ATTR Amyloid Cardiomyopathy: Characterization of Extracellular Vesicles as Potential Stratifiers and Prognostic Biomarkers of the Disease

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1. INTRODUCTION

Amyloidosis is still considered a rare disease; however, it remains underdiagnosed, suggesting that its true prevalence is much higher. The etiology of the disease involves the deposition of amyloid proteins in different organs, including the heart. Recent data report a progressive increase in the prevalence of this disease in the United States, rising from 18 cases per 100,000 persons per year to 55.2 cases per 100,000 persons per year, reinforcing the concept of limited diagnosis[1]. Amyloid protein formation occurs through cleavage, denaturation, or overproduction of abnormal proteins, generating oligomers that may exhibit an antiparallel β-pleated sheet configuration, forming insoluble amyloid fibrils (7 to 10 nm in diameter) that accumulate in the extracellular space of tissues and may lead to organ dysfunction[2].

Although several tissues and organs may be affected due to systemic amyloidosis, cardiac involvement is the main cause of morbidity and mortality related to this condition[3,4]. Five types of amyloidosis can affect the heart: immunoglobulin light-chain amyloidosis (AL), transthyretin amyloidosis (TTR), serum amyloid A protein, apolipoprotein AI, and immunoglobulin heavy-chain amyloidosis. However, AL and TTR amyloidoses account for more than 95% of all cardiac amyloidosis cases[5]. Although AL amyloidosis is more aggressive, TTR amyloidosis tends to be diagnosed later due to its slower progression and because it is clinically well tolerated. As a consequence, 39% of patients are diagnosed at an advanced stage, presenting inability to walk, heart failure, or sudden cardiac death[1,6].

Transthyretin (TTR) is a 127-amino-acid transport protein, expressed mainly by the liver. Under physiological conditions, it circulates as a

homotetramer; however, due to genetic mutations or aging, these tetramers become destabilized, releasing monomeric TTR and enabling misfolding[7]. Transthyretin amyloidosis encompasses two forms of the disease: age-related protein degeneration (wild-type ATTR) and familial transthyretin amyloidosis (variant TTR)[8].

Patients with cardiac amyloidosis frequently present heart failure due to increased filling pressures with preserved ejection fraction, resulting from progressive thickening of the biventricular walls and diastolic dysfunction due to reduced cardiac compliance[1,3,4]. Despite the common clinical manifestations of cardiac amyloidosis, recent studies have shown that TTR-CA may present a distinct clinical phenotype: it occurs in older individuals, with manifestations such as carpal tunnel syndrome 4 to 5 years before the diagnosis of CA, small-fiber motor neuropathy, low-voltage electrocardiogram, atrial fibrillation, low-flow aortic stenosis with significant left ventricular hypertrophy, and heart failure disproportionate to the degree of aortic stenosis[5,9,10,11].

Although there are multiple consolidated diagnostic methods for TTR-CA, its early diagnosis remains a major challenge because its clinical symptoms overlap with those of other diseases[12,13,14]. In recent years, extracellular vesicles (EVs) have emerged as biomarkers for several diseases, including cancers and cardiovascular diseases, through their quantification and biological characterization[15,16,17]. These properties contain important information, as EVs participate in intercellular communication by transferring biological content in the form of proteins, lipids, nuclear material, miRNAs, and non-coding RNAs from their cells of origin. Thus, they may contribute to remote functional effects and

promote homeostasis and immunity in physiological or pathological processes, including inflammatory responses[18],[19].

According to their size, EVs are classified as microvesicles (0.1 to 1 μm) or exosomes (<0.1 μm) and are present in most body fluids. They can be protective—promoting the removal of harmful cellular components and supporting cell survival—or deleterious, favoring the signaling of mechanisms harmful to the heart, potentially contributing to heart failure development[20,21,22]. Growing evidence shows that EVs play essential roles in intercellular communication among cardiac tissue cells and are involved in regulating cardiomyocyte hypertrophy, apoptosis, and cardiac fibrosis[23]. These are pathophysiological features often modulated during cardiac remodeling in amyloid cardiomyopathy.

However, although several studies have demonstrated the involvement of EVs in acute ischemic events and heart failure, the evaluation of EV profiles and characterization in TTR-CA remains poorly understood. To date, the few published studies on this topic have shown that patients with AL amyloidosis, who often present renal failure as a consequence of the deposition disease, had urinary exosomes carrying high amounts of light-chain oligomers compared with patients with nephropathies of other etiologies[24,25]. Another study demonstrated that EVs isolated from the brains of mice induced with Alzheimer's disease contributed to neuronal death, the transport of neurotoxins, and the perpetuation of the overexpression of β-amyloid precursor protein that accumulated over time[26]. These data reinforce that, just as changes in EV concentration and biological composition occur in other cardiovascular diseases and cardiomyopathies, such modulation is also expected in amyloid cardiomyopathy.

Conversely, there is evidence showing that protective exosomes may bind to non-vesicular proteins in each physiological environment, thereby reducing their circulating concentration. Therefore, it is evident that EVs may play an ambiguous role, exerting protective or deleterious effects[27],[28]. Thus, we believe that EVs may serve as biomarkers of disease progression in cardiac amyloidosis.

The rationale for this project is rooted in the underdiagnosed nature of cardiac amyloidosis, a condition often considered rare and usually identified only at advanced stages of life, when clinical symptoms have already become evident. In light of this reality, there is an urgent need to develop earlier diagnostic methods capable of detecting the disease before severe clinical outcomes occur.

In this challenging context, the analysis of the proteomic signature present in extracellular vesicles emerges as a promising approach. These vesicles, released by cells, carry information that reflects their cellular origin and play a crucial role in intercellular communication. Thus, the proposed research aims to identify specific biomarkers present in extracellular vesicles that can be detected in the early stages of cardiac amyloidosis. It is expected that this approach will not only enable more effective therapeutic interventions, but also provide substantial improvements in the quality of life of individuals affected by this debilitating and often overlooked pathology.

2. OBJECTIVE

2.1 Primary Objective

The primary objective of this study is to quantify and characterize serum extracellular vesicles (EVs) in patients with different forms of transthyretin-related

amyloidosis (TTR) and to investigate their role as a biomarker of cardiac involvement progression.

2.2 Secondary Objectives

- Investigate the relationship between the quantity and characteristics of serum EVs and ventricular function in different study groups of patients with TTR-CA.
- Evaluate the association of serum EVs with cardiac imaging findings, including cardiac magnetic resonance, echocardiography, and technetium-pyrophosphate scintigraphy, across the different patient groups.
- Perform statistical analyses, such as linear or logistic regression, to identify significant predictors and assess the predictive value of EVs in relation to parameters of cardiac dysfunction/amyloidosis, as well as to investigate potential associations among these parameters.

3. METHODOLOGY

3.1 Study Site and Sampling

This prospective, single-center study will be conducted at the Clinical Unit of Cardiomyopathy of the Heart Institute (InCor – HCFMUSP), located at the Hospital das Clínicas of the University of São Paulo School of Medicine (HCFMUSP), in São Paulo, Brazil. This center is nationally and internationally recognized for its excellence in the treatment of cardiac diseases and has a complete and adequate infrastructure for the conduct of scientific studies.

Sixty patients will be selected from those treated at the Clinical Unit of Cardiomyopathy of InCor – HCFMUSP and will be subdivided into three distinct groups, as described below:

- **TTR-CA + DM (n=20):** TTR cardiac amyloidosis with myocardial dysfunction;
- **TTR-A (n=20):** TTR amyloidosis with neurological involvement;
- **TTR-Gen (n=10):** patients who carry the genotype but do not present the phenotype of TTR amyloidosis;
- **CTL (n=20):** Healthy individuals;

Volunteer selection will be based on the study's inclusion criteria. Eligible patients will be invited to participate and will receive a detailed explanation regarding the objectives, procedures, potential risks, and benefits of the study. Participation will be voluntary and subject to the signing of the Informed Consent Form (ICF) by the participant or by a close family member if the patient is incapacitated. The limited sample size is a direct consequence of the rarity of the disease under investigation.

3.2 Diagnosis of Transthyretin Amyloid Cardiomyopathy

Patients with TTR cardiac amyloidosis will be diagnosed based on a combination of imaging methods and clinical assessments. These methods include:

- **Electrocardiogram (ECG):** For evaluation and detection of possible electrical abnormalities.

- **Echocardiography with Tissue Doppler and Strain Analysis:** This technique will allow the assessment of myocardial thickening, increased interatrial septal thickness, enlargement and increased volume of the left atrium, as well as the identification of diastolic dysfunction.

3.3 Inclusion Criteria

• Adult patients aged 18 years or older;

• Confirmed diagnosis of transthyretin-related cardiac amyloidosis (TTR-CA), with or without myocardial dysfunction, according to established diagnostic criteria;

• Willingness to comply with study procedures and requirements;

• Ability to provide written informed consent;

3.4 Exclusion Criteria

• Presence of other significant cardiac conditions that may interfere with the study outcomes, such as severe coronary artery disease or major valvular disease;

• Inability to provide informed consent or to participate in the required clinical assessments and examinations;

3.5 Study Design

After participation in the study is confirmed, the initial clinical examinations will be scheduled. Volunteers will be evaluated regarding their personal and family health history, and the variables to be collected are described in the study's Case Report Form (CRF).

3.6 Collection and Biochemical Analysis of Myocardial Injury Markers

Approximately 10 mL of whole blood will be collected from all study participants in dry tubes for the analysis of biochemical markers of myocardial injury, such as CK-MB, troponin, and BNP. The tubes will then be kept for one hour at 37°C to allow clot retraction. Next, the tubes will be centrifuged at 2,000 rpm for 10 minutes, and the serum will be separated, aliquoted into microtubes, and stored in an ultra-freezer at −80°C for subsequent biochemical analysis.

3.7 Serum Preparation and Storage

Approximately 10 mL of whole blood from all study participants will be collected in a dry tube. The tubes will then be kept for one hour at 37°C to allow coagulum retraction. Next, they will be centrifuged at 2,000 rpm for 10 minutes, and the serum will be separated, aliquoted into microtubes, and stored in an ultra-freezer at –80°C.

3.8 Isolation of Extracellular Vesicles (EVs)

The isolation of EVs will follow the mitochondrial separation procedure described by Higuchi et al[29]. Serum samples will be diluted (1:5) in a dense, sugar-rich medium containing 200 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, and 0.5 g/L BSA (pH 7.2), and incubated at room temperature for one hour. Samples will then be centrifuged for 12 minutes at 9,500 g, and the supernatants will be collected for further processing.

3.9 Morphological Analysis of EVs by Transmission Electron Microscopy (TEM)

Morphological evidence of EVs will be obtained using Transmission Electron Microscopy (TEM). Sample embedding will follow the rapid procedure described by Duarte et al[30]. etect exosomes and microvesicles present in the supernatant after phase separation, 400 µL of the supernatant will be fixed by adding 1 mL of 3% glutaraldehyde at 4°C for 3 hours, followed by postfixation with a reduced osmium solution (700 µL of 1% osmium tetroxide + 700 µL of 3% potassium ferricyanide) at 4°C for 30 minutes, and centrifugation at 13,000 g for 5 minutes at 12°C. This procedure allows pellet formation, as lipid-rich exosomes and microvesicles become heavier after fixation. The pellet will then be collected and washed in a solution containing 0.9% NaCl + 360 mOsm/kg sucrose, and incubated in 0.5% uranyl acetate for 3 hours at 4°C.

The pellets will then be dehydrated for 10 minutes in 70% ethanol, 5 minutes in acidified 2,2-dimethoxypropane with 0.1 N HCl, and 2 minutes in 4% copper sulfate in acetone. Infiltration will be performed using a mixture of EponEMbed 812 resin and araldite 502 resin (1:1), and polymerization will take place in an oven at 100°C for 1 hour. Blocks will be sectioned using an ultramicrotome at 60–70 nm thickness and placed on palladium-coated copper–nickel grids (200 mesh) (Electron Microscopy Sciences, Hatfield, PA, USA).

3.10 Protein Signature of EVs (LC/MS/MS)

The proteomic profiling of serum EVs will be performed using liquid chromatography coupled with mass spectrometry (LC/MS). After isolation, the supernatant containing EVs will undergo protein extraction with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors without EDTA. Protein concentration will be determined via spectrometry using the Bradford method[31]., according to a standard BSA calibration curve.

An aliquot corresponding to 100 µg of total protein from each sample will be incubated with sample buffer containing ¼ β-mercaptoethanol, and heated at 100°C for 5 minutes for protein denaturation. Samples will then be subjected to 10% polyacrylamide gel electrophoresis at 140 V for 1 hour. After electrophoresis, gels will be rinsed with deionized water, proteins will be fixed, and the gel will be stained for 1 hour using Coomassie Blue R-350. Protein bands will be excised, recovered, and digested with trypsin following the protocol described by Lundby et a[32]. Extracted peptides will be analyzed on a high-resolution LC/MS system, allowing identification of proteins of interest.

3.11 Quantification and Characterization by Nanosight – NTA (Nanoparticle Tracking Analysis)

A Serum EV characterization (size, concentration, and protein signature) will be performed using a highly sensitive nanoparticle tracking method (Nanosight – NTA). Using a NanoSight NS300 ultramicroscope equipped with a high-sensitivity sCMOS camera (Malvern Instruments, Ltd., Malvern, UK), particle detection will be carried out by light scattering when irradiated by a laser

beam, and particle size will be determined according to the Stokes–Einstein equation, based on Brownian motion.

Serum-derived EVs will be diluted (1:50) in particle-free PBS (0.02 µm filtered) to achieve a concentration within the recommended measurement range (1–10×10⁸ particles/mL). Immunolabeling of predefined proteins will follow antibody datasheet recommendations, and detection will be performed using the appropriate fluorophore–laser wavelength combination.

Image acquisition settings will include: automatic camera level, manual threshold and focus adjustment, 25°C measurement temperature, 5 runs at 30 fps, and 60-second recordings. Scattered light images will be automatically analyzed using NTA software (version 3.4). Light-scattering dot plots and movement-velocity graphs will then be generated, enabling quantification of unlabeled and/or immunolabeled particles (Malvern Panalytical, Ltd., Malvern, UK).

3.12 Statistical Analysis

Statistical analyses will be performed using GraphPad Prism 6. Each variable will first be assessed for normality using the Kolmogorov–Smirnov distance test, and classified as parametric or non-parametric.

Parametric (normally distributed) data will be presented as mean ± standard deviation, and group comparisons will be performed by ANOVA, followed by Tukey’s post-hoc test. Non-parametric (non-normal) data will be presented as median and interquartile range, and comparisons will be made using the Kruskal–Wallis test, followed by Dunn’s post-hoc test.

Spearman's correlation test will be performed to assess potential associations between EV characteristics and the degree of myocardial dysfunction across groups, as well as imaging findings. A p-value < 0.05 will be considered statistically significant.

3.13 Ethical Considerations

The principal investigator will ensure that Informed Consent (Appendix 1) is obtained in accordance with international ethical and regulatory guidelines established by the ICH (International Council for Harmonisation) for Good Clinical Practice and the ethical principles defined by the Brazilian National Health Council Resolution No. 466/2012.

This study will be submitted for approval by the Research Ethics Committee (CAPPesq) via Plataforma Brasil. All ethical considerations involving human research defined by Resolution 466/2012 will be followed.

All collected data will be treated confidentially and in compliance with the Brazilian General Data Protection Law (LGPD). After study completion, data will be anonymized and analyzed by the InCor research team. Upon final analysis, results are expected to be published in scientific journals.

3.14 Risk and Benefit Assessment of the Clinical Protocol

3.14.1 Risks

During participation, some procedures may cause mild discomfort or minimal risks. ECG may cause mild skin adhesion sensation or, rarely, allergic reactions to electrode adhesives. Echocardiography is painless, although the conductive gel may cause cold or wet sensations, with rare gel-related allergic reactions. Technetium pyrophosphate scintigraphy involves slight discomfort during injection and minimal radiation exposure, with very low risk of allergic reactions to the radiopharmaceutical. MRI may be uncomfortable for individuals with claustrophobia, and gadolinium contrast carries risks for patients with renal impairment. Blood collection may cause needle puncture discomfort, bruising, and post-collection dizziness.

3.14.2 Benefits

Expected benefits include comprehensive cardiac health evaluation through advanced imaging and diagnostic tools that enable early detection of abnormalities related to cardiac amyloidosis and other conditions.

Participation also contributes to the advancement of scientific knowledge on cardiac amyloidosis and its early diagnosis.

Investigating extracellular vesicles as potential biomarkers may lead to more sensitive and specific diagnostic methods and more targeted and effective therapies, benefiting not only participants but also the broader medical community and future patients.

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