV hospitalization and mortality (class of recommendation I, level of evidence B) (3). The 2022 ACC/AHA/HFSA guidelines recommend tafamidis also in patients with NYHA class III symptoms and add that tafamidis should be considered in select patients (52).

Acoramidis, also known as AG10, binds with high affinity and selectivity TTR in human serum. It is designed to mimic the structure of the protective T119M mutation and forms hydrogen bonds with the same serine residues at position 117 that stabilize the T119M variant. It has proven efficacy in stabilizing TTR both for Val122Ile mutation and TTRwt amyloidosis without significant adverse effects (53). Acoramidis 800 mg twice a day vs. placebo is currently being investigated in ATTRibute-CM, a phase 3 randomized controlled trial enrolling patients with symptomatic ATTR-CM (NCT03860935). ATTRibute-CM did not meet its primary endpoint at month 12 with a mean observed 6MWD decline of 9 meters and 7 meters for the acoramidis and placebo arms, respectively. However, an improvement in KCCQ-OS and a decline in Nt-proBNP concentrations as well as in serum TTR concentrations were observed (54). Considering that tafamidis showed a significant reduction in mortality only after 18 months, the investigators included a co-primary endpoint in a longer timeframe, a hierarchical combination of all-cause mortality, frequency of cardiovascular-related hospitalization and change in the total distance walked in 6 min over a 30 months period.

3.3. TTR disruption/resorption

As both TTR stabilizers and silencers do not influence advanced ATTR-CMs, drugs defined as ATTR degraders are supposed to be the only ones that may lead to pathology regression, targeting already deposited amyloid fibrils.

Doxycycline proved able to remove TTR amyloid deposits, and tauroursodeoxycholic acid (TUDCA) reduced non fibrillar-TTR and other markers associated with pre-fibrillar TTR (**Figure 1**). Combined administration of these drugs had synergic effects in removing amyloid deposits in transgenic TTR mice models, in early disease stages (55). A phase II, open-label study demonstrated that doxycycline (100 mg twice/day) and TUDCA (250 mg three times/day) administered continuously for 12 months in patient with TTR-amyloidosis (FAP and/or CM) stabilized the disease for at least 1 year. Only 2 patients out of 20 discontinued the therapy, one due to gastric pain and the other because of persistent nausea and loss of appetite (36). Another phase II study conducted on 28 patients with ATTR-CM with a combination of doxycycline and ursodeoxycholic acid (UDCA), showed less benefits and a high discontinuation rate due to treatment failure, side effects and voluntary dropouts. The study was divided into two parts, the first one with a 12-month period treatment with doxycycline 200 mg/day for 4 weeks with intermittent discontinuation for 2 weeks, associated with UDCA 750 mg/day; the second consisted in a withdrawal period in which disease progression was monitored. The high rate of treatment failure may indicate that doxycycline discontinuation may decrease the efficacy of the drugs and that TUDCA may be more effective than UDCA (37). However, the results remain difficult to be interpreted (22). ATTR degraders showed better results in younger patients with less advanced disease (56). Doxycycline plus TUDCA or UDCA are currently not approved by EMA or FDA due to the small number of patients included in the studies and the controversial results (57). Wider RCTs are needed to assess effectiveness of the treatment.

3.4. Gene editing

Clustered regularly interspaced short palindromic repeats and associated Cas9 endonuclease (CRISPR-CAS-9), enabling targeted *in vivo* genome editing, could be an emerging therapy in ATTR amyloidosis. A recent report described the partial results of a phase I, open-label, multicentre trial on an *in vivo* gene-editing therapeutic agent, called NTLA-2001, showing promising results. NTLA-2001 is based on the CRISPR-Cas9 system and comprises a lipid nanoparticle encapsulating messenger RNA for Cas9 protein and a single guide RNA targeting TTR. This preliminary analysis was conducted on 6 patients with ATTRv-PN. NTLA-2001 was administered on a single escalating dose of 0.1 or 0.3 mg per kilogram. To avoid inflammatory reactions patients were pre- treated with glucocorticoids and antihistamines. Twenty-eight days after the injection, mean reduction in serum TTR protein concentration from baseline was 52% with 0.1 mg/Kg and 87% with 0.3 mg/Kg. Therapy was well tolerated and no patients experienced serious adverse events (32). Recruiting in the study is still ongoing with a target of 72 patients (NCT04601051). This study will evaluate safety, tolerability, pharmacokinetics and pharmacodynamics of NTLA-2001 in ATTRv-PN and in both ATTRv-CM and ATTRwt-CM. Estimated primary completion date is March 2025. NTLA-2001

has a clear advantage compared to siRNA-based and ASO-based therapies since the knockdown is expected to be permanent after a single injection not requiring serial infusions.

3.5. Monoclonal antibodies

In the last years anti-TTR humanized monoclonal antibodies (mAb) were also investigated (**Figure 2**) (13). In preclinical studies on ex vivo and *in vivo* models these antibodies could promote clearance of amyloid fibrils by selectively targeting ATTR aggregates and promoting macrophage-mediated phagocytosis. Importantly, these antibodies did not react with native TTR tetramers but detected misfolded proteins, by targeting epitopes more exposed in TTR dissociative monomers, non-native oligomers, and/or aggregates (58, 59).

PRX004 is an investigational mAb designed to prevent fibril formation by specifically targeting and clearing the misfolded forms of the TTR protein found in ATTR-CM. Its safety, tolerability, pharmacokinetics, pharmacodynamics, and maximum tolerated dose are currently being evaluated in a phase 1 study (NCT03336580). NI006 is another investigational human mAb that targets TTR amyloid. A phase 1 trial with NI006 evaluating its safety, pharmacokinetic profile and exploratory outcome measures of efficacy in patients with hereditary or wild-type ATTR-CM is ongoing (NCT04360434) (13).

4. ATTR symptomatic treatment

4.1. Heart failure

The main goal of symptomatic therapy in patients with amyloid CM is to improve QoL and well-being. The supportive treatment in patients with signs or symptoms of HF is based on maintenance of euvolaemia and consists in loop diuretics (usually furosemide) (3, 60). The optimal balance between diuretic therapy and fluid status may be challenging to achieve due to the reduced stroke volume typical of restrictive cardiomyopathies (61).

The use of neurohormonal modulators, namely beta-blockers, angiotensin converting enzime inhibitors (ACE-Is), angiotensin receptor blockers (ARBs) and angiotensin receptor neprilysin inhibitor (ARNI) is controversial in those patients presenting with HF and reduced ejection fraction (62). Indeed, on one hand patients with CA may have a significant neurohormonal activation similarly to non-amyloidotic HF patients (63). On the other hand, these drugs may be not tolerated due to the development of hypotension (62, 64). Beta-blockers may be poorly tolerated also because of conduction disturbances or decreasing cardiac output with consequent exercise intolerance when cardiac output becomes critically dependent on heart rate (35, 65). In a recent study including 309 consecutive patients with ATTR-CM, there was no association of neurohormonal blockade use with survival (64). In the 2021 ESC position paper on the diagnosis and treatment of cardiac amyloidosis, it is recommended to avoid beta-blocker and ACE-i/ARBs as they exacerbate hypotension, particularly when amyloid autonomic dysfunction is present (2). Similar indications can be found in other major documents from scientific societies on cardiac amyloidosis (60). Nevertheless, about one third of patients in the ATTR-ACT trial were on beta-blockers or ACEi/ARB (48).

Conversely, use of MRA is not generally contraindicated and it is recommended (Class I) in Canadian Cardiovascular Society/Canadian Heart Failure Society (CCS/CHFS) cardiac amyloidosis' guidelines even in the absence of specific studies (66). MRAs are already approved for the treatment of HFrEF (Class I) and HFmrEF (Class IIb) (3) and recent studies focused on the use of spironolactone in HFpEF. Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist (TOPCAT) is a phase III, multicenter, international, randomized, double-blind, placebo controlled trial conducted on 3,445 patients that showed that adding spironolactone to existing therapy in patient with HF and LVEF ≥45% did not significantly reduce the incidence of the primary outcome of death from CV causes, aborted cardiac arrest or hospitalization for HF. However, HF hospitalizations were significantly reduced in the spironolactone group. An analysis from TOPCAT trial showed that the subset of patients with structural and functional echocardiographic features typical of cardiac amyloidosis, despite having a worse prognosis, experienced similar benefits from spironolactone therapy to other patients (67).

The diagnosis of CA was an exclusion criterion in major clinical trials on sodium-glucose-cotransporter-2 (SGLT2) inhibitors. A series of 15 consecutive patients with ATTR-CA and diabetes treated with SGLT2 inhibitors by the diabetologists has been reported without significant adverse effects (68). Due to their cardiorenal benefits, the robust safety and tolerability, with clinical trial data reporting minimal effects on blood pressure, glycaemia-related adverse events, and no excess in acute kidney injury (69, 70), it appears reasonable that SGLT2 inhibitors may be well tolerated and might be helpful, at least due to their add-on diuretic effect and by reducing diuretic resistance. Currently, no RCTs specifically investigated safety and benefits of HF therapies, namely neurohormonal antagonists, SGLT2 inhibitors, in patients with cardiac amyloidosis.

Similarly, the oral soluble guanylate cyclase stimulator vericiguat may be considered in patients with HFrEF, NYHA class II-IV who have had worsening HF despite other evidence based medical therapies in patients with HFrEF (71). However, CA was an exclusion criteria of the VICTORIA trial and vericiguat has never been tested in patients with ATTR-CM.

4.2. Atrial fibrillation and anticoagulation

Atrial fibrillation (AF) seems to be more common in cardiac amyloidosis than in the general population. Sanchis et al. reported an overall AF prevalence of 44% among patients with both AL and ATTR-CM (72). The prevalence is even higher in patients with ATTRwt-CM and has been estimated around 70% (73–75). However, previous studies have shown no relationship

between AF and prognosis in ATTR-CM (72, 73). This might be explained by the frequent absence of atrial contraction at echocardiographic assessment, defined as "atrial electromechanical dissociation" (AEMD) even in sinus rhythm (SR) (76). These patients presenting AEMD have, indeed, a poorer prognosis than CA patients in SR but with an effective atrial contraction and have similar prognosis to those with AF (76).

As a consequence of atrial dysfunction and enlargement, patients with ATTR-CA have a higher risk of left atrial thrombosis even when in SR. This may also be related to the amyloid deposition into the atrial wall, and the cardiotoxic damage of atrial cardiomyocytes by amyloid precursors (77). Among patients with CA and AF/atrial flutter, anticoagulation is indicated regardless of CHA2DS2-VASc score as in these patients it is not associated with the probability of LAA thrombosis. The role of anticoagulation in patients with CA and sinus rhythm as well as the choice between vitamin K antagonists (VKAs) and direct oral oral anticoagulants (DOACs) are other unmet needs that should require a RCT. A few retrospective studies showed no differences between DOACs and VKAs in embolic events, bleeding risk and overall mortality (78–80). A study by Mentias et al. analysed 551 patients with amyloidosis and new diagnosis of AF and found a decreased risk of mortality, ischemic stroke and major bleeding events when DOAC was prescribed, compared to VKA (81). Transesophageal echocardiogram should be performed in all patients before cardioversion of atrial arrhythmias since the chance of intracardial thrombus remains high, even among patients receiving adequate anticoagulation (82, 83).

Management of AF in cardiac amyloidosis is complicated due to restrictive physiology and the frequent association with autonomic dysfunction. Patients often do not tolerate rate control therapies (beta-blockers, calcium-channel blockers and digoxin) as they may exacerbate hypotension and they reduce the compensatory heart rate, which is the main driver of cardiac output (84). As a result, rhythm control strategy may be preferred. However, Mints et al. found no mortality benefit with antiarrhythmic drugs vs. rate control strategy (73). Evidence regarding transcatheter ablation of atrial arrythmias are limited to date. El-Am et al. investigated outcome of direct-current cardioversion (DCCV) for atrial arrhythmias in patients with CA. Although the success rate of restoring sinus rhythm was high, tachyarrhythmias and bradyarrhythmias complicating DCCV were significantly more frequent in CA patients compared with control patients (82). In all these studies, the rate of AF recurrence was higher than in the general population. More recently, Donnellan et al. has shown that rhythm control strategies, including antiarrhythmic drugs, ablation and DCCV, were more effective when performed early in the course of the disease (85).

4.3. Rhythm disturbances and devices

As a consequence of myocardial tissue infiltration, ATTR-CM patients are more prone to develop rhythm disturbances, such as atrio-ventricular blocks, sick sinus syndrome or atrial fibrillation with bradycardia, needing pacemaker implantation (2, 7). Predictors of pacemaker implantation have been recently described in a large cohort of patients (19). The indications for pacemaker implantation are the same as in patients without CA in the absence of specific evidence (60).

As ATTR-CM is a restrictive cardiomyopathy, stroke volume may be markedly reduced and chronic right ventricular apical pacing can result in left ventricular dyssynchrony. Cardiac resynchronisation therapy (CRT) may be considered if a high burden of pacing is expected (5, 61). Nonetheless, the benefits of CRT have been established in patients with non-amyloidotic HF and further investigations are warranted in CA. An implantable cardioverter defibrillator (ICD) should be used in secondary prevention with standard indications (60). ICD implantation in primary prevention is controversial as sudden cardiac death may be caused by electromechanical dissociation as seen in studies conducted mainly on AL-CM patients. Nevertheless, selected patient may benefit from ICD placement (22, 60, 86–88).

4.4. Aortic stenosis

Among patients with proven TTR-CA, up to 16% had moderate or severe aortic stenosis (AS). ATTR-CM has a prevalence ranging from 4% to 29% among patients with severe AS, with a higher prevalence of the low-flow low-gradient phenotype (89–92). Notably, amyloid deposition did not worsen prognosis of patients undergoing transcatheter aortic valve replacement (TAVR) (90, 91). Further studies are needed to assess the best treatment options in these patients.

5. Final remarks

Increased awareness in ATTR-CM has led to the development of new disease-modifying therapies, to an earlier diagnosis in the course of the disease and to a better management of the patients. (93) Ioannou et al. performed a retrospective analysis using data from the National Amyloidosis Centre, London, between 2002 and 2021 with the aim to characterize changes in the clinical phenotype of patients diagnosed with ATTR-CA over the past 20 years. They showed a progressive increase in the referrals. This was accompanied by a greater number of ATTR-CA diagnoses ($n = 35$ in the first period, 2002–2006; $n = 968$ in the last period, 2017–2021), predominantly of the wild-type form. Importantly, a greater proportion of patients were diagnosed at an early-stage over time. This was associated with a progressive decrease in mortality during the study period (2007–2011 vs. 2012–2016: hazard ratio, 1.57 [95% CI, 1.31–1.89], $P < 0.001$; and 2012–2016 vs. 2017–2021: hazard ratio, 1.89 [95% CI, 1.55–2.30], $P < 0.001$) (94).

Patients in earlier milder disease stages are less likely to experience events (death, heart failure admissions, arrhythmia) in clinical trials. This might explain why the ATTR-ACT trial was successful (as patients enrolled some years ago may have been

sicker, with the placebo group experiencing more events), whereas the more recent ATTRibute-CM testing acoramidis failed its primary endpoint at 12 months. Of note, a mortality benefit with tafamidis was only seen at 18 months, as opposed to 12 months, which again might in part explain the failure of AG10 to meet primary end point at 12 months. As a result of the above, there is now a need of wider RCTs. While the ATTR-ACT trial enrolled <500 patients, more recent studies had to extend the population included. The ongoing eplontersen' CARDIO-TTRansform has significantly expanded its recruitment target. The initial recruitment target was below 1,000, but the trial sponsors have recognised the importance of the changing phenotype over time and have rightfully opted to expand recruitment significantly to 1,400 in an attempt to increase the power of the study. The earlier diagnosis of the disease, the discovery of an appropriate therapy and the consequent reduction in overall mortality can explain the negative results of some recent trials.

Nowadays, the only approved treatment for ATTR-CM is tafamidis, a TTR stabilizer, that has shown important benefits on survival and QoL in the ATTR-ACT trial. Tafamidis exceeds conventional cost- effectiveness thresholds. The high cost of tafamidis prevents certain patients from accessing treatment, particularly in privatised healthcare systems. Furthermore, the high cost has led to tafamidis not being publicly funded in countries with publicly funded free healthcare. Such countries include UK and Australia. This high cost barrier to these patients accessing life changing medication is extremely important, and really emphasises the need for stringent, thoughtful and appropriately powered clinical trial design so that ultimately patients can get access to affordable treatment.

Of note, as the first benefits of tafamidis on prognosis have been shown after 18 months of treatment, it should be started soon in the natural course of the disease, therefore an early diagnosis remains crucial. Two TTR silencers are approved only for patients with ATTRv polyneuropathy and possible concomitant cardiomyopathy: patisiran and inotersen. TTR silencers and stabilizers prevent amyloid formation but have no effects on already deposited fibrils. TTR degraders can remove fibrils and bring a regression of the pathology but have more adverse effects and no effective phase III trial was yet conducted. Hypothetically, an association between stabilizers/silencers and degraders may be a good strategy, but there is no clear evidence yet. New anti-TTR humanized antibodies could be an alternative in fibrils removal, mediated by phagocyte activation. CRIPSR-CAS9, an innovating gene-editing therapy used in hereditary pathologies could be a real revolution in the natural course of ATTR-CM with first studies showing promising results.

Symptomatic therapies, except for diuretic therapy in decompensated HF, are largely not assessed. ACEi/ARB/ARNI and beta-blockers are generally not recommended even in patients with reduced LVEF. Conversely, spironolactone showed a potential benefit in patients enrolled in the TOPCAT trial with suspected CA. SGLT2 inhibitors deserve further investigations. Anticoagulation in patients in both sinus rhythm and atrial fibrillation remain a major unmet need.

Author contributions

DT and GB equally contributed to ideation and writing the first draft of the paper. Other authors revised the manuscript. MM contributed to ideation and revision of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor (AP) declared a past co-authorship with the authors (DT and MM).

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Final farewell to Claudio Rapezzi: observation, deduction and knowledge in medicine

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Many colleagues have known him as a brilliant mind and prominent member of the cardiological and amyloidosis community, many others as a trustful and distinguished partner in research with flashes of extraordinary intelligence and an exceptional ability for lateral thinking, and many more as a close friend with extremely sharp irony and culture. However, Professor Claudio Rapezzi was primarily a mentor with the unique ability to ignite the minds and the hearts of young researchers and colleagues with his scientific passion.

Although he was aware of his exceptional qualities, Prof. Rapezzi was extremely humble, he loved spending time in his small office writing of science, and despite being extremely busy, his door was always open to everyone. He had a natural disposition in human relationships, promoting a safe environment for young physician to improve their medical knowledge. He was a progressive thinker and, as a teacher, he embodied the qualities of curiosity, critical thinking, observation, passion and creativity (**Figure 1**). Meeting Professor Rapezzi marked a fundamental moment in the career of many young researchers. The path of those who had the privilege to be supervised by or to collaborate with him was influenced in many different and sometimes unexpected ways.

William Osler once said "*The whole art of medicine is in observation… but to educate the eye to see, the ear to hear and the finger to feel takes time, and to make a beginning, to start a man on the right path, is all that you can do*". Professor Claudio Rapezzi dedicated his life to the study of medicine and became a master in the art of observation. During a career spanning almost 50 years, he has deeply transformed the cardiological and the amyloidosis community worldwide.

With his unique qualities of curiosity, imagination and immense culture, he was the exemplar of the physician and detective character with his ability to spot inconsistencies in challenging clinical scenarios in a contemporary version of Sherlock Holmes (1). He shared with the cardiology community the "red flag approach" in cardiomyopathies and, particularly, in amyloidosis (2, 3). Indeed, one of the mottos he loved and was used to share was "When you have eliminated the impossible, whatever remains, however improbable, must be the truth" (The Sign of the Four, 1890), and this is exactly how he was often teaching his students and peers how to approach the suspicion and identification of rare cardiac diseases.

He was among the first researchers to understand the key value of carpal tunnel syndrome as an early clinical marker of future development of cardiac amyloidosis (4, 5).

FIGURE 1
Professor Claudio Rapezzi during a discussion at the "Advances in Heart Failure, Cardiomyopathies and Pericardial Diseases" held in Trieste (Italy).

Professor Rapezzi considered electrocardiography as the longest-running tool for non-invasive tissue characterisation. Through his expert interpretation, Professor Rapezzi was able to explore the presence of amyloid deposition in the heart and to predict what endomyocardial biopsy and cardiac magnetic resonance would have demonstrated many years later (6, 7). Professor Rapezzi was very passionate on dissecting the heterogeneous clinical phenotype of ATTR amyloidosis (8, 9). His classification of ATTR clinical phenotypes (cardiac, neurological and mixed) has been implemented worldwide by clinicians, researchers and pharmaceutical companies. He coordinated the Transthyretin Amyloid Outcome Survey (THAOS), with the final aim of understanding and characterizing the natural history of ATTR amyloidosis (10). In early 2000s, Professor Claudio Rapezzi was a pioneer in investigating the clinical use of scintigraphy with bone tracers for the diagnosis of cardiac amyloidosis (11, 12). Ten years later, that intuition paved the way for the development of a non-invasive algorithm for the diagnosis of transthyretin cardiac amyloidosis in an international collaboration with the National Amyloidosis Centre (London, UK) which has substantially changed the paradigm for diagnosing ATTR cardiac amyloidosis and has dramatically reduced the need for invasive endomyocardial biopsy (13–15). Ideally, every diagnosis made without the need for cardiac biopsy is a tribute to the fine intelligence and original intuition of Professor Claudio Rapezzi and those who collaborated with him over time.

Professor Rapezzi coordinated the international phase 3 Safety and Efficacy of Tafamidis in Patients With Transthyretin Cardiomyopathy (ATTR-ACT) trial of tafamidis (16), which is the only drug ever tested in ATTR cardiac amyloidosis to have impacted survival. In 2018, he presented the results of the ATTR-ACT at the European Society of Cardiology Congress held in Munich and ignited the audience with his passion and culture (**Figure 2**). Professor Rapezzi considered tafamidis as a drug of "firsts", highlighting four themes:

- The first drug to show efficacy in ATTR cardiac amyloidosis;
- The first example of precision medicine in the treatment of a cardiomyopathy;
- The first drug to show efficacy in heart failure with preserved ejection fraction;

- The first drug without anti-neurohormonal activity to show efficacy in heart failure;

The ATTR-ACT study has transformed the treatment of ATTR cardiac amyloidosis and revolutionised the lives of patients suffering from this increasingly diagnosed cause of heart failure; in no small way, this is thanks to Professor Rapezzi.

On top of his undisputed scientific expertise, Professor Rapezzi was highly considered for his critical approach to the methodology of advancing medical knowledge. The question of "nosology" and its application in cardiomyopathies was central in Professor Rapezzi's vision, who was a great estimator of the writer Umberto Eco and the philosopher Karl Popper. A recent stimulating example is represented by the impossible interview between Sherlock Holmes and David Sackett about the fundamental question "how much can we trust the guidelines?" (17). Professor Rapezzi's pupils will never forget his positive approach to "error in medicine" as a source of thinking and a unique opportunity for advancing medical understanding. In this field, Professor Rapezzi coordinated an international group of researchers with the aim of defining criteria for classification of cardiomyopathies in 2008 (18). Understanding that the boundaries of restrictive cardiomyopathy have become increasingly blurred, he recently identified the limitations of the classification of restrictive cardiomyopathy encountered in clinical practice today and proposed a new definition of this specific form of heart disease (19).

Professor Rapezzi dedicated the last years of his career to the foundation of the Italian Network for Cardiac Amyloidosis (20), with the aim of promoting collaboration among Italian centres involved in the care of patients with suspected or confirmed cardiac amyloidosis. The legacy of Professor Rapezzi continues today with the many ideas and research questions that awaits to be explored by his pupils disseminated worldwide: the role of electrocardiography in the contemporary care of patients with cardiac amyloidosis (21), the cardiomyopathy-oriented interpretation of echocardiographic findings (22, 23), the association between cardiac amyloidosis and aortic stenosis, the many questions on the mechanisms of bone tracers binding to the amyloid infiltrated heart (24), the clinical usefulness of tafamidis in ATTR-CM patients with NYHA functional class III symptoms, the correlation between genotype and phenotype

FIGURE 2
Professor Rapezzi presenting the results of the ATTR-ACT study at the European society of cardiology congress held in Munich.

in ATTR amyloidosis (25), gender differences in ATTR-CM (26–28), the new frontiers in treatment strategies and the possibility of combination therapy (29).

Professor Claudio Rapezzi was a giant of the amyloid field. It is worth asking to ourselves "What the amyloidosis culture in Italy and in the world would have been without Professor Rapezzi?". How will the field keep progressing without him?". Most of his former collaborators and peers were actually wondering what innovations his brilliant mind was conceiving when he showed up at the 2022 International Society of Amyloidosis meeting in Heidelberg.

We will never forget his legendary humour and immense culture during his scientific talks or chairing sessions in the countless meetings across the world.

From his legacy, we should all start approaching the medical field by dissecting the overall picture into its multiple and detailed features, like he was used to do when he referred to the Pink Floyd Prism album cover when talking about the different aspects and heterogeneity of amyloidosis.

We are close to his beloved Marinella, friends and colleagues in Ferrara, Bologna and all around the world.

He will be greatly missed and will continue being an inspiration for the next generations.

Author contributions

AP, GS, CCQ, MF and JDG conceived, drafted and edited the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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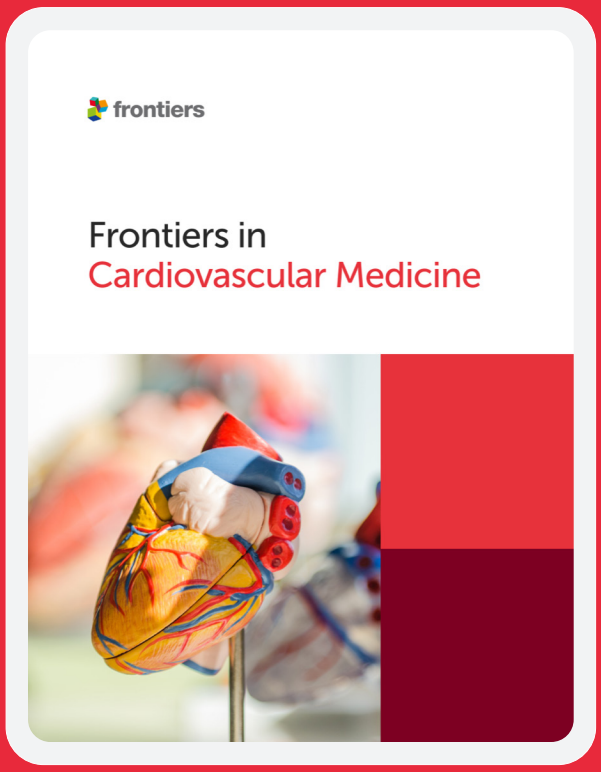

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