# Non-synonymous polymorphisms in the *HRC* and *ADRB1* genes may be associated with all-cause death in patients with non-ischemic heart failure

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**Abstract.** Sudden cardiac death (SCD) is an unpredictable and common mode of death in patients with heart failure (HF). Alterations in calcium handling may lead to malignant arrhythmias, resulting in SCD, and variants in calcium signaling-related genes have a significant association with SCD. Therefore, the aim of the present retrospective cohort study was to investigate the association of Ser96Ala [histidine-rich calcium-binding protein (*HRC*)], Ser49Gly [β1-adrenergic receptor (*ADRB1*)], Arg389Gly (*ADRB1*) and Gly1886Ser [ryanodine receptor 2 (*RYR2*)] polymorphisms with serious arrhythmic events and overall mortality in patients with HF with reduced left ventricular ejection fraction of non-ischemic etiology. In total, 136 patients with HF underwent physical examination, routine laboratory tests, non-invasive assessment of cardiac function and an invasive electrophysiological study. The primary outcome was the occurrence of serious arrhythmic events, set as either SCD or appropriate implantable cardioverter-defibrillator (ICD) therapy, and the secondary outcome was all-cause death. During a median follow-up of 37 months, arrhythmic events occurred in 26 patients (19%) and 41 patients (30%) died. Patients carrying the Ser allele of the Ser96Ala polymorphism in *HRC* had worse survival than those with the Ala/Ala genotype (log-rank P=0.043). Despite the difference in survival time, the Ala/Ala genotype was not associated with all-cause death in the regression analysis [unadjusted hazard ratio (HR)=0.17; 95% CI, 0.02-1.21]. Regarding the Ser49Gly and Arg389Gly polymorphisms in *ADRB1*, homozygosity for the major alleles at both sites (Ser49Ser and Arg389Arg) was associated with a two-fold increased risk of all-cause death compared with the other genotype combinations (unadjusted HR=1.98; 95% CI, 1.02-3.82). However, this association was lost after controlling for clinical covariates. No association was observed for the Gly1886Ser polymorphism in *RYR2*. Overall, the present findings are concurrent with the hypothesis that the Ser96Ala (*HRC*), Ser49Gly (*ADRB1*) and Arg389Gly (*ADRB1*) polymorphisms may be associated with HF prognosis. In particular, the Ser96Ala polymorphism might aid in risk stratification and patient selection for ICD implantation.

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*Abbreviations:* *ADRB1*/β1-AR, β1-adrenergic receptor; EPS, electrophysiological study; HF, heart failure; HRC, histidine-rich calcium-binding protein; ICD, implantable cardioverter-defibrillator; LVEF, left ventricular ejection fraction; PCR, polymerase chain reaction; RyR2, ryanodine receptor 2; SCD, sudden cardiac death; SR, sarcoplasmic reticulum; VA, ventricular arrhythmia; VF, ventricular fibrillation; VT, ventricular tachycardia

*Key words:* HF, SCD, arrhythmia, ICD, calcium, polymorphism, *HRC*, *ADRB1*, *RYR2*

## Introduction

Heart failure (HF) is a clinical syndrome resulting from structural and functional abnormalities of the heart, which result in elevated intracardiac pressures and inadequate cardiac output at rest and/or during exercise (1). Chronic HF is classified according to the left ventricular ejection fraction (LVEF) into HF with reduced EF (LVEF ≥40%), HF with mildly reduced EF (LVEF 41-49%) and HF with preserved EF (LVEF ≥50%) (2). HF is a major public health problem due to its high mortality rate, recurrent hospitalizations and its association with a low quality of life (1,3). In recent years, several randomized controlled trials have provided new evidence that can change the management of patients with HF with mildly reduced or preserved EF, with the introduction of new medical therapies, such as the sodium-glucose co-transporter 2

inhibitor (dapagliflozin or empagliflozin), thus reducing the risk of HF hospitalizations and cardiovascular death (2). Meanwhile, up to 40% of deaths among patients with HF are sudden and unexpected, resulting from an interplay between a vulnerable substrate (created by conditions such as aging and myocardial scars resulting from acute myocardial infarction) and multiple transient factors that trigger a fatal event (4). In this regard, implantable cardioverter-defibrillators (ICDs) are effective at correcting potentially lethal ventricular arrhythmias (VAs) and are recommended for the primary prevention of sudden cardiac death (SCD) and all-cause mortality in patients with symptomatic HF of ischemic etiology and LVEF ≤35% (1). However, the overall survival benefit of ICD therapy in patients with HF of non-ischemic etiology is unclear, as it is restricted to some subgroups of patients or even not recommended in others (5-8). Therefore, the risk stratification of SCD in patients with non-ischemic HF remains challenging. In this scenario, genetic variants have emerged as potential biomarkers of arrhythmia and SCD risk (9,10), especially those located in genes that code for calcium-signaling proteins responsible for excitation-contraction (9,11-13).

Histidine-rich calcium-binding protein (HRC) is a luminal sarcoplasmic reticulum (SR) protein that regulates calcium handling by mediating its uptake, storage and release (14). HRC protein levels are downregulated in human and experimental HF, and either the overexpression or ablation of HRC results in aberrant calcium cycling, leading to the development of HF upon aging or after pressure overload-induced stress (14). The gene encoding human HRC, located on chromosome 19q13.3 (15), is expressed in striated muscles and arteriolar smooth muscle cells (14). The Ser96Ala (286A>C; rs3745297) common variant in exon 1 of the *HRC* gene is functionally linked to protein activity in normal and failing hearts (16-19). The Ala allele was first identified as causing malignant VAs in patients with idiopathic dilated cardiomyopathy (20), which has not been fully confirmed in patients with predominantly ischemic cardiomyopathy (21,22).

β1-adrenergic receptor (β1-AR/*ADRB1*) is a G-protein-coupled receptor predominantly found in the myocardium, where it regulates cardiomyocyte contraction and relaxation, heart rate and atrioventricular conduction (23). In HF, sustained sympathetic adrenergic activation leads to desensitization and downregulation of β1-AR, ryanodine receptor 2 (RyR2) dysfunction and calcium leak, with these last two abnormalities exacerbating cardiac dysfunction, ventricular arrhythmogenesis and SCD (23). The Ser49Gly (145A>G; rs1801252) and Arg389Gly (1165C>G; rs1801253) variants in the *ADRB1* intronless gene, located on chromosome 10, exert functional effects on agonist-promoted downregulation and response to β-blockers (24). The Gly49 allele is associated with greater receptor desensitization and downregulation than the Ser49 allele (25,26), whereas the Gly389 allele is associated with decreased G-protein coupling of β1-AR and decreased cardiac contractility compared with the Arg389 allele (25-27). Both polymorphisms have been reported to be associated with HF prognosis in some studies but not in others (24,26).

RyR2 is a calcium-release channel protein located in the SR membrane that is essential for excitation-contraction coupling in cardiac muscle (28). Post-translational modifications in RyR2 increase its activity (and hence calcium leak) in failing hearts in humans and experimental HF (29). These alterations in RyR2 can induce VAs and may also contribute to supraventricular defects, such as sinus node dysfunction and atrial fibrillation (29). Human RyR2 is encoded by a large gene on chromosome 1q43 (28), and the Gly1886Ser (5656G>A; rs3766871) polymorphism in exon 37 is located in the functional domain of the tetrameric channel (30), which is involved in excitation-contraction coupling and the binding of physiological ligands (31). The Ser allele has been reported to be associated with VAs (32) and SCD (33) in patients with HF.

The aim of the present study was to investigate whether Ser96Ala (*HRC*), Ser49Gly (*ADRB1*), Arg389Gly (*ADRB1*) and Gly1886Ser (*RYR2*) polymorphisms were associated with serious arrhythmic events and all-cause death in a cohort of patients with non-ischemic HF with reduced LVEF in Southern Brazil.

## Materials and methods

*Study design and population*. A retrospective cohort study of 136 adults with HF and reduced LVEF (≤40%) of non-ischemic etiology was performed. Patients were enrolled between March 2011 and November 2016 at the Heart Failure and Cardiac Transplant Clinic, Clinical Hospital of Porto Alegre (HCPA) (Porto Alegre, Brazil), a tertiary care university hospital. The ejection fraction was assessed by two-dimensional transthoracic echocardiography, and non-ischemic cardiomyopathy was defined as the absence of atherosclerotic coronary lesions >75% on coronary arteriography, or absence of necrotic or ischemic areas on single-photon emission computed tomography or cardiac magnetic resonance. Patients were not included in the study if they had a history of SCD with resuscitation, cardiogenic syncope, sustained ventricular tachycardia (VT), advanced cerebrovascular disease or a life expectancy of <1 year due to non-cardiovascular diseases.

At baseline, the patients underwent a comprehensive clinical and laboratory evaluation consisting of physical examination, routine laboratory tests, non-invasive assessment of cardiac function (two-dimensional echocardiography, 12-lead resting electrocardiogram, 24-h Holter monitoring and cardiopulmonary exercise test) and an invasive electrophysiological study (EPS), as previously described (34,35). The present study was approved by the Research Ethics Committee of HCPA (Institutional Review Board no. 0000921) under CAAE no. 93278918.9.0000.5327 and consolidated review no. 2.824.527. All subjects provided written informed consent before data collection, blood collection and clinical evaluation.

*Follow-up and outcomes*. Patients were followed up on regular outpatient visits at 3 and 6 months, and every 6 months thereafter. Patients who failed to return were contacted by telephone, received home visits or were followed up indirectly through their relatives. The primary outcome of the present study was the occurrence of serious arrhythmic events, defined as SCD or appropriate ICD therapy, whereas the secondary outcome was all-cause death. ICD shocks were considered appropriate if they were caused by VT or ventricular fibrillation (VF). The outcomes were adjudicated independently by two investigators who were blinded to the baseline assessment and genotype data. The adjudication of outcomes was based on clinical history,

statements from family members, review of ICD or pacemaker electrograms, hospital charts and death certificates. The outcome data were updated in November 2020.

*DNA isolation and genotyping*. At the beginning of the EPS, a 20-ml sample of peripheral venous blood was collected from each subject in EDTA-containing tubes for the analysis of biomarkers potentially associated with HF outcomes. Samples were then centrifuged at 1,258 x g for 15 min at 4˚C at The Cardiovascular Research Laboratory of HCPA. Plasma was aspirated with a micropipette, aliquoted and stored in microtubes at -70˚C, while the blood cells were kept at -20˚C until DNA isolation. Genomic DNA was isolated using a salting out procedure (36), quantified by spectrophotometry (NanoDrop 1000; NanoDrop; Thermo Fisher Scientific, Inc.), diluted to a concentration of 10 ng/$\mu$l and stored at -20˚C until genotyping.

Genotyping of *HRC*, *ADRB1* and *RYR2* polymorphisms was performed by quantitative polymerase chain reaction (qPCR) using pre-designed commercial assays containing specific primers and hydrolysis probes (TaqMan® Genotyping Assay, ID numbers C__11506744_1_, C__8898508_10, C__8898494_10 and C__27513673_20 for the Ser96Ala, Ser49Gly, Arg389Gly and Gly1886Ser polymorphisms, respectively; cat. no. 4351379; Thermo Fisher Scientific, Inc.). Amplification reactions were carried out in a final volume of 10 $\mu$l containing 20 ng genomic DNA, 1X TaqMan Genotyping Master Mix (cat. no. 4371355; Thermo Fisher Scientific, Inc.) and 1X TaqMan SNP Genotyping Assay (cat. no. 4351379; Thermo Fisher Scientific, Inc.). Reaction plates were loaded into a qPCR thermal cycler (StepOnePlus Real-Time PCR System; Thermo Fisher Scientific, Inc.) and heated for 10 min at 95˚C, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. The fluorescence data and amplification plots were analyzed using automated allele-calling software (SDS 2.1; Thermo Fisher Scientific, Inc.).

To improve genotyping accuracy, a DNA sample of each genotype (also collected from the present study patients) was used in all PCR runs as a positive control. The investigators who performed the genotyping were blinded to the clinical and laboratory data, and the amplification plots were visually checked by two investigators. The genotyping success rate ranged from 89% (Arg389Gly) to 97% (Ser49Gly and Gly1886Ser), and 116 (85%) samples were successfully genotyped for all four polymorphisms. Approximately half of the samples were re-genotyped for each polymorphism and the concordance rate was 100%. Genotyping data generated during the present study and a minimal clinical dataset are available in a public repository (https://doi.org/10.6084/m9.figshare.22929857).

*Statistical analysis*. Data are presented as the mean ± standard deviation, median (25-75th percentiles), absolute frequency (%) or relative frequency. After checking for normal distribution using the Shapiro-Wilk test, continuous data were compared between groups using the unpaired Student's t-test or Mann-Whitney U test. Categorical data, including genotype and allele frequencies, were compared between groups using the Pearson $\chi^2$ test, likelihood-ratio $\chi^2$ test (used for contingency tables larger than 2x2 with >20% of the cells having expected counts <5) or Fisher's exact tests, as appropriate. Analysis of adjusted residuals was performed where appropriate to examine how different cells contributed to the $\chi^2$ values. Allele frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium were evaluated using the goodness-of-fit $\chi^2$ test (37).

The association of the four polymorphisms in the *HRC*, *ADRB1* and *RYR2* genes with serious arrhythmic events and all-cause death was also evaluated using Kaplan-Meier survival analysis. Survival curves obtained for different genotypes were compared using the log-rank test, and hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox regression with the backward stepwise (Wald) procedure in the multivariable analysis. Statistical analyses were performed using SPSS (version 18; SPSS, Inc.) and WinPEPI (version 11.50) (38) statistical software. Two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Baseline characteristics of the study population*. The majority of the 136 subjects included in the present study were middle-aged or elderly (≥40 years old; 84%), male (58%) and white (75%). Furthermore, the majority were in New York Heart Association functional classes (3) I and II (81%), had a conduction disorder (65%) and had severe ventricular dysfunction caused by idiopathic, hypertensive or alcoholic cardiomyopathy (75%). Regarding the pharmacological treatment of HF, most patients were using β-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, spironolactone and/or digoxin (Table I).

*Follow-up and outcomes*. During a median follow-up of 37 months (25-75th percentiles, 25-68 months), 11 patients (8.1%) died of cardiovascular causes, 8 patients (5.9%) underwent heart transplantation and 3 patients (2.2%) had cardiac syncope. Furthermore, 1 patient died during transplant surgery. The primary outcome occurred in 26 patients (19.1%; 17 had SCD and 9 had appropriate ICD therapy), whereas the secondary outcome (all-cause death) occurred in 41 patients (30.1%). A total of 86 patients (63.2%) experienced neither primary nor secondary outcomes.

Table I summarizes the baseline clinical and demographic characteristics of the population of the present study stratified by primary and secondary outcomes. Patients with serious arrhythmic events had lower levels of lymphocytes, higher levels of uric acid, larger left ventricular diameters, longer QRS duration and HV interval, and a higher frequency of conduction disorder, non-sustained VT and periodic breathing during exercise than those without the primary outcome. Patients who died from any cause were older, had lower body mass index, lower levels of lymphocytes, higher levels of uric acid and creatinine, lower LVEF, larger left ventricular diameters, longer QRS duration and HV interval, lower peak oxygen uptake ($VO_2$), higher $VE/VCO_2$ slope, and had a higher frequency of ventricular pacing, conduction disorder, and periodic breathing during exercise compared with those who were alive. Tables SI and SII provide a more detailed description of the baseline profile of the present study population stratified by the primary and secondary clinical outcomes, respectively.

Table I. Clinical characteristics of the study population stratified by the primary and secondary clinical outcomes.

| | | Serious arrhythmic events | | | All-cause death | | |
|---|---|---|---|---|---|---|---|
| Characteristic | All patients (n=136) | Without (n=110) | With (n=26) | P-value | Alive (n=95) | Deceased (n=41) | P-value |
| Age, years | 55.0±13.0 | 54.9±12.7 | 55.6±14.3 | 0.794 | 53.2±12.8 | 59.2±12.7 | 0.005 |
| Male | 79 (58.1) | 64 (58.2) | 15 (57.7) | >0.999 | 50 (52.6) | 29 (70.7) | 0.076 |
| White[a] | 98 (74.8) | 79 (75.2) | 19 (73.1) | >0.999 | 68 (74.7) | 30 (75.0) | >0.999 |
| New York Heart Association class | | | | | | | |
| I | 57 (41.9) | 44 (40.0) | 13 (50.0) | 0.640 | 38 (40.0) | 19 (46.4) | 0.730 |
| II | 53 (39.0) | 44 (40.0) | 9 (34.6) | | 39 (41.1) | 14 (34.1) | |
| III | 26 (19.1) | 22 (20.0) | 4 (15.4) | | 18 (18.9) | 8 (19.5) | |
| Heart failure etiology | | | | | | | |
| Idiopathic | 60 (44.1) | 50 (45.5) | 10 (38.5) | 0.353 | 48 (50.5) | 12 (29.3) | 0.108 |
| Hypertensive | 25 (18.4) | 22 (20.0) | 3 (11.5) | | 16 (16.8) | 9 (21.9) | |
| Alcoholic | 17 (12.5) | 14 (12.7) | 3 (11.5) | | 9 (9.5) | 8 (19.5) | |
| Others | 34 (25.0) | 24 (21.8) | 10 (38.5) | | 22 (23.2) | 12 (29.3) | |
| Physical examination | | | | | | | |
| Systolic blood pressure, mmHg | 119.4±21.9 | 119.8±21.7 | 117.9±23.0 | 0.701 | 120.0±21.5 | 118.0±22.9 | 0.557 |
| Diastolic blood pressure, mmHg | 74.5±12.8 | 74.6±12.5 | 74.0±14.3 | 0.511 | 75.4±13.0 | 72.4±12.3 | 0.143 |
| Body mass index, kg/m$^{2}$[b] | 28.2±6.0 | 28.4±6.0 | 27.6±6.1 | 0.465 | 29.0±6.2 | 26.4±5.1 | 0.019 |
| Routine laboratory tests | | | | | | | |
| Hemoglobin, g/dl | 13.3±1.6 | 13.4±1.6 | 13.2±1.7 | 0.565 | 13.4±1.5 | 13.2±1.9 | 0.468 |
| Lymphocytes, mm$^{3}$ | 1,975 [1,542-2,465] | 2,015 [1,652-2,520] | 1,530 [905-2,335] | 0.008 | 2,010 [1,660-2,520] | 1,780 [1,190-2,330] | 0.043 |
| Creatinine, mg/dl | 0.98 [0.79-1.34] | 0.96 [0.75-1.17] | 1.05 [0.88-1.53] | 0.070 | 0.93 [0.72-1.15] | 1.09 [0.93-1.54] | 0.001 |
| Sodium, mEq/l[e] | 140.1±2.7 | 140.0±2.9 | 140.4±2.1 | 0.544 | 140.4±2.7 | 139.4±2.6 | 0.054 |
| Potassium, mEq/l | 4.6±0.4 | 4.6±0.4 | 4.5±0.4 | 0.521 | 4.6±0.4 | 4.6±0.4 | 0.288 |
| Uric acid, mg/dl[d] | 7.2 [5.9-9.0] | 6.9 [5.7-8.7] | 8.7 [7.1-9.9] | 0.004 | 7.0 [5.7-8.7] | 8.0 [6.6-9.8] | 0.028 |
| LDL cholesterol, mg/dl[d] | 106±36 | 106±38 | 105±28 | 0.830 | 107±36 | 104±37 | 0.650 |
| Echocardiography | | | | | | | |
| LV ejection fraction, %[e] | 27.5±8.0 | 28.0±8.1 | 25.4±7.2 | 0.131 | 28.6±8.1 | 25.1±7.3 | 0.023 |
| LV end-diastolic diameter, mm[f] | 67.9±8.8 | 66.6±8.1 | 73.1±9.7 | 0.001 | 66.6±8.1 | 71.0±9.6 | 0.007 |
| LV end-systolic diameter, mm[e] | 59.1±9.1 | 58.1±8.5 | 63.6±10.3 | 0.010 | 57.7±8.2 | 62.5±10.4 | 0.006 |
| Left atrium diameter, mm[e] | 47.4±6.6 | 47.3±6.8 | 47.9±5.9 | 0.641 | 46.2±6.1 | 50.1±7.0 | 0.001 |
| Electrocardiography | | | | | | | |
| Rhythm | | | | | | | |
| Normal sinus rhythm | 108 (79.5) | 89 (81.0) | 19 (73.1) | 0.666 | 79 (83.1) | 29 (70.8) | 0.007 |
| Atrial fibrillation | 21 (15.4) | 16 (14.5) | 5 (19.2) | | 15 (15.8) | 6 (14.6) | |
| Ventricular pacing | 7 (5.1) | 5 (4.5) | 2 (7.7) | | 1 (1.1) | 6 (14.6) | |

Table I. Continued.

| Characteristic | All patients (n=136) | Serious arrhythmic events | | | All-cause death | | |
|---|---|---|---|---|---|---|---|
| | | Without (n=110) | With (n=26) | P-value | Alive (n=95) | Deceased (n=41) | P-value |
| QRS duration, msec | 120 [102-157] | 118 [100-153] | 143 [112-174] | 0.025 | 114 [100-150] | 140 [115-173] | 0.001 |
| Conduction disorder | 88 (64.7) | 64 (58.2) | 24 (92.3) | 0.002 | 50 (52.6) | 38 (92.7) | <0.001 |
| 24-h Holter monitoring | | | | | | | |
| Non-sustained VT[e] | 47 (34.8) | 32 (29.4) | 15 (57.7) | 0.013 | 32 (34.0) | 15 (36.6) | 0.929 |
| Cardiopulmonary exercise testing | | | | | | | |
| $VO_2$ peak, ml/kg/min | 17.9±4.9 | 18.1±4.8 | 17.0±5.3 | 0.287 | 18.6±5.0 | 16.2±4.5 | 0.009 |
| $VE/VCO_2$ slope[g] | 41.3±12.0 | 40.2±10.8 | 46.6±15.8 | 0.107 | 38.9±9.9 | 47.2±14.7 | 0.005 |
| Periodic breathing during exercise, % | 24 (17.6) | 15 (13.6) | 9 (34.6) | 0.020 | 12 (12.6) | 12 (29.3) | 0.037 |
| Electrophysiological study | | | | | | | |
| Induced VT or VF | 16 (11.8) | 12 (10.9) | 4 (15.4) | 0.508 | 11 (11.6) | 5 (12.2) | >0.999 |
| HV interval, msec[h] | 52.6±10.5 | 51.4±9.7 | 58.0±12.3 | 0.020 | 51.5±10.3 | 55.4±10.7 | 0.042 |
| Heart failure medication | | | | | | | |
| β-blocker | 132 (97.1) | 106 (96.4) | 26 (100.0) | >0.999 | 92 (96.8) | 40 (97.6) | >0.999 |
| ACE inhibitor or ARB | 132 (97.1) | 108 (98.2) | 24 (92.3) | 0.165 | 92 (96.8) | 40 (97.6) | >0.999 |
| Spironolactone | 93 (68.4) | 76 (69.1) | 17 (65.4) | 0.896 | 68 (71.6) | 25 (61.0) | 0.308 |
| Digoxin | 111 (81.6) | 88 (80.0) | 23 (88.5) | 0.407 | 75 (78.9) | 36 (87.8) | 0.326 |
| Amiodarone | 8 (5.9) | 7 (6.4) | 1 (3.8) | >0.999 | 7 (7.4) | 1 (2.4) | 0.435 |

Data are presented as the mean ± standard deviation, median [25-75th percentiles] or absolute number (%). [a]Missing data for 5 patients without serious arrhythmic events (4 who were alive and 1 who died). [b]Missing data for 1 patient with serious arrhythmic events who died. [c]Missing data for 1 patient without serious arrhythmic events who died. [d]Missing data for 5 patients without serious arrhythmic events who were alive and 1 patient with serious arrhythmic events who died. [e]Missing data for 1 patient without serious arrhythmic events who were alive. [f]Missing data for 2 patients without serious arrhythmic events who were alive. [g]Missing data for 13 patients (8 without serious arrhythmic events, 5 with serious arrhythmic events; 7 were alive and 6 have died). [h]Missing data for 7 patients (5 without serious arrhythmic events, 2 with serious arrhythmic events; 2 were alive and 5 have died). LDL, low-density lipoprotein; LV, left ventricular; VT, ventricular tachycardia; $VO_2$, oxygen consumption; $VE/VCO_2$, ventilatory equivalent for carbon dioxide; VF, ventricular fibrillation; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

Table II. Genotype and allele frequencies of *HRC*, *ADRB1* and *RYR2* polymorphisms stratified by the primary and secondary clinical outcomes.

| | | Serious arrhythmic events | | | All-cause death | | |
|---|---|---|---|---|---|---|---|
| Polymorphism | All patients | Without | With | P-value | Alive | Deceased | P-value |
| Ser96Ala, *HRC* | 128 | 106 | 22 | | 90 | 38 | |
| Genotype | | | | | | | |
| Ser/Ser, AA | 58 (45.3) | 51 (48.1) | 7 (31.8) | 0.364 | 40 (44.4) | 18 (47.4) | 0.060 |
| Ser/Ala, AC | 53 (41.4) | 42 (39.6) | 11 (50.0) | | 34 (37.8) | 19 (50.0) | |
| Ala/Ala, CC | 17 (13.3) | 13 (12.3) | 4 (18.2) | | 16 (17.8)[a] | 1 (2.6)[a] | |
| Allele | | | | | | | |
| Ser, A | 0.660 | 0.679 | 0.568 | 0.215 | 0.633 | 0.724 | 0.211 |
| Ala, C | 0.340 | 0.321 | 0.432 | | 0.367 | 0.276 | |
| Ser49Gly, *ADRB1* | 132 | 106 | 26 | | 91 | 41 | |
| Genotype | | | | | | | |
| Ser/Ser, AA | 89 (67.4) | 69 (65.1) | 20 (76.9) | 0.312 | 58 (63.7) | 31 (75.6) | 0.172 |
| Ser/Gly, AG | 40 (30.3) | 34 (32.1) | 6 (23.1) | | 30 (33.0) | 10 (24.4) | |
| Gly/Gly, GG | 3 (2.3) | 3 (2.8) | 0 (0.0) | | 3 (3.3) | 0 (0.0) | |
| Allele | | | | | | | |
| Ser, A | 0.826 | 0.811 | 0.885 | 0.296 | 0.802 | 0.878 | 0.184 |
| Gly, G | 0.174 | 0.189 | 0.115 | | 0.198 | 0.122 | |
| Arg389Gly, *ADRB1* | 121 | 99 | 22 | | 84 | 37 | |
| Genotype | | | | | | | |
| Arg/Arg, CC | 64 (52.9) | 53 (53.5) | 11 (50.0) | 0.471 | 42 (50.0) | 22 (59.5) | 0.557 |
| Arg/Gly, CG | 48 (39.7) | 40 (40.4) | 8 (36.4) | | 36 (42.9) | 12 (32.4) | |
| Gly/Gly, GG | 9 (7.4) | 6 (6.1) | 3 (13.6) | | 6 (7.1) | 3 (8.1) | |
| Allele | | | | | | | |
| Arg, C | 0.727 | 0.737 | 0.682 | 0.575 | 0.714 | 0.757 | 0.598 |
| Gly, G | 0.273 | 0.263 | 0.318 | | 0.286 | 0.243 | |
| Gly1886Ser, *RYR2* | 132 | 106 | 26 | | 91 | 41 | |
| Genotype | | | | | | | |
| Gly/Gly, GG | 119 (90.1) | 94 (88.7) | 25 (96.2) | 0.415 | 81 (89.0) | 38 (92.7) | 0.606 |
| Gly/Ser, GA | 12 (9.1) | 11 (10.4) | 1 (3.8) | | 9 (9.9) | 3 (7.3) | |
| Ser/Ser, AA | 1 (0.8) | 1 (0.9) | 0 (0.0) | | 1 (1.1) | 0 (0.0) | |
| Allele | | | | | | | |
| Ser, G | 0.947 | 0.939 | 0.981 | 0.385 | 0.940 | 0.963 | 0.615 |
| Gly, A | 0.053 | 0.061 | 0.019 | | 0.060 | 0.037 | |

Data are expressed as absolute number (%) or relative frequency. [a]Frequencies that deviate significantly from expected in the analysis of adjusted residuals. *ADRB1*, β1-adrenergic receptor; *HRC*, histidine-rich calcium-binding protein; *RYR2*, ryanodine receptor 2.

*Association between polymorphisms and outcomes*. The frequency distributions of *HRC*, *ADRB1* and *RYR2* polymorphisms are shown in Tables II and SIII. The genotype frequencies of the four studied polymorphisms were in accordance with the Hardy-Weinberg equation in the overall population and in the subgroups stratified by primary and secondary clinical outcomes. The differences observed in the genotype and allele frequencies between the groups with and without serious arrhythmic events and between the subjects who died and those who were alive did not reach statistical significance (Table II). Moreover, the genotype and allele frequencies of all polymorphisms did not differ between white and non-white patients with HF (Table SIII), and the four polymorphisms were not associated with the primary and secondary outcomes in the analyses stratified by self-declared ethnicity (data not shown).

However, the analysis of adjusted residuals indicated that the Ala/Ala genotype of the Ser96Ala polymorphism in *HRC* was more frequent than expected under the null hypothesis (that is, assuming that the genotypes are not associated with the outcome) among the patients who were alive (Table II). Notably, carriers of the major allele (Ser/Ser or Ser/Ala) had worse survival rates than those homozygous for the Ala allele during follow-up (Fig. 1). Despite this difference, the Ala/Ala

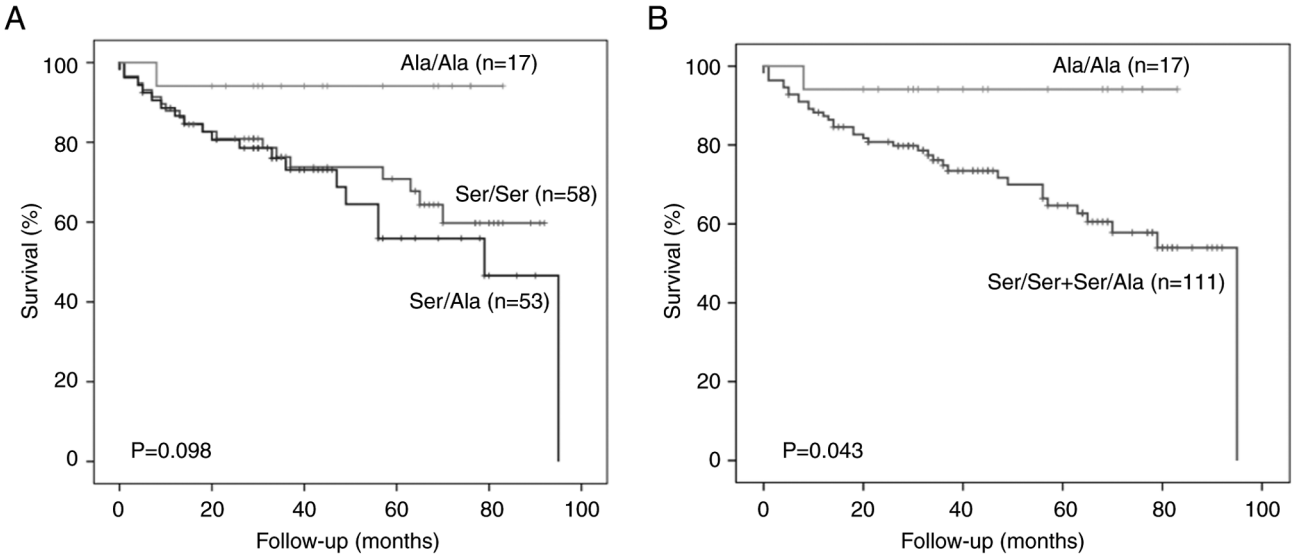


Figure 1. Kaplan-Meier survival estimates of all-cause death according to the (A) genotypes of the Ser96Ala polymorphism and the (B) carriers of the Ser96 allele in the histidine-rich calcium-binding protein gene.

genotype was not associated with all-cause death in regression analysis (unadjusted HR=0.17; 95% CI, 0.02-1.21; P=0.076). To further examine the potential association of the Ser96Ala polymorphism in *HRC* with cardiac dysfunction, an exploratory analysis of the association between this gene variant and individual clinical outcomes (SCD, cardiovascular-related death, heart transplantation, cardiac syncope and appropriate ICD therapy) was explored. Notably, homozygotes for the Ala allele had more appropriate ICD shocks than carriers of the Ser allele [3/17 (17.6%) vs. 3/111 (2.7%), respectively; P=0.031], whereas 7/8 patients who underwent heart transplantation were homozygous for the Ser allele (data not shown).

Regarding the Ser49Gly and Arg389Gly polymorphisms in the *ADRB1* gene, neither polymorphism was individually associated with arrhythmic events or all-cause death (Table II). However, patients homozygous for the major alleles at both sites (Ser49Ser and Arg389Arg) had a higher rate of all-cause death than those with other genotype combinations (Ser49Ser + Arg389Gly, Ser49Ser + Gly389Gly, Ser49Gly + Arg389Arg, Ser49Gly + Arg389Gly, or Gly49Gly + Arg389Arg; Fig. 2). Carrying the four major alleles was associated with a two-fold increased risk of all-cause death, but this association was lost in the multivariate analysis after controlling for covariates that were also associated with this outcome in the univariate analysis (Table III).

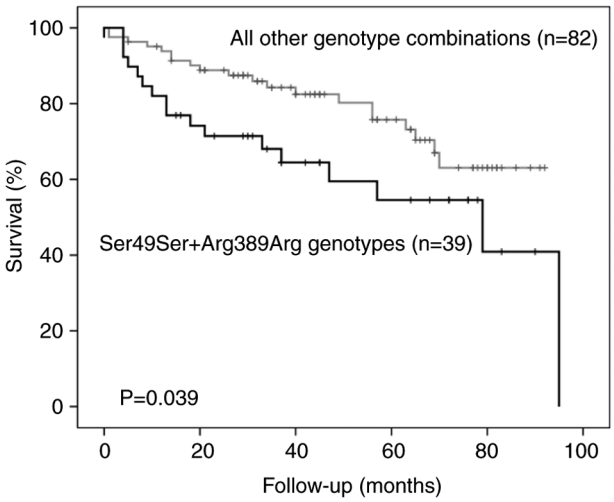


Figure 2. Kaplan-Meier survival estimates of all-cause death according to the genotype combinations of the Ser49Gly and Arg389Gly polymorphisms in the β1-adrenergic receptor gene.

## Discussion

In the present study, evidence of an association of Ser96Ala (*HRC*), Ser49Gly (*ADRB1*), Arg389Gly (*ADRB1*) and Gly1886Ser (*RYR2*) polymorphisms with the combined outcome of SCD or appropriate ICD therapy was not found in outpatients with non-ischemic HF from Southern Brazil. However, the Ser96Ala polymorphism appeared to be associated with all-cause death and appropriate ICD therapy when the outcomes were analyzed individually. Moreover, the Ser49Gly and Arg389Gly polymorphisms were found to be jointly associated with all-cause death in the univariate analysis.

Regarding the number of appropriate ICD shocks and the Ser96Ala polymorphism, the findings of the present study are in line with those reported in a large cohort of predominantly white patients with ischemic cardiomyopathy from the Genetic Risk Assessment of Defibrillator Events multicenter study. In this previous study, carriers of the Ala allele experienced more appropriate shocks than those homozygous for the Ser allele. However, in contrast to the findings of the present study, the secondary endpoint of death, heart transplant, or ventricular assist device use was not associated with the Ser96Ala polymorphism (21). Similarly, in a study of patients with idiopathic dilated cardiomyopathy from Greece, carriers of the Ala allele were more likely to need ICD due to a history of sustained VT or VF prior to study entry than those homozygous for the Ser allele. During follow-up, homozygotes for the Ala allele were more susceptible to life-threatening ventricular arrhythmic events (including SCD and episodes

Table III. Cox regression analysis for all-cause death (secondary outcome).

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variable[a] | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Ser49Ser + Arg389Arg genotypes | 1.98 | 1.02-3.82 | 0.043 | 1.62 | 0.80-3.27 | 0.181 |
| Body mass index, kg/m$^2$ | 0.92 | 0.87-0.98 | 0.012 | 0.91 | 0.85-0.97 | 0.004 |
| LVEDD, mm | 1.06 | 1.02-1.09 | 0.004 | 1.07 | 1.02-1.12 | 0.003 |
| $VO_2$ peak, ml/kg/min | 0.92 | 0.86-0.98 | 0.012 | 0.87 | 0.81-0.94 | <0.001 |

[a]Age at study entry (years), QRS duration (msec), ventilatory equivalent for carbon dioxide, periodic breathing during exercise (%) and HV interval (msec) were also entered in the initial model and were removed during the backward stepwise (Wald) procedure. HR, hazard ratio; CI, confidence interval; LVEDD, left ventricular end-diastolic diameter; $VO_2$, oxygen consumption.

of unstable VT or VF) and cardiac death from any cause than Ser allele carriers (20). However, another study observed no association between the Ser96Ala polymorphism and VA in a cohort of Caucasian patients with predominantly ischemic cardiomyopathy from Southern Italy (22). In addition to the Ser96Ala polymorphism being poorly studied in the clinical setting in humans, studies are heterogeneous in terms of population, HF etiology and outcomes evaluated (20-22), which may explain the partially discrepant findings regarding the existence and direction of the association between this polymorphism and HF prognosis. Nevertheless, two of the three previous studies (20,21) and the present study have found evidence suggesting that the Ser96Ala polymorphism in *HRC* is associated with life-threatening arrhythmias and/or death.

The causal relationship between the Ser96Ala polymorphism and calcium handling in cardiomyocytes has already been demonstrated in cell and animal models (14,16-19). Substitution of Ala for Ser at position 96 has been reported to result in altered interactions of HRC with the major SR calcium cycling proteins (16), aberrant calcium transients and increased calcium leak, thus leading to more frequent calcium sparks and calcium waves (14), which in turn trigger aftercontractions and delayed afterdepolarizations (14,16). These electrical phenomena may be the underlying mechanisms of the increased propensity to VAs (17-19), decreased cardiomyocyte contractility (18,19), increased ventricular automaticity under stress conditions (19) and increased mortality (18,19), even in the absence of structural remodeling (18) or concomitant cardiac disease (19). Taken together, these studies show that Ser96 is critical for the cardioprotective effect of HRC (16-19).

In general, the present results regarding the two polymorphisms in *ADRB1* are in accordance with those obtained from previous studies by our research group in other cohorts of patients with HF with ischemic and non-ischemic etiology (39-41). In the first study by Biolo *et al* (39), the Ser49Gly polymorphism was reported to not be associated with non-sustained VT on Holter monitoring or death. By contrast, homozygous patients for the Gly389 allele had less non-sustained VT and a higher HF-related survival rate than the carriers of the Arg389 allele. In another cohort of patients with HF using ICDs for primary and secondary prevention, the Arg389Gly polymorphism was not associated with the risk of appropriate shocks (41). A meta-analysis of studies published between 2003 and 2011, including patients with HF who were predominantly white and of non-ischemic etiology from Sweden, Denmark, Germany, Italy, the Netherlands, the United Kingdom, the USA and China, revealed that neither of the two polymorphisms of the *ADRB1* gene were associated with all-cause mortality or combined endpoints comprising death, heart transplantation and hospitalization (42). Such a lack of association of the Ser49Gly and Arg389Gly polymorphisms with adverse outcomes in patients with HF has also been reported in other studies carried out in France (43), the USA (44,45) and Japan (46), regardless of the use of ICD (45). In addition, the Arg389Gly polymorphism has been reported to not be associated with the incidence of malignant tachyarrhythmias (fast VT/VF), appropriate ICD shocks or cardiac death in Caucasian patients with HF 12 months after undergoing cardiac resynchronization therapy device implantation (47). In another study of Caucasian patients with HF, the Gly49 allele carriers were shown to have a higher risk of having appropriate ICD shocks than the homozygotes for the Ser49 allele, while the Arg389Gly polymorphism was not associated with arrhythmic events (48).

A potential explanation for the discrepancies across the studies may lie in the interaction between the Ser49Gly and Arg389Gly polymorphisms, ethnicity and the use (and dose) of β-blockers, as reported by several authors (39,49-53). For example, in a large cohort of African American and Caucasian patients with HF with both ischemic and non-ischemic etiologies, the Gly allele of the Arg389Gly polymorphism was associated with decreased survival in Caucasians only among those not taking β-blockers (51). In the present study, 132 of 136 patients (97%) were taking β-blockers. If the Ser49Gly or Arg389Gly polymorphism has a small effect on survival in patients with HF, it may have been overcome by treatment with β-blockers. Apart from the interaction between *ADRB1* polymorphisms and β-blockers, a joint effect of adrenergic receptor gene variants on event-free survival has been recurrently reported (40,41,44,46,52). For example, in one of our previous studies, a combination of the Thr164Ile (β2-adrenergic receptor), Gly49Gly and/or Gly389Gly genotypes was independently associated with lower all-cause and HF-related mortality than other genotypes (40). In line with these studies, the present study also found that patients carrying both the

Ser49Ser and Arg389Arg genotypes had a lower survival rate than those with other genotype combinations, indicating the synergistic effect of multiple common gene variants with small effect sizes on HF prognosis.

As for the Gly1886Ser polymorphism in the *RYR2* gene, the findings of the present study were partially in agreement with those obtained in a large study conducted in Han Chinese patients with chronic HF of ischemic or idiopathic etiology, in which the Gly1886Ser polymorphism was not associated with VAs among patients with HF or with HF severity, as defined by LVEF. However, patients carrying the Ser allele had an increased risk of heart transplantation and SCD (33). The results of the present study also partially differed from those reported in an Italian study of Caucasian patients with HF with both ischemic and non-ischemic etiologies who underwent primary or secondary prevention ICD implantation, in which the Ser allele was associated with an increased risk of VT/VF. However, during follow-up, 30 patients died, and only one patient was heterozygous for the Ser allele (32). In addition to having only two previous reports investigating the association of the Gly1886Ser polymorphism with HF prognosis (32,33), these studies and the present study have included patients with different inclusion criteria, HF etiologies, degrees of severity and composite endpoints, which makes the results not directly comparable.

In summary, the present study supported previous evidence that genetic variants in calcium handling and signaling genes are associated with HF prognosis. Beyond the understanding of the molecular basis of arrhythmic events and death in patients with HF with non-ischemic etiology, the findings of the present study support further research aimed at identifying new non-invasive (or minimally invasive) predictive biomarkers of arrhythmia and death that could be useful to improve the risk stratification and selection criteria for ICD implantation. For example, the Ala96 allele of the Ser96Ala polymorphism in *HRC* is a common variant in the general population and seems to have no deleterious effects in normal cardiomyocytes. However, it becomes arrhythmogenic under stress conditions such as in the failing heart (17). In the present study, almost all patients homozygous for the Ala96 allele were alive after a follow-up of 80 months, although they had more appropriate ICD shocks than the carriers of the Ser allele. This reinforces the idea that patients carrying certain alleles or genotypes are likely to benefit the most from ICD implantation. However, the main limitation of the present study was the small sample size. Although no patient was lost during follow-up, not all were using ICD, and not all DNA samples could be genotyped for the four polymorphisms. This reduced the power to detect associations of low magnitude, while the positive associations detected in the crude analyses should be considered suggestive rather than conclusive. Therefore, the findings of the present study need to be replicated in future studies.

In conclusion, the present retrospective cohort study of patients with non-ischemic HF provided suggestive evidence that the Ser96Ala polymorphism in *HRC* may be associated with all-cause death and appropriate ICD therapy, whereas the Ser49Gly and Arg389Gly polymorphisms in *ADRB1* may be jointly associated with all-cause death. This search for an improved prediction of HF prognosis is important for clinical practice, as the overall survival benefit of ICD therapy in patients with HF of non-ischemic etiology is still uncertain, and the conventional criteria used to indicate the implantation of this type of device are not sufficient to identify patients who are likely to benefit from this intervention. Randomized clinical trials, including patients undergoing contemporary treatment for non-ischemic HF and the presence of high-risk genetic polymorphisms, may indicate a subgroup of patients with an improved cost-benefit ratio for ICD implantation.

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**Availability of data and materials**

The genotyping datasets generated and/or analyzed during the current study are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.22929857). The data are available from the corresponding author upon reasonable request.

**Authors' contributions**

BMM, MP, MA, LER and KGS conceived the present study. BMM and MP enrolled the patients, acquired clinical data and updated the database. LER supervised the study clinical stages. MA supervised DNA isolation. TMT and BLSP performed genotyping. KGS supervised genotyping. TMT updated the database. LER and KGS confirm the authenticity of all the raw data. KGS performed the statistical analyses. LER and KGS contributed to funding resources. KGS wrote and edited the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by The Research Ethics Committee of HCPA (Institutional Review Board-IRB: 0000921; CAAE no. 93278918.9.0000.5327 and consolidated review no. 2.824.527; Porto Alegre, Brazil). Written informed consent was obtained from all the participants.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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