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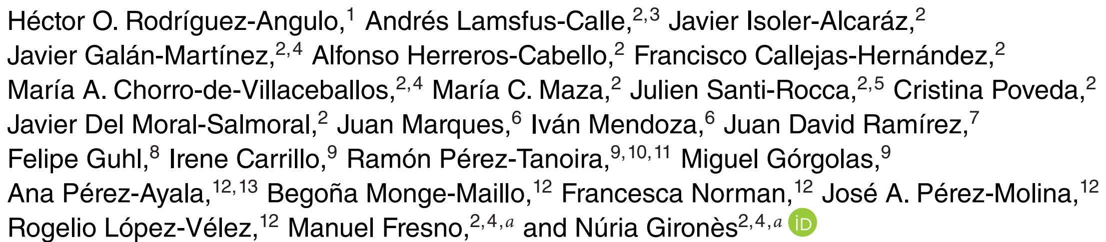
Original Article

Autoantibodies against the immunodominant sCha epitope discriminate the risk of sudden death in chronic Chagas cardiomyopathy



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In Chagas disease (ChD) caused by *Trypanosoma cruzi*, new biomarkers to predict chronic cardiac pathology are urgently needed. Previous studies in chagasic patients with mild symptomatology showed that antibodies against the immunodominant R3 epitope of sCha, a fragment of the human basic helix-loop-helix transcription factor like 5, correlated with cardiac pathology. To validate sCha as a biomarker and to understand the origin of anti-sCha antibodies, we conducted a multicenter study with several cohorts of chagasic patients with severe cardiac symptomatology. We found that levels of antibodies against sCha discriminated the high risk of sudden death, indicating they could be useful for ChD prognosis. We investigated the origin of the antibodies and performed an alanine scan of the R3 epitope. We identified a minimal epitope MRQLD, and a BLAST search retrieved several *T. cruzi* antigens. Five of the hits had known or putative functions, of which phosphonopyruvate decarboxylase showed the highest cross-reactivity with sCha, confirming the role of molecular mimicry in the development of anti-sCha antibodies. Altogether, we demonstrate that the development of antibodies against sCha, which originated by molecular mimicry with *T. cruzi* antigens, could discriminate electrocardiographic alterations associated with a high risk of sudden death.

Keywords: *Trypanosoma cruzi*; Chagas; cardiomyopathy; prognosis; immunodominant epitope; alanine scan

Introduction

Chagas disease (ChD) caused by the protozoan parasite *Trypanosoma cruzi* affects 7–8 million people

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worldwide. Although ChD is endemic in Latin America, there is a high prevalence in nonendemic countries as a result of migration, mainly in the United States and Spain.[1,2]

It is characteristic of the disease that the chronic phase remains asymptomatic for many decades, developing into cardiomyopathy in about 30% of the chronic cases. There are several serological and molecular diagnostic tools available based on total parasite homogenates, antigenic fractions as the trypomastigote excreted/secreted antigens,[3] and glycoconjugates.[4] Besides, parasite DNA[5] and cDNA[6] expression libraries were used in screenings that allowed the identification of most of the immunodominant parasite antigens. Combined with other antigens, they were used to design new-generation tests, such as the recently evaluated Architect Chagas (Abbott Laboratories)[7] and the Multi-cruzi test (InfYnity Biomarkers).[8]

However, the infection can lead to sudden death (SD), mainly as a result of cardiac arrhythmia or heart failure that can be caused by different mechanisms during the lifespan of the patients, as parasite persistence, neurogenic damage, and/or autoimmunity,[9,10] for which no predictive tools are available. Biomarkers of cardiac damage and inflammation commonly used for the prognosis of other cardiomyopathies that have been tested in chagasic patients (ChP), but there is no single molecule that meets all the required expectations to implement in endemic countries (reviewed in Ref. 11). There are also promising studies evaluating the efficacy of treatment by PCR, and enzyme-linked immunosorbent assay (ELISA) but still better biomarkers are needed (reviewed in Ref. 12).

On the other hand, many autoantigens are recognized by antibodies present in the serum of ChP[11] as a result of molecular mimicry (similarity of amino acid sequences between parasite and host antigens).[10,13,14] The β1-adrenergic[15] and muscarinic M2[16] receptors, and cardiac myosin[17,18] are the targets of autoantibodies in ChD and likely related to cardiac pathology.

The sCha autoantigen was identified by screening of an expression library of the Jurkat T cell line with sera from ChP. sCha is a short form of Cha, an isoform of TCFL5 (transcription factor like 5), a transcription factor of the basic helix-loop-helix (bHLH) family.[19] The sCha reactive immunodominant epitope was mapped by overlapping peptide-scanning ELISA to its N-terminal, named R3 (MRQLDTNVER), and showed a positive correlation with cardiac symptomatology.[20,21] Moreover, specific antibodies against the R3 peptide purified from the patient's sera cross-reacted with parasite antigens.[20] Thus, to understand how anti-sCha antibodies are raised in patients, further experiments were needed.

Here, we analyzed different cohorts of patients in a multicenter study, including patients with more severe cardiac symptomatology, to investigate the usefulness of sCha as a discriminatory biomarker of cardiomyopathy. Finally, we performed fine mapping of the R3 immunodominant peptide and identified a minimal five-amino-acid epitope with a high percent of similarity with a *T. cruzi* antigen that triggered sCha cross-reactive antibody production.

Methods

Patient ethics statement

The study was approved by the Ethical Committees of Hospital Ramón y Cajal and Fundación Jimenez Diaz in Madrid (Spain) (Ref. FUN-BEN-2007-01 and 10/2016, respectively); by the local and Institutional Review Board and the Ethics Research Committee of the World Health Organization (WHO) as part of the BENEFIT trial (Benznidazole Evaluation for Interrupting Trypanosomiasis) for ClinicaAboodShaio, Hospital de la Policía, Fundación Cardiovascular de San Gil Santander (Colombia); by the Ethical and Scientific Committee of the Tropical Medicine Institute for the Cardiology Service of Tropical Medicine Institute (Central Venezuelan University, Caracas, Venezuela) (Ref. CEC-IMT 01/20014). Patients were diagnosed with two different serological tests (ELISA IgG, and/or indirect hemagglutination, and/or indirect immunofluorescence), according to WHO criteria. All patients signed informed consent. Data on human subjects were analyzed anonymously, and clinical investigations have been conducted according to the Declaration of Helsinki.

Animal ethics statement

This study was carried out in strict accordance with the European Commission legislation on the protection of animals used for scientific purposes (2010/63/EU). The protocol for the treatment of the animals was approved by the Comité de Ética de la Dirección General del Medio Ambiente de

la Comunidad de Madrid (Spain; permits PROEX 21/14 and PROEX 148/15). Animals had unlimited access to food and water. They were euthanized in a CO_2 chamber, and all efforts were made to minimize their suffering.

Human sera

Patients from Bolivia with no alterations in their electrocardiogram (ECG) (n = 115), presence of bundle branch block (BBB) (n = 27), and sinus block (SB) (n = 9) were recruited at Hospital Ramón y Cajal; and patients with cardiac symptoms (n = 8) or not (n = 15) and treated (n = 29) or not (n = 38) with benznidazole were recruited at the *Fundación Jiménez Díaz*. Patients from Venezuela, comprising Chagas-negative cardiac controls (n = 9) and Chagas-positive cardiac patients (n = 35), were recruited. Patients from Colombia, with (n = 47) and without (n = 13) ectopic arrhythmias were recruited. The exclusion criteria involved the presence of other serious infectious, organic, and psychiatric pathologies in patients from Bolivia, other infectious diseases and age under 18 and above 75 years in patients from Venezuela, and the presence of other acute, hepatic, renal, or psychiatric diseases, and age under 40 and above 50 years in patients from Colombia.

Recombinant sCha purification

Recombinant sCha was purified from bacteria transformed with a pQE-70 sCha cDNA plasmid and purified by nickel nitrilotriacetic acid (Ni-NTA, QIAGEN) chromatography following the manufacturer's directions. Protein concentration was determined using the bicinchoninic acid (BCA) assay.

T. cruzi *parasites*

The Y *T. cruzi* strain was obtained from Dr. David (Harvard Medical School, Boston, MA) and the VFRA strain from ChagasEpiNet VII Framework Consortium. *T. cruzi* trypomastigotes were obtained from Vero cell (ATCC number CCL-81) cocultures. *T. cruzi* epimastigotes were cultured in liver infusion tryptose medium. One hundred eighty million parasites were centrifuged to 1600 *g* for 10 min, and pellets were washed twice with phosphate-buffered saline (PBS), frozen at −80 °C, and defrosted three times. Extracts were subjected to three cycles of a 30-s sonication on ice followed by 30 s of resting on ice. Protein concentration was determined using the BCA assay.

Synthetic peptides

The sequences of the synthetic peptides from Genscript are shown in Table 1 and Tables S1 and S3 (online only).

Indirect ELISA assay

MAXISORP 96-well multi-well plates (NUNCLON) were covered with recombinant sCha or *T. cruzi* extract (5 μg per well) for 16 h at 4 °C. Unspecific binding was blocked with PBS, 0.2% Tween-20, 3% skim dehydrated milk for 1 h at room temperature (RT). After two washes of 10 min with washing buffer (PBS, 0.2% Tween-20, and 1% skim dehydrated milk), the membranes were incubated with 1:100 dilution of different patients' sera for 2 h at RT, and subsequently with 1:10,000 dilution of goat anti-human IgG coupled to peroxidase (Thermo Scientific) for 1 h at RT. After five washes of 10 min with washing buffer, 100 μL of *O*-phenylenediamine (OPD, Sigma-Aldrich) was added following the manufacturer's directions for colorimetric detection of antibodies against sCha. Finally, after 30 min incubation, O.D. at 450 nm was measured in a plate reader (EL340, Microtek instruments).

The competitive ELISA assay

Competitive ELISA was performed as the indirect ELISA described above with modification; that is, diluted sera from patients were preincubated with the peptides at the indicated concentrations for 1 h at RT.

B cell epitope definition

For the normalization of the peptide competition data, the O.D. values were expressed as a percentage of the noncompeted control for each serum analyzed. The overall median percentage was calculated among values in which competitor peptides were added. Percentage values above the median were considered as positive and thus important for antibody recognition.

Anti-PPDC19 antibody

Five BALB/c mice were immunized four times with the PPDC19 peptide coupled to keyhole limpet hemocyanin (KLH) every 2 weeks at Genscript Inc. For the first three immunizations, 25 μg of KHL-PPDC19 was emulsified with complete Freund's adjuvant. The last immunization was done using 50 μg of KLH-PPDC19 emulsified with incomplete Freund's adjuvant. After 2 weeks, mice were

Table 1. Candidate R3H10 cross-reactive antigens

T. cruzi antigen	Tritrypdb ref (above) NCBI ref (below)	(%)	Alignment with R3H10 of sCha
ENO	TcCLB.504105.140 XP_819700.1	80	R3H10 MRQLDTNVER : : . :: . . . ENO MRDLDGTPNK
HSlVU	TcCLB.510819.60 XP_812843.1	70	R3H10 MRQLDTNVER : : . : : . . HSlVU MRELDNYIIG
ABCT	TcCLB.510943.80 XP_812776.1	70	R3H10 MRQLDTNV-ER . : : : : : : ABCT LRQLDECVLEY
PPDC	TcCLB.510903.50 XP_809549.1	70	R3H10 MRQLDTNVER . : : : : :. PPDC LRQLDTKDVV
GP63	TcCLB.510363.290 XP_808833.1	60	R3H10 MRQLDTNVER : : : : . : GP63 MRQLIHVTSR

Note: Amino acid sequences of the peptides, percent of similarity of the first five N-terminal amino acids (%), and alignments with R3H10 of sCha are shown. ABCT, ABC transporter; ENO, enolase; GP63, glycoprotein 63; HSIVU, chaperone; PPDC, phosphonopyruvate decarboxylase.

euthanized, blood was collected, and serum was obtained. Mice sera were pooled together for performing ELISA and western blot assays.

Western blot analysis

Twenty micrograms of parasite lysates and 0.1, 0.5, 0.75, and 1 μg of purified recombinant sCha were resolved in 13% SDS-PAGE gel and transferred to 0.22-μm pore nitrocellulose membrane (Bio-Rad) that were blocked in 5% BSA-TBST (bovine serum albumin; Tris-buffered saline with 0.1% Tween-20) for 1 h at RT and incubated with 1:1000 dilution of anti-PPDC antibody (GeneScript) for 16 h at 4 °C and subsequently incubated with 1:2000 dilution of horseradish peroxidase–conjugated secondary anti-mouse antibody (Cell Signaling Technology) during 1 h at RT. Luminescence signal was detected using SuperSignal West Dura Substrate (Pierce Biotechnology) using Amersham Imager 600 (GE Healthcare).

Statistical analysis

Data are shown as the mean ± SEM and box and whiskers. Statistical significance was evaluated by Student's t-test when two groups were compared, one-way ANOVA posttest for trend ($^{*}P \leq 0.05$ and $^{**}P \leq 0.01$), and the area under the ROC curve (AUC), as indicated, using GraphPad Prism® 8.00 software (La Jolla, CA).

Results

sCha as a discriminatory biomarker

We assessed the potential of sCha as a prognosis biomarker in several cohorts from different hospitals, countries, and stages of the disease. For this, it was necessary to organize the data according to the different stratification methodology used in the different health centers where serum samples were collected. Patients were classified according to the following four criteria: general (treatment response and cardiac disease presence/absence) and arrhythmia (tachy- and bradyarrhythmias) evolution.

First, the reactivity against *T. cruzi* was compared versus sCha. In a cohort from Venezuela, the distribution of antibody levels against *T. cruzi* in Chagas-positive patients (Fig. 1A) did not exactly match with sCha (Fig. 1B). While only three patients were negative for *T. cruzi,* seven were negative for sCha (Fig. 1C). Besides, the sCha data showed a normal frequency distribution in Chagas-positive patients, but *T. cruzi* data did not (Fig. S1, online only). Moreover, symptomatic patients from Bolivia showed higher levels of anti-sCha antibodies than the nonsymptomatic ones (Fig. 1D). Further analysis showed that the AUC was 0.726 ± 0.103, with a 95% confidence interval (CI) of 0.523–0.929, and a

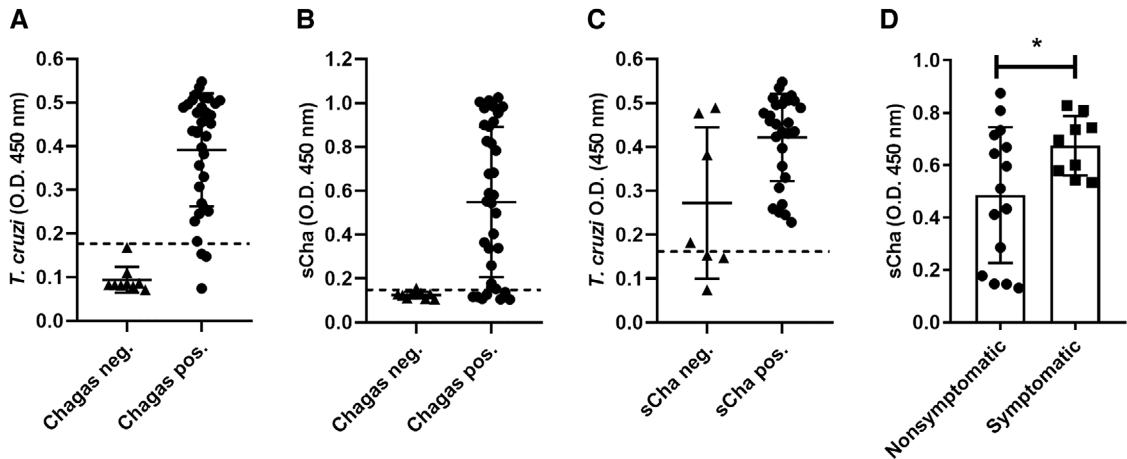

Figure 1. Comparison of the sCha and *T. cruzi* ELISA in cardiac patients negative and positive for Chagas. (A) The *T. cruzi* ELISA showed 91.43% sensitivity and 100% specificity with a cutoff value of 0.17. (B) The sCha ELISA showed 77.14% sensitivity and 100% specificity with a cutoff value of 0.16. (C) *T. cruzi* ELISA values in sCha-negative and -positive patients. Chagas-negative cardiac controls ($n = 9$) and Chagas-positive cardiac patients ($n = 35$) were from Venezuela. Cutoff, sensitivity, and specificity of the ELISAs were calculated using the area under the ROC curve with a 95% interval of confidence in GraphPad Prism 8.0. (D) The sCha ELISA of nonsymptomatic ($n = 16$) and symptomatic ($n = 9$) patients from Bolivia (Fundación Jiménez Díaz). Statistical significance was determined by Student's *t*-test with Welch's correction ($^*P \leq 0.05$).

P value of 0.069, suggesting the potential of sCha as a discriminatory biomarker of cardiac alterations.

Further analysis was performed in Venezuelan patients taking into account clinical histories. Figure 2 shows the results from patients with a clinical history of tachyarrythmias (premature ventricular beats and different grades of ventricular tachycardias). The ectopic ventricular activity was assessed according to the Lown grading system,[22] in which the "0" stage represents patients without ectopic activity and "5" represents patients with the R-on-T phenomenon (malignant ventricular arrhythmias). Patients in 0–2 classifications are at low risk, and in 3–5 classifications, they are at high risk of SD. Venezuelan patients with a high risk of SD (Lown classification ≥ 3) did not show significant differences in O.D. against *T. cruzi* versus the ones with a low risk of SD (Fig. 2A) but showed a significantly higher anti-sCha response (Fig. 2B). Further analysis showed that the AUC for sCha was 0.717 ± 0.084, with a 95% CI of 0.553–0.881, and a significant *P* value of 0.023. In this case, the cutoff value within the 95% CI gave 0.548 with 64.71% sensitivity and 61.9% specificity. To the contrary, the AUC for *T. cruzi* was 0.611 ± 0.102, with a 95% CI of 0.412–0.810, and a *P* value of 0.287. The comparison between sCha and *T. cruzi* AUC analysis showed the value of the anti-sCha ELISA in the discrimination of electrocardiographic alterations associated with a high SD risk. On the other hand, Colombian patients were grouped according to the presence/absence of ectopic beats, a potentially abnormal cardiac pattern that eventually could be related to SD. Equally, patients with ectopic beats registered higher anti-sCha antibodies than nonectopic and cardiac controls (Fig. S2, online only). Further analysis showed that the AUC for sCha was 0.679 ± 0.096, with a 95% CI of 0.490–0.868, and a significant *P* value of 0.049. In this case, the cutoff value within the 95% interval confidence gave 0.506 with 69.23% sensitivity and 27.66% specificity. Finally, Bolivian patients (Fig. 3) were organized according to the presence or absence of the right BBB of the anterior subdivision or SB, an almost pathognomic sign in ChP with cardiac involvement (Fig. S3, left panel, online only). Also, patients from Colombia were analyzed using the sCha ELISA and represented according to the absence or presence of ectopic arrhythmias (Fig. S3, right panel, online only). In both groups of patients, there was a significant trend to an elevation of anti-sCha response depending on BBB and SB appearance, respectively.

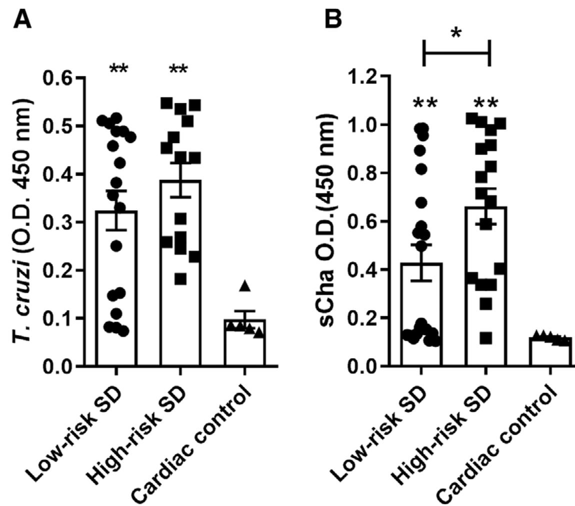

Figure 2. sCha reactivity and risk of sudden death and tachycardia. Comparison between low ($n = 21$) and high risk ($n = 17$) of sudden death Chagas-positive cardiac patients and Chagas-negative cardiac controls ($n = 5$) from Venezuela in *T. cruzi* (A) and sCha (B) ELISA. Statistical significance was determined by Student's *t*-test (*$P \leq 0.05$; **$P \leq 0.01$).

These trends were not observed in the *T. cruzi* ELISA.

Electrocardiogram interval analysis

ECGs were recorded 5 years after serum collection in those Venezuelan patients who remained adhered to the study. A more detailed analysis of the ECG intervals allowed us to separate the patients according to the percentiles of the length of PR, QRS, and QTc intervals. In such a sense, the clinical upper limit of PR interval (for considering first AV degree block) is >200 ms, QRS upper limit is 120 ms, and QTc is 450 milliseconds.[23] In this work, we set the upper limit at 75th percentile, in order to consider those patients with intervals near the upper limit (PR interval = 205.4 ms, QRS interval = 114 ms, and QTc interval = 409.4 milliseconds). Patients with prolonged PR intervals showed significantly higher levels of anti-sCha antibodies, but no significant differences were observed with the *T. cruzi* ELISA (Fig. 3A). Significant differences were observed between short and long QRS intervals for both the *T. cruzi* and sCha ELISAs (Fig. 3B), and no significant differences in the QTc interval length in any case (Fig. 3C).

Fine mapping of the R3 epitope of sCha

Interestingly, while constructing the expression plasmid for recombinant sCha purification, we realized that locating the 6xHis-Tag at the N-terminus of the protein abolished antibody recognition (unpublished). This suggested that essential amino acids could be localized at the N-terminus of the R3H10 peptide. Thus, to test this hypothesis, we performed an alanine scan of R3H10. We optimized a competitive ELISA assay utilizing the R3 peptide, named from now on as R3H10 (MRQLDTNVER) and R3H19 (MRQLDTNVERRALGEIQNV) at 0.1, 1, 10, and 100 μg/mL in serum from a patient with high levels of antibodies (Fig. 4A). The results showed that 10 and 100 μg/mL peptide concentrations gave similar levels of competition with both peptides. Then, we tested the alanine pepscan 10 amino acid peptides (Table S1, online only) at 10 and 100 μg/mL obtaining similar results (Fig. 4B). Therefore, we decided to utilize the lowest peptide concentration (10 μg/mL) for analyzing more patients' sera with the alanine pepscan R3H10 peptides.

Figure 5A shows boxplots of a competitive ELISA with the alanine scan peptides corresponding to 30 ChP from Bolivia, normalized as percentages of the control, and indicates that peptides corresponding to 4 out of 10 residues mutated to alanine (M, R, L, and D) were necessary for antibody binding. But eight different patterns were observed according to individual results (Table S2, online only). Of those, in 50% of the patients,

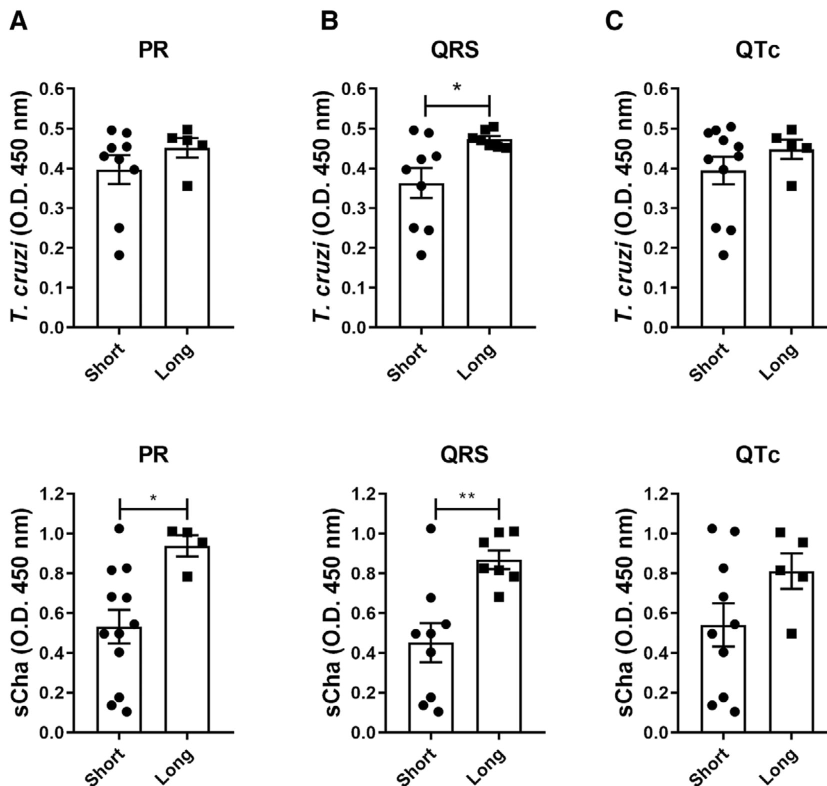

Figure 3. Electrocardiographic (ECG) intervals and anti-sCha antibodies. ECGs were recorded in a group of patients from Venezuela ($n = 16$) 5 years after the serologic analysis was performed. Short and long interval classes and levels of anti–*T. cruzi* and anti-sCha antibodies are represented according to PR (A), QRS (B), and QTc (C) intervals. Statistical significance was determined by Student's *t*-test ($^*P \leq 0.05$; $^{**}P \leq 0.01$).

the pattern was MRXLDXXXXX, in 16.7%, it was MRQLDXXXXX (66.7% overall involving the first five N-terminal residues), and in 33.3%, it involved C-terminal residues (TNVER). Moreover, residues M, R, L, and D mutated to alanine were the most frequent above the median percentage of the competitive ELISA, showing higher optical densities (O.D.s) and percentages than the other residues (Fig. 5B). The Logo graphical representation of the patterns observed in a multiple sequence alignment showed that the minimal consensus sequence was MRQLD (Fig. 5C).

MRQLD sequence cross-reactive T. cruzi *antigens*

To find *T. cruzi* antigens with similarity with the MRQLD epitope, we performed BLAST searches in GenBank, using the reference CL Brener genome. However, the shorter the sequences, the more retrieved sequences using BLAST, and the more difficult it is to choose candidates. Since for a low percentage of patients, additional amino acids (TNVER) were necessary for antibody recognition, we searched for sequences similar to R3H10 of sCha using BLAST, and among the retrieved sequences, we focused on those with more similarity considering the minimal epitope (MRQLD). Table 1 shows the percent of similarity, as well as the sequence alignments to R3H10 of the selected *T. cruzi* antigens identified in RefSeq genome strain CL Brener: enolase (ENO), chaperone (HSlVU), ABC transporter (ABCT), phosphonopyruvate decarboxylase (PPDC), and glycoprotein 63 (GP63). TritrypDB BLAST searches of complete amino acid sequences of the candidates showed orthologues in the *Trypanosomatida* order. BLAST searches of 19 amino acid peptides containing the cross-reactive sequences (named ENO19, HSlVU19, ABCT19, PPDC19, and GP6319) were performed (Table S3, online only) in TritrypDB and NCBI.

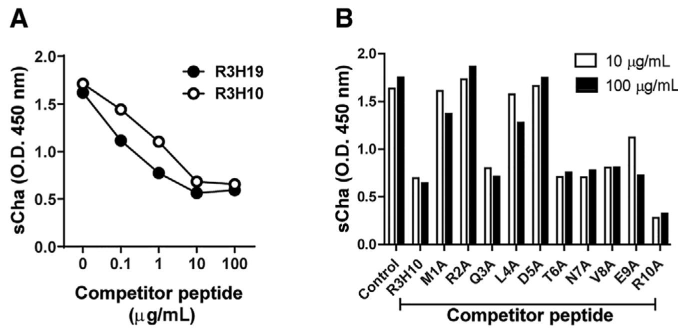

Figure 4. Reactivity against sCha and alanine scan mapping optimization by competitive ELISA. The competitive ELISA of sCha using sera from a Bolivian patient and using different concentrations of R3H19 and R3H10 peptides (A). The competitive ELISA of sCha using sera from a Bolivian patient and different concentrations of the alanine scan R3H10 peptides (B).

Interestingly, BLAST searches of ENO19 showed hits with 100% identity and 100% coverage to *Trypanosoma* spp., and to many bacterial species, while ABCT19 to *T. cruzi* and *T. grayi* (Supplementary Data, online only). Only HSlVU19, PPDC19, and GP6319 showed >95% identity and 100% coverage to different *T. cruzi* strains (Table S3, online only), suggesting that these peptides are *T. cruzi*–specific.

Validation of MRQLD cross-reactive candidate T. cruzi *antigens*

To demonstrate cross-reactivity of antibodies against sCha with the parasite antigen, synthetic peptides corresponding to the candidate sequences (Table S3, online only) at different concentrations (0.1, 1, 10, and 100 μg/mL) were tested in competitive ELISAs using a positive patient serum (Fig. 6A).

Peptides at 10 μg/mL were able to compete for the binding of the antibody to sCha; thus, 15 patients' sera from Bolivia (treated at Hospital Ramón y Cajal, Madrid, Spain) were analyzed under the same conditions. Figure 6B shows that ABCT and PPDC peptides were the ones that competed more significantly for antibody binding to sCha, followed by the HSlVU chaperone. No significant competition was observed with ENO and GP63 peptides. We aimed to immunize mice with a *T. cruzi* cross-reactive candidate antigen to demonstrate antibody cross-reactivity. Thus, to induce a stronger antibody response in mice, longer amino acids were synthesized: 19 amino acid peptides of sCha (R3H19), ABCT (ABCT19), and PPDC

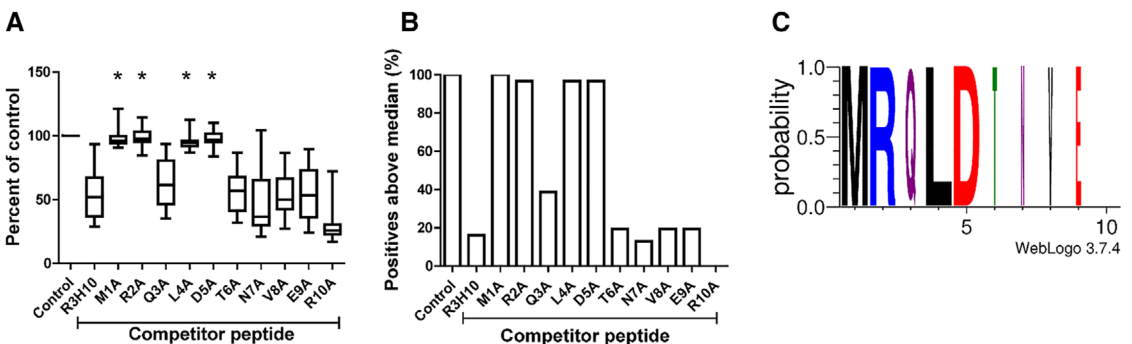

Figure 5. Alanine scan in a group of patients. (A) Competitive ELISA using 10 μg/mL of the alanine scan of R3H10 peptides was performed in a group of patients from Bolivia (Hospital Ramón y Cajal, Madrid, Spain) ($n = 30$); data are represented in box plots of the percent inhibition with respect to the control of each patient's sera (100%); statistical significance was determined by Student's t-test (*$P \leq 0.05$). (B) Percent of patients for which the mutated amino acids abolished anti-sCha antibody peptide competition. (C) The consensus sequence for antibody recognition after sequence alignment of individual patient's pattern generated using WebLogo3.[31]

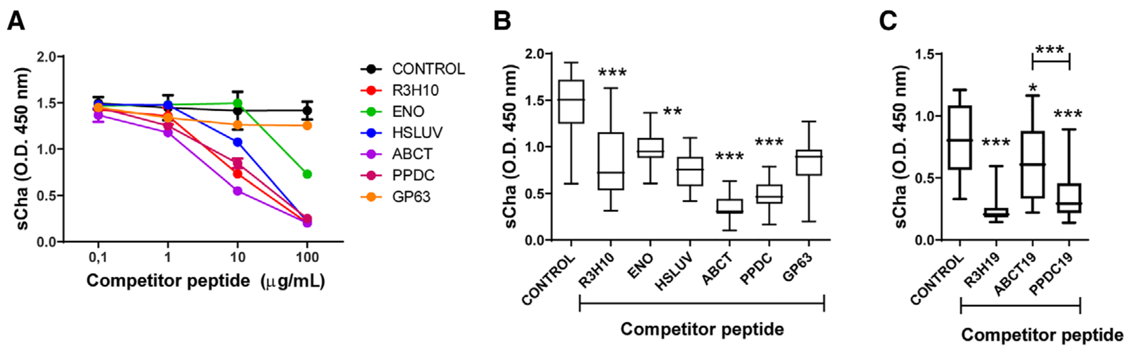

Figure 6. Competitive ELISA with cross-reactive *T. cruzi* antigen candidates. (A) Competitive ELISA using peptides at different concentrations using a single patient's serum. (B) The competitive ELISA of a group of patients from Bolivia (from Hospital Ramón y Cajal, Madrid, Spain) ($n = 15$) with 10 amino acid peptides; results are presented in box plots; statistical significance was determined by Student's *t*-test (**$P \leq 0.01$ and ***$P \leq 0.001$). (C) The competitive ELISA of a group of patients from Venezuela ($n = 24$) with 19 amino acid peptides; results are presented in box plots; statistical significance was determined by Student's *t*-test (*$P \leq 0.05$ and ***$P \leq 0.001$).

(PPDC19) were synthesized (Table S3, online only). These peptides were first tested in a group of 24 ChP from Venezuela. Figure 6C shows that the PPDC19 peptide competed more significantly than the ABCT19 peptide for the binding of antibodies to sCha, indicating that PPDC could be a good candidate for the generation of sCha cross-reactive antibodies.

Antibodies against T. cruzi *PPDC19 recognize human sCha*

BALB/c mice were immunized with the PPDC19 peptide coupled to KLH as described in Methods. Figure 7A shows that the anti-PPDC19 serum raised in mice was able to recognize sCha in ELISA. The titer of the anti-PPDC19 serum in the sCha ELISA was about 9×10^{-4}. Moreover, competition with 10 µg/mL of the immunizing PPDC19 peptide caused a 29% reduction in binding to sCha at 1:100 anti-PPDC19 serum dilution. On the other hand, the ABCT19 peptide was able to reduce 57% of binding to sCha, but at a much higher serum dilution (1:10,000). However, the R3H19 peptide was able to reduce 45% the binding of anti-PPDC antibody to sCha at 1:1000 serum dilution, indicating their cross-reactivity with anti-PPDC19 antibody and the sCha autoantigen. Cross-reaction of anti-PPDC19 antibody between PPDC19 and sCha was also demonstrated by western blot analysis (Fig. 7B). Moreover, anti-PPDC19 specifically recognized a few *T. cruzi* proteins in cell-derived trypomastigotes lysates (Fig. 7B).

Discussion

The identification of biomarkers of progression and/or treatment response is a major research priority in ChD. One of the principal problems for clinicians who deal with ChD is the broad range of probabilities in clinical outcomes in asymptomatic patients. It is well known that only 30% of patients will develop chronic complications, but, until now, there are no reliable methods for predicting chronic outcomes. Nonprevious symptomatic SD is the principal cause of death in ChP. Electrocardiographic rhythm changes are predictors of both disease severity and outcome,[24] but suppose clinical suspicion or previous diagnosis of abnormalities and implies specialized cardiology units that are largely not available in the poorest endemic areas. Additionally, in nonendemic countries, blinded diagnostic surveys for arrhythmias are required to avoid additional expenses.

Our results indicate the usefulness of the sCha autoantigen as an SD high-risk discriminant biomarker in multicenter cohorts from different countries and cardiac symptomatology. High levels of anti-sCha antibodies showed the ability in a retrospective way to identify patients with arrhythmias, especially the different kinds of tachyarrhythmias included in this study. We were able to set a

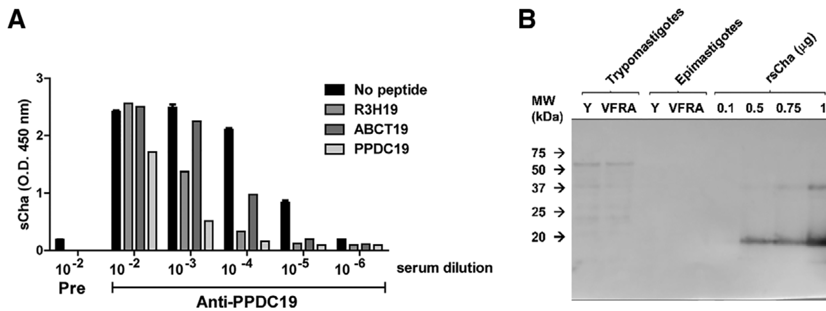

Figure 7. Generation of cross-reactive antibodies. (A) Serum from mice immunized with the PPDC19 synthetic peptide was assayed using competitive ELISA in serial dilutions using R3H19, ABCT19, and PPDC19 at 10 μ/mL competitor peptides. (B) Western blot analysis of 20 μg of trypomastigote and epimastigote lysates of the Y and VFRA strains and 0.1, 0.5, 0.75, and 1 μg of recombinant sCha (rsCha); the mouse anti-PPDC19 serum recognized parasite proteins of various sizes and rsCha (20 kDa band).

threshold for sCha autoantibodies in patients with SD risk in 0.548 (64.71% sensibility and 61.90% specificity), while in the conventional anti–*T. cruzi* antibodies screening, the differences between low- and high-risk patients were not statistically significant. These results reinforce the idea about the utility of the anti-sCha ELISA test to discriminate pathology in ChD. Moreover, after 5 years, ECG records indicated that anti-sCha antibody levels were significantly higher in those patients with prolonged PR intervals, a sign of atrioventricular block. Thus, since anti-sCha antibodies may be determined by ELISA or even in a multiple antigen blot assay test, it could be used as an alternative to ECG analysis, which is not always possible in endemic areas.

Unfortunately, since no ECG records were taken at the time of serum collection, the prognostic value of anti-sCha antibodies is unknown and deserves further study. For other causes of SD, there are genetic markers[25] and serum markers of cardiac extracellular matrix turnover that can be used as prognosis biomarkers,[26] but these markers are significantly more expensive and require more equipment and trained personal. If the prognosis value of anti-sCha antibodies is demonstrated in future studies, it will be very useful as an alternative to the described expensive markers.

As reviewed by Cortes-Serra *et al.*,[11] many biomarkers, including autoantibodies, have been associated with ChD cardiomyopathy, whose levels increase with symptomatology, but most of them are unable to distinguish between ChD cardiomyopathy and other cardiomyopathies. Contrarily, autoantibodies against sCha are Chagas cardiomyopathy–specific, not described for any other cardiomyopathy, and not present in healthy individuals.

The mechanism underlying the generation of autoantibodies in human ChD was unknown (reviewed in Ref. 13). We determined that the MRQLD sequence was minimal for antibody binding to sCha. The PPDC19 peptide containing the LRQLD sequence, with 80% identity and 100% similarity, competed most significantly for antibody binding to sCha. Antibodies against PPDC19 raised in mice cross-reacted with sCha, suggesting that PPDC is one of the *T. cruzi* antigens but likely not the only one responsible for the generation of cross-reactive antibodies. We previously reported that human antibodies against human R3H10 recognized a *T. cruzi* antigen of about 36 kDa in western blot analysis.[20] We demonstrated here the cross-reactivity in the opposite direction, antibodies to PPDC of *T. cruzi* cross-reacted with sCha.

Interestingly, a *T. cruzi* genome-scale B cell epitope prediction identified the QRMSNASG**G**GGG**GMRQNE** peptide (C6_30), where bold letters represent key amino acids for antibody binding, as a candidate to differentiate patients infected with different strains of *T. cruzi* in ELISA, with 95.8% sensitivity, 88.5% specificity, and 92.7% accuracy.[27] As can be observed, the last five C-terminal amino acids of the C6_30 peptide (…MRQNE) present 60% identity and 80% similarity with the MRQLD epitope of sCha. Moreover, a recent report on Chagas diagnosis using bacterial display random 12-mer peptide library and next-generation sequencing revealed highly antigenic epitopes that permit the development of

high-performance multiplex serological assays.[28] Interestingly, a total of 48 positives out of 58 samples (82.7%) from different cohorts showed high reactivity against the peptide [KR]MRXID. Again, the last five C-terminal amino acids present 60% identity and 80% similarity with the MRQLD epitope of sCha. This coincidence from three different laboratories, using strikingly different approaches, indicates the immunodominance of the R3H5 and R3H5-like epitopes and their value in ChD diagnosis.

On the other hand, *TCFL5* encodes several isoforms, but not sCha, suggesting that sCha could be generated by proteolytic cleavage. Indeed, cleavage predictions using the PROSPER (Protease Specificity Prediction Server)[29] showed that matrix metalloprotease 9 (MMP9) might cleave the sequence "…NRSR/MRQLD…" and expose the MRQLD cryptic epitope. MMP9 is involved in cardiac remodeling, and it is increased in chronic infection.[30] Thus, likely the infection reveals the presence of the cryptic epitope through cleavage of Cha by MMP9. Interestingly, the sequences described by Mendes *et al.*[27] (QRMSNASGGGGGGMRQNE) and Kamath *et al.*[28] ([KR]MRXID) are also predicted to be targets of MMP9, rendering the sequences MRQNE and MRXID, respectively.

Taken together, our results allowed us to identify the origin of autoantibodies against sCha by a molecular mimicry mechanism between the immunodominant R3H5 epitope and the PPDC peptide, and even though their pathological role was not evaluated, they are without doubt good discriminating biomarkers of ECG alterations associated with a high risk of sudden death.

Author contributions

M.F. and N.G. presented the concept. M.A.C.-de-V. and M.C.M. performed methodology and experimentation. A.L.-C., J.I.-A., J.G.-M., A.H.-C., and C.P. performed the experiments and verified the results. H.O.R.-A., J.S.-R., J.D.R., and A.P.-A. were involved in clinical data curation. A.L.-C., A.H.-C., F.C.-H., and J. del M.-S. performed database searches. J.M., I.M., F.G., M.G., J.A.P.-M., R.L.-V., M.F., and N.G. supervised the study. I.C., R.P.-T., A.P.-A., B.M.-M., F.N., J.M., I.M., F.G., M.G., J.A.P.-M., and R.L.-V. provided samples from patients. N.G. and H.O.R.-A. carried out formal analysis and investigation. N.G., M.F., and H.O.R.-A. prepared the original draft. A.L.-C., J.S.-R., and J.D.R. reviewed and edited the article. H.O.R.-A. and N.G. were involved in data visualization. M.F. and N.G. were involved in funding acquisition. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Frequency distribution of the O.D. values of *T. cruzi* and sCha ELISAs.

Figure S2. Arrhythmias in Colombian patients. Nonectopic ($n = 47$) and ectopic ($n = 13$).

Figure S3. sCha reactivity and bradycardia.

Table S1. Sequences of the R3H10 alanine scan peptides.

Table S2. Sequences of the R3H10 alanine scan peptides.

Table S3. Sequences of the 19 amino acid synthetic peptides.

Table S4. Results of the TritrypDB and NCBI BLAST searches of the 19 amino acid peptides.

Competing interests

The authors declare no competing interests.

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