ORIGINAL ARTICLE

Association of Anti-β1 and Anti-M2 Antibodies with Autonomic Nervous System Modulation in Patients with Chronic Chagas Cardiomyopathy

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Abstract

Background: Different immune mechanisms of myocardial damage involved in the pathophysiology of Chagas disease coexist with high titers of autoantibodies induced by *T. cruzi*. There are few studies in the literature about the adaptive role of anti-β1 and anti-M2 antibodies in chronic Chagas cardiomyopathy (CCC).

Objectives: To evaluate the association between anti-β1 and anti-M2 antibodies with heart rate variability (HRV) parameters on 24h Holter monitoring and the rate-pressure product (RPP) on cardiopulmonary exercise testing (CPET).

Methods: Anti-β1 and anti-M2 antibody titers were measured by enzyme-linked immunosorbent assay (ELISA) in 64 patients affected by CCC. Analysis of HRV was performed through the time-domain indices NNs, mean NN, SDNN, SDANN, SDNN index, NNNs, RMSSD, and pNN50. Spearman's correlation coefficient was used to assess the association between antibody titers and numerical variables. The Mann–Whitney test was used for comparison between two groups. Multiple linear regression was used to identify independent variables capable of explaining anti-β1 and anti-M2 antibody titers at the 5% significance level.

Results: On 24h Holter, during the period of greatest parasympathetic activation (2:00-6:00 a.m.), an inverse association was found between anti-β1 titers and SDNN (r_s=-0.13, p=0.041, n=43), as well as a direct association between anti-M2 titers and SDANN (r_s=0.317, p=0.039, n=43). Regarding CPET variables, anti-β1 titers were directly associated with RPP (r_s=0.371, p=0.005, n=56). The subgroup of patients with a normal chronotropic response showed higher anti-β1 titers than the subgroup with an impaired response (p=0.023). RPP was an independent explanatory variable for anti-β1 titers, although with a low coefficient of determination (R^2=0.147).

Conclusion: The findings of this study suggest that, in patients with CCC, anti-β1 and anti-M2 antibodies may affect HRV parameters. RPP was directly associated with higher anti-β1 titers.

Keywords: Chagas Disease/ physiopathology; Antibodies, Bispecific; Anti-B1, Anti-M2; Antibody, Formation; Autonomic Nervous System.

Introduction

Chagas disease (CD) is one of the four leading causes of death from infectious and parasitic diseases in Brazil.[1-3] The elucidation of its pathogenesis was marked by pivotal studies that evaluated cross-reactivity between the ribosomal P protein of *Trypanosoma cruzi* and human ribosomal proteins (molecular mimicry), as well as by advances in the knowledge of the structure of β-adrenergic receptors, used as antigens to screen for specific anti-β-adrenergic antibodies in chronic Chagas cardiomyopathy (CCC).[4,5]

Köerbele[6] advocates that CCC is essentially neurogenic, while most authors note it is characterized by irreversible

damage caused by the presence of autoantibodies.[7,8] Recent studies have called into question the primary nature of autonomic nervous system abnormalities. Endomyocardial biopsies show continuous and progressive destruction of myocardial fibers with cardiac remodeling, as well as the presence of inflammatory and neuronal mediators in Chagas-related HF and HF of other etiologies.[9-11]

The extent of myocardial damage leads to progressive ventricular dilatation with impairment of cardiac receptors and consequent autonomic dysfunction, which can predate the onset of ventricular dysfunction and occur at all stages of the disease.[12-14]

Anti-β1 and anti-M2 antibodies detected early in the natural history of the disease seem to exert effects on cardiac electrogenesis through an agonistic action similar to that of adrenaline and acetylcholine, respectively. The various forms of Chagas heart disease cannot be distinguished by high serum levels of these antibodies, nor by markers of left ventricular function.[15-19]

In heart failure (HF) of other etiologies, the presence of anti-β1 autoantibodies causes prolonged activation of these cardiac receptors, inducing cell dysfunction, arrhythmias, and apoptosis[12,13,18,19,20], but in CD, some patients with CCC exhibit low levels of norepinephrine, unlike those with HF in the same functional class but of non-Chagas etiologies.[21,22]

Agonists for receptors coupled pleiotropically to multiple cell signaling pathways may preferentially signal some pathways over others, i.e., display bias towards certain signals. The agonist-like and allosteric effects of autoantibodies and autonomic modulation in CD warrant further investigation.

Within this context, the present study investigated the association of high titers of anti-β1 and anti-M2 antibodies in CCC with autonomic dysfunction as assessed by heart rate variability (HRV) and the behavior of the rate-pressure product (RPP) during cardiopulmonary exercise testing (CPET).

Methods

This was a cross-sectional study of 64 patients with CCC recruited from the outpatient cardiomyopathy clinic at the Instituto Nacional de Cardiologia (INC). This convenience sample was followed for a period of 3 years and 8 months, and is part of an extensive database created as part of a doctoral research project.[23-24]

The study sample was approved by the INC Research Ethics Committee, and all patients read and signed an informed consent form.

Inclusion criteria were age over 18 years and positive serology by two methods (hemagglutination and ELISA).[25] Patients with pacemakers, on stem cell therapy, with insulin-dependent diabetes mellitus, stage IV hypertension, chronic kidney disease (serum creatinine ≥ 2.5 mg/dL), anemia (hemoglobin < 9.0 mg/dL), thyroid disease, or heart valve disease with hemodynamic compromise were excluded from the sample.

All patients received optimized clinical treatment for HF according to their NYHA functional class. Drugs with β-adrenergic action, vasodilators, diuretics, statins, angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), and antiarrhythmics were prescribed as per routine practice.

Anti-cardiac receptor response

Anti-β1 (adrenergic) and M2 (muscarinic) receptor antibody titers were determined by means of an enzyme immunoassay (ELISA). The colorimetric response was measured by reading the optical density at a wavelength of 450 nm in a Molecular Devices microplate reader.[26]

Twenty-four-hour Holter monitoring

Patients were fitted with a *Cardio Flash* monitor (Cardio Sistemas Comercial e Indústria Ltda.) for evaluation of HRV. Autonomic modulation,[27] expressed by HRV parameters in the time domain (milliseconds), was evaluated by specific software. Data on cardiac cycle fluctuations were obtained, and the indices related to these fluctuations were represented by the average over the whole recording period (24h) and by periods of the circadian cycle of greater sympathetic (1 p.m. to 5 p.m.) and parasympathetic (2 a.m. to 6 a.m.) activity. The parameters of interest were NNs, mean NN, SDNN, SDANN, SDNN index, NNNs, RMSSD, and pNN50. Normal limits were based on the published literature.[28] The parameters associated with greatest risk of arrhythmic events or death are: SDANN < 40 ms, SDNN < 50 ms, RMSSD <15 ms, and pNN50 < 1%.

Cardiopulmonary exercise test

Exercise testing was performed on an Imbrasport ATL treadmill (Comércio de Aparelhos Eletrônicos Ltda.). Fifty-six patients underwent CPET, all on prescribed

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therapy. The ramp, Bruce, and Naughton protocols were used. Functional aerobic capacity was assessed by VO_2max and the *rate-pressure product (RPP = HR x SBP)*. The blood pressure response during the test was used as an indicator of the heart's inotropic response. *Chronotropic incompetence* (CI) was defined as the patient's inability to reach 85% of the maximum HR predicted for their age (220 - age; *CI = predicted HR - achieved HR × 100 / HR).*[28-30]

Statistical analysis

Nonparametric methods were used, as antibody titers were not normally distributed (demonstrated by rejection of the assumption of normality according to the Kolmogorov–Smirnov test). The level of significance was set at 5% ($p < 0.05$). Statistical analysis was performed in the SAS® System software environment, version 6.11 (SAS Institute, Inc., Cary, North Carolina), and in SPSS version 17.0.

The observed data were expressed descriptively as median and interquartile range (IQR) or absolute (n) and relative (%) frequency.
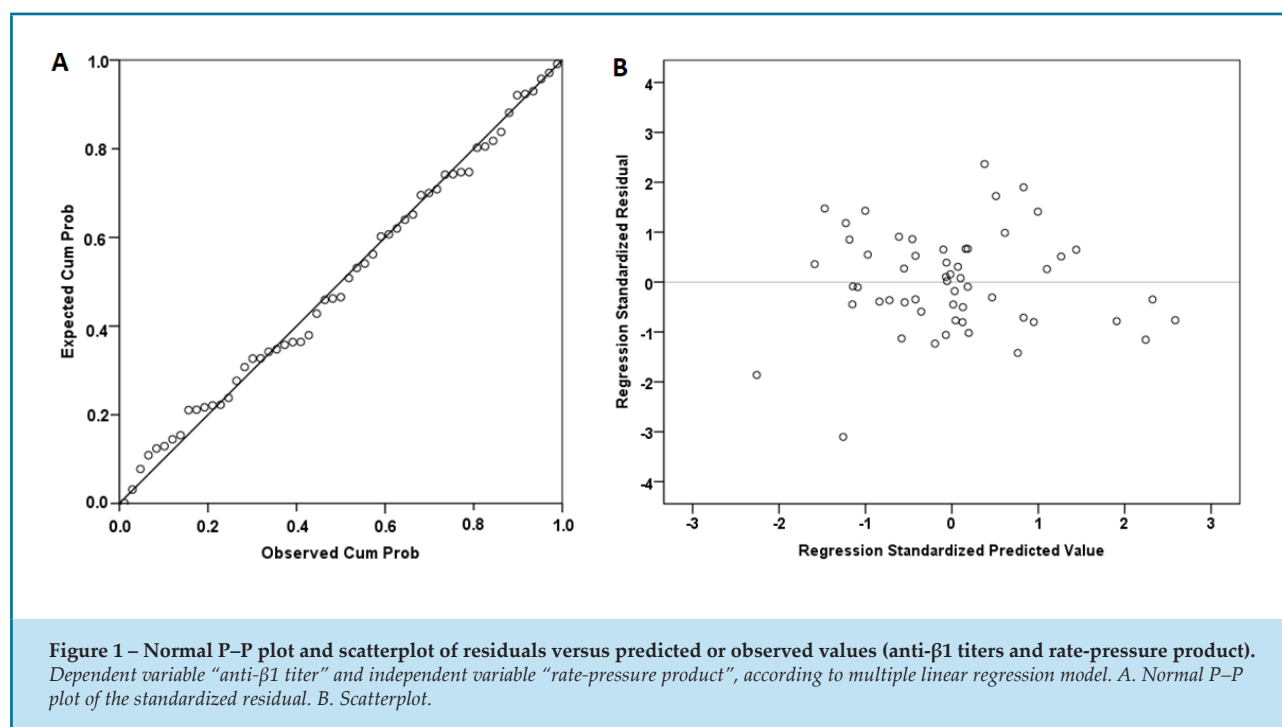
The association of anti-β1 and anti-M2 antibody titers with numerical variables was analyzed by Spearman's correlation coefficient, while the Mann–Whitney test was used for comparisons between two groups.

Multiple linear regression (MRL) was used to identify independent variables to explain anti-β1 and anti-M2 titers. Log-transformed data were used for the titers. As shown in Figure 1, regarding anti-β1 titers and RPP, the P-P plot does not provide any evidence contradicting the assumption of normality of residuals, and the scatterplot of residuals as a function of the standardized predicted values is quite random, validating the assumption that the residuals are independent and identically distributed. The Kolmogorov-Smirnov test did not reject the hypothesis of normality of the residuals (p = 0.71).

Results

Sample profile

A total of 64 patients with CCC were included. The median age was 63 years (IQR 13.6). The sample was predominantly female (70%); 39 patients were white (61%), and 14 were black (22%). All but one were migrants (n=63, 98%), many of whom had left their states of origin (mostly Bahia, Minas Gerais, and Pernambuco) more than 30 years before. A history of poor housing conditions was found in 80% of the sample.

Figure 1 – Normal P–P plot and scatterplot of residuals versus predicted or observed values (anti-β1 titers and rate-pressure product).
Dependent variable "anti-β1 titer" and independent variable "rate-pressure product", according to multiple linear regression model. A. Normal P–P plot of the standardized residual. B. Scatterplot.

Infection occurred via the natural route in 63 patients (98%) and via blood transfusion in 1 patient (2%). The social history of the sample was characterized by high rates of smoking, alcoholism, and illiteracy.

Only 1 patient had a diagnosis of diabetes. Most (78%) were in NYHA class I or II, with a median EF of 55% by Simpson's method. *(Table 1).*

Anti-cardiac receptor antibodies

Anti-β1 and anti-M2 antibody titers were evaluated in 64 patients. The median titer of anti-β1 antibody was 0.986 (IQR 0.528), and the anti-M2 titer, 1.275 (IQR 0.625). Considering the cut-off values defined in a previous study comparing a population with and without Chagas disease[26], 42.2% of patients were reactive for anti-β1 (>1.054) and 45.3% for anti-M2 antibodies (>1.297).

Table 1 – Sample profile at baseline	Total sample
Characteristic	**(N=64)**
Age in years, median (IQR)	63 (13.6)
Female sex, n (%)	45 (70)
Ethnicity, n (%)	
White	39 (61)
Black	11 (17)
Other	14 (22)
BMI in kg/m², median (IQR)	26 (3.7)
State of origin, n (%)	
BA	18 (28)
MG	16 (25)
PB	10 (16)
Other	20 (31)
Smoking, n (%)	20 (31)
Alcoholism, n (%)	33 (52)
Educational attainment – primary education, n (%)	1 (2)
Time away from endemic area in years, median (IQR)	30 (20.3)
History of living in a stucco house, n (%)	51 (80)
Type 2 diabetes mellitus, n (%)	1 (2)
Systolic blood pressure in mmHg, median (IQR)	120 (20)
Heart rate in bpm, median (IQR)	60 (13.8)
NYHA functional class, n (%)	
I	29 (45)
II	21 (33)
III	14 (22)
Ejection fraction (Simpson's method), %, median (IQR)	55 (21)
Atrial fibrillation, n (%)	5 (8)
Medications for heart failure, n (%)	
ACEI or ARB	51 (80)
β-blocker	24 (38)
Mineralocorticoid receptor antagonist	14 (22)

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Twenty-four-hour Holter monitoring and cardiopulmonary exercise testing

During the study, only 43 patients underwent 24-hour Holter monitoring and 56 patients underwent CPET. Reasons for this relatively low prevalence included scheduling difficulties, referral for cell therapy and subsequent loss to follow-up at the outpatient cardiomyopathy clinic, or pacemaker placement before CPET could be performed.

On 24-hour Holter monitoring, a significant inverse correlation was observed between anti-β1 titers and the SDNN index in the period from 2 to 6 a.m. (r_s=0.313; p=0.041; n=43); — the higher the anti-β1 titer, the lower the expected value of the SDNN index from 2 to 6 a.m. — and a significant, direct correlation between M2 titers and SDANN from 2 to 6 a.m. (r_s=0.317; p=0.039; n=43) — the higher the anti-M2 titer, the higher the SDANN value. (Figure 2; Table 2).

On analysis of CPET results, a significant direct correlation was observed between anti-β1 titer and the RPP (r_s=0.371; p=0.005; n=56): the higher the anti-β1 titer, the higher the RPP. (Figure 2). On multiple linear regression, the RPP was the only independent variable capable of explaining anti-β1 antibody titer at the 5% significance level (p=0.016). The overall explanatory power of the model, however, was low (R^2 = 0.142), i.e., only 14.2% of the variability in anti-β1 antibody titers was explained by the RPP.

The subgroup of patients with a normal chronotropic response had higher anti-β1 titers than the subgroup with an impaired chronotropic response (p=0.023). The subgroup with a normal inotropic response had higher anti-M2 titers than the subgroup with an impaired inotropic response (p=0.044). Chronotropic and inotropic responses were analyzed in only 51 and 54 patients, respectively, because they
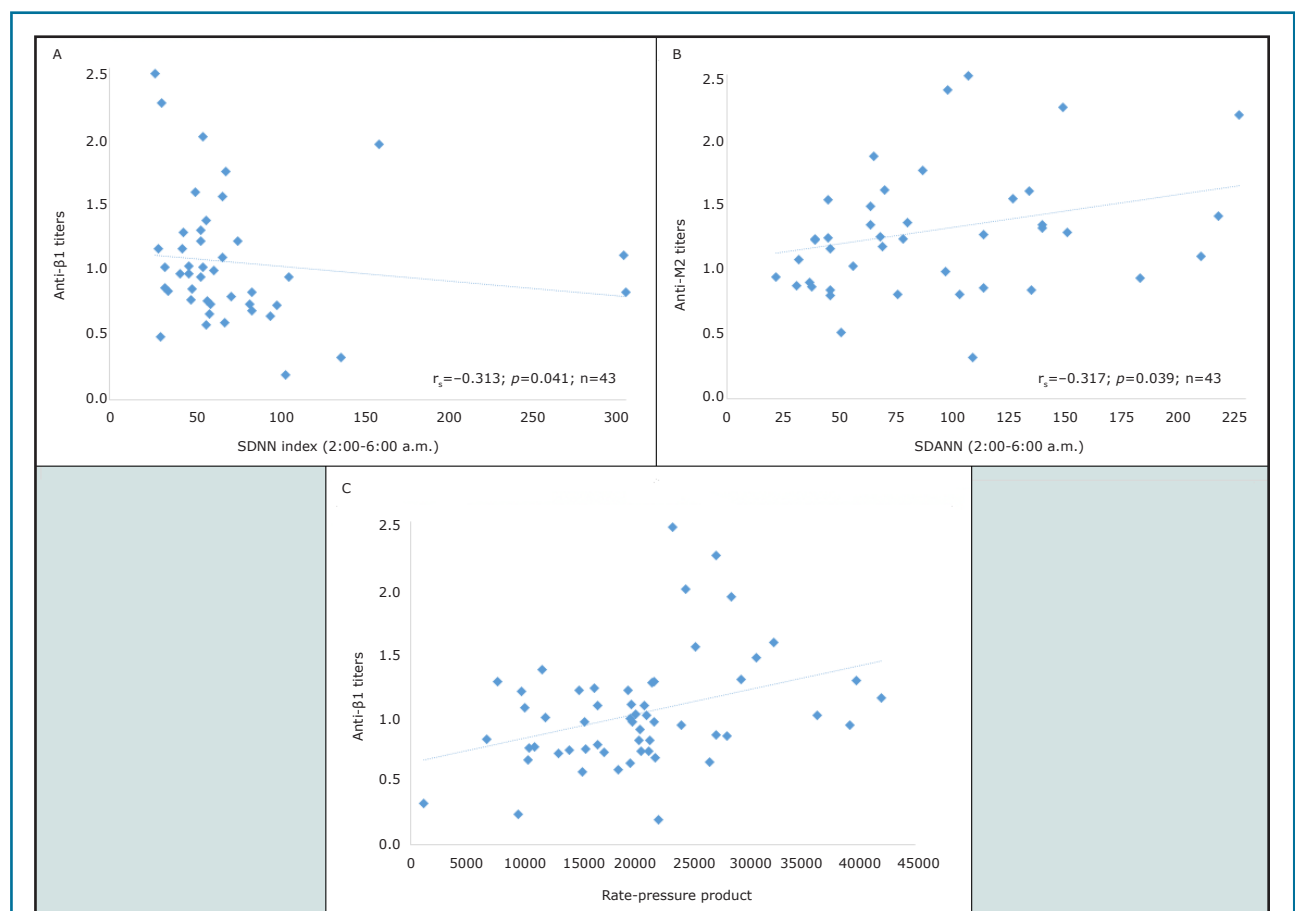

Figure 2 – Correlation between antibodies and Holter and cardiopulmonary exercise stress test variables
r_s: Spearman correlation coefficient. A. Correlation between anti-β1 antibody titer and SDNN index on 24h Holter monitoring. B. Correlation between anti-M2 antibody titer and SDANN on 24h Holter monitoring. C. Correlation between anti-β1 antibody titer and rate-pressure product on cardiopulmonary exercise test.

were not reported by the CPET service and were not entered in the database. *(Table 3).*

In an exploratory analysis of antibody titers and NYHA functional class, higher anti-β1 titers were observed in patients with better functional class (median: 1.019 in class I; 0.998 in class II; 0.884 in class III).

Discussion

Autoimmune reactions directed at G protein-coupled receptors and the production of anti-β1 and anti-M2 autoantibodies acting on these receptors are often associated with changes in HRV, with a consequent increase in morbidity and mortality in patients with HF.[31,32] Decreased HRV is considered an independent risk factor for arrhythmic events and all-cause mortality. Some authors have shown that a lower HRV implies a loss of cardioprotective activity of the parasympathetic system. These changes in HRV occur both in patients without functional LV abnormalities and in those with regional wall motion abnormalities and an EF lower than 50%.[12,13,16,31,33,34]

Data obtained over the last three decades suggest that at least some cases of dilated cardiomyopathy (DCM) could be secondary to an autoimmune disease caused by viruses or protozoa, or even by infections that induce autoimmunity in predisposed individuals.[13,16,19]

Table 2 – Correlation between anti-β1 and anti-M2 autoantibody titers and numerical variables of 24-hour Holter monitoring

Variable		Anti-β1 titer			Anti-M2 titer	
	n	r_s	*p*-value	r_s	*p*-value
NNs, 2:00–6:00 a.m.	43	0.067	0.67	-0.177	0.26
Mean NN, 2:00–6:00 a.m. (ms)	43	-0.101	0.52	0.033	0.83
SDNN, 2:00–6:00 a.m. (ms)	43	-0.122	0.44	0.283	0.07
SDANN, 2:00–6:00 a.m. (ms)	43	-0.052	0.74	**0.317**	**0.039**
SDNN index, 2:00–6:00 a.m. (ms)	43	**-0.313**	**0.041**	0.072	0.65
NNNs, 2:00–6:00 a.m.	43	0.078	0.62	-0.184	0.24
RMSSD, 2:00–6:00 a.m. (ms)	43	-0.273	0.08	-0.003	0.99
pNN50, 2:00–6:00 a.m.	43	-0.218	0.16	0.015	0.92

r^s: Spearman correlation coefficient. NNs: number of N–N intervals considered normal. Mean NN: average over all evaluated cardiac cycles. SDNN: standard deviation of all N–N cycles measured during the recording. SDANN: standard deviation of the mean of the N–N intervals in all 5-min segments on the record. SDNN index: mean of the standard deviation of the N–N intervals of all 5-min segments on the record. NNNs: number of adjacent normal intervals measured during the recording. RMSSD: root mean square of successive differences between adjacent N–N intervals during the recording. pNN50: percentage of heart rate variability greater than 50 ms between successive normal cycles during the recording.

Table 3 – Comparison between anti-β1 and anti-M2 autoantibody titers and cardiopulmonary exercise test variables

Variable	Category	n	Anti-β1 titer		Anti-M2 titer	
			Median (IQR)	*p*-value	Median (IQR)	*p*-value
Chronotropic response	Normal	22	1.107 (0.86 – 1.35)	**0.023**	1.270 (0.99 – 1.58)	0.71
	Impaired	29	0.836 (0.73 – 1.06)		1.259 (0.95 – 1.45)	
Inotropic response	Normal	49	1.002 (0.78 – 1.26)	0.17	1.288 (1.04 – 1.62)	**0.044**
	Impaired	5	0.758 (0.63 – 1.11)		1.075 (0.82 – 1.15)	

IQR: Interquartile range. Mann–Whitney test.

Autoantibodies that modulate the function of receptors that transduce the regulation of heart rate and contractility by the autonomic nervous system may be implicated in DCM pathogenesis. They are also present in HF of other etiologies and atrial tachyarrhythmias. Whether these autoantibodies can actually cause HF or are just an epiphenomenon in the course of disease, however, remains unknown.[35]

In the last decade, humoral autoimmunity against β1 adrenergic and cholinergic receptors has come to be considered one of the probable causes of chronic HF. Therapeutic concepts have been directed towards this pathogenic process, and have shown promise in the treatment of end-stage DCM.[10,24]

In CD, evidence suggests that the continuous action of both anti-β1 and anti-M2 autoantibodies is implicated in Chagas cardiomyopathy. Studies in rodents subjected to immunization with peptides representing the sequence of the second extracellular loop of the β1 adrenergic receptor developed left ventricular dilatation and dysfunction, with consistent evidence of chronic stimulation and desensitization of β1 adrenoceptor signaling, effects that were reversible upon removal of the antibodies. These studies support the hypothesis that β1 adrenoceptor G protein-coupled autoantibodies are a decisive cofactor in chronic HF.[36,37]

The present study showed that increased anti-β1 and anti-M2 receptor antibody titers seem to alter nighttime HRV (2:00 to 6:00 a.m.) and exert modulating effects on membrane receptors. A decrease in sympathetic activity was observed during this period in patients with high anti-β1 antibodies, paradoxically to their expected agonist effect. The higher the anti-β1 antibody titer, the lower the SDNN (a marker of sympathetic activity), and the higher the anti-M2 titer, the greater the SDANN, which also reflects sympathetic activity. These changes occurred at night, where an exacerbation of parasympathetic activity could be a relevant trigger in precipitating atrioventricular blocks, symptomatic bradycardia, and syncope.

Considering that both anti-β1 and anti-M2 antibodies behave as allosteric modulators via signal transduction, in settings where sympathetic activity is exacerbated or the parasympathetic activity is blunted, they would act in a modulatory manner to maintain overall balance. The youngest patient in this study was 33 years old and had high titers of anti-β1 antibodies, while the oldest was 87 and had high concentrations of both anti-β1 and anti-M2 antibodies.

These antibodies seem to exert both an inhibitory and a stimulating effect on muscarinic or adrenergic receptors. Whether these effects play any role in a protective response against nighttime tachyarrhythmias and/or bradyarrhythmias through signal transduction remains unclear.

Classically, the stimulation of adrenergic receptors consists of a signaling cascade involving the G_s (stimulatory) protein and activation of adenylyl cyclase, which is accompanied by an increase in cyclic AMP (cAMP) and PKA (cAMP-dependent protein kinase).[38]

Constant stimulation of β1 adrenergic receptors alters the Ca^{2+}-dependent PKA signaling pathway and regulates the Ca^{2+} current by mechanisms involving Ca^{2+}/calmodulin-dependent protein kinase II, leading to myocyte apoptosis and changes in cardiac adaptation mechanisms, which in turn lead to ventricular remodeling.[20,23]

Some proteins have allosteric and modulating properties and bind to their substrates non-competitively, with the ability to produce a conformational change in protein structure. Proteins are dynamic compounds, and have multiple functional or conformational states. A signaling molecule interacts with its receptor and activates cellular mechanisms, producing a second signal or change in activity in the cellular protein, altering the metabolic activity of the target cell.[16,38]

In HF of other etiologies, the presence of anti-β1 antibodies is associated with greater morbidity, and some authors believe that these antibodies can be considered predictors of mortality. The CIBIS-ELD study used a novel ELISA approach to investigate the relationship between anti-β1 antibodies in a cohort of HF patients and found that patients with an ejection fraction < 50% and a diastolic diameter > 55 mm had higher heart rates and significantly higher anti-β1 antibody titers.[39]

Beta-blocker therapy, despite being highly effective, is insufficient to prevent clinical deterioration in the presence of anti-β1 autoantibodies.[40] In CD, there is evidence showing that patients with CCC have low levels of norepinephrine, unlike their counterparts at the same functional class but with HF of non-Chagas etiologies.[21]

This study also assessed whether anti-β1 and anti-M2 antibodies would have influenced physiological responses during exercise in patients with CD.

Some CPET variables are markers of severity, including HR reduction in the first minute of recovery and a decline in blood pressure during exercise.

However, these parameters were not associated with the presence of anti-β1 or anti-M2 antibodies. It is well known that initial exertion-induced tachycardia depends on suppression of vagal activity, while further increases in HR depend on autonomic (specifically, sympathetic) increments according to exercise intensity.[30]

In this study, a normal chronotropic response and RPP were found to be associated with high anti-β1 titers. Patients with a normal inotropic response had high anti-M2 titers. The agonistic action of these antibodies on β adrenergic receptors explains the increase in HR and blood pressure. The increase in RPP was attributed to the additional positive allosteric modulating action of the anti-β1 adrenergic antibody during exercise, acting as a ligand that modifies the action of an orthosteric agonist, endogenous activator, or antagonist by binding to the allosteric site of the receptor.[38]

Higher anti-M2 titers reflect a modulating action or interaction with other signaling pathways (signal transduction), which stimulate sympathetic action in the heart and other systems that participate in the ANS signaling cascade.[38] Anti-M2 antibodies appear to exert both negative and positive allosteric modulating actions. Both anti-β1 and anti-M2 antibodies modulate the cascade of adaptive ANS responses during exercise.[41,42]

Considering the findings of previous studies on autoantibodies,[37] at least three distinct effects of these antibodies on their respective receptors must be considered: they modulate binding of true ligands; they activate various effector pathways; and they modulate the disposition of the receptor and simultaneous binding to the receptor. However, how autoantibodies cause changes in receptor activity and how this relates to the pathogenesis and severity of CCC remains unclear.

Whether the presence of circulating antibodies has any role in the clinical outcome of patients with CCC is still an open matter; future clinical trials are needed to assess the prognostic nature of these antibodies. Some authors have shown that immune cross-reactivity is not always harmful, and a balanced parasite/host relationship may exist, allowing the disease to remain clinically silent for many years.[11,19]

In Chagas' heart disease, myocardial remodeling seems to be related to etiopathogenic mechanisms linked to neuronal activation. The prognosis is worse compared to that of other cardiomyopathies, with a disturbingly high incidence of sudden death secondary to arrhythmias, and current therapeutic approaches are based on results obtained in heart diseases of another etiologies.[12,13,16]

Including measurement of anti-β1 and anti-M2 autoantibody in the routine workup of patients with CD, plus exercise testing and 24-hour Holter monitoring, would create a panel for assessment of the whole spectrum of HRV behavior and its relationship with autoantibodies in the long-term follow-up of these patients.

This study has some limitations, including: **1)** the absence of a control group for comparison of findings; **2)** the fact that the sample was not separated into groups by NYHA functional class for analysis of HRV and autoantibody behavior according to the degree of cardiac remodeling; **3)** the fact that autoantibody titers were not compared with those measured in cardiomyopathies of other etiologies; **4)** the lack of functional testing of anti-β1 and anti-M2 antibodies (in a Langendorff preparation) from the serum of these patients for comparison with HRV behavior and measurement of a potential active role of these antibodies in ANS modulation, since functionally active antibodies can modulate vagal action even in the absence of structural heart disease.[43-45] Finally, since autoantibodies are part of the immune background of heart disease, **5)** the lack of tools and diagnostic criteria specific for CCC makes it difficult to interpret the data and monitor the natural history of these patients.

Conclusion

The data presented herein show that anti-β1 and anti-M2 autoantibodies seem to exert a modulating effect on the autonomic nervous system, altering nighttime measures of HRV (SDNN and SDANN). There was an inverse association between anti-β1 antibodies and sympathetic activity, and a direct association between anti-M2 and sympathetic activity during this period.

The rate-pressure product correlated significantly with anti-β1 antibody titers, although as an independent variable (5%), the association was weak.

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Author contributions

Conception and design of the research: Cunha DM, Cunha AB. Acquisition of data: Cunha DM, Cunha AB. Analysis and interpretation of the data: Cunha DM. Statistical analysis: Noé R. Writing of the manuscript: Cunha DM, Tzirulnik PC. Critical revision of the manuscript for intellectual content: Cunha AB, Cunha DM.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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