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Small-RNA sequencing identifies serum microRNAs associated with abnormal electrocardiography findings in patients with Chagas disease

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SUMMARY

Background: Chagas disease, caused by the parasite Trypanosoma cruzi (T. cruzi), affects around 6–7 million people in Latin America and hundreds of thousands in the United States and Europe. A main complication of chronic Chagas disease is cardiomyopathy, possibly manifesting as arrhythmias, heart failure, or sudden cardiac death. Understanding the link between T. cruzi infection and cardiomyopathy is essential for early diagnosis and adequate treatment.

Methods: We sequenced small RNAs in serum samples from 228 Chagas patients recruited in Chile, Bolivia and Italy. After bioinformatic processing of sequencing data to quantify serum miRNA expression, robust logistic regression was applied to identify miRNAs differentially expressed in Chagas patients with abnormalities in electrocardiography (ECG), bundle-branch block on ECG, and high Kuschnir scores. We also investigated the association between genotype-based miRNA expression and the risk of abnormal ECG findings.

Findings: As reported, the risk of abnormal ECG findings was higher in male patients and increased with age. Three miRNAs showed lower serum expression levels in patients with abnormal ECG: miRNA-101-3p, miRNA-576-3p and miRNA-629-5p ($p < 0.0002$), especially in patients with high Kuschnir scores. The expression of miRNA-629-5p was negatively correlated with the CCL5 expression ($p = 3.7\times10^{-8}$), a chemokine frequently reported in Chagas disease. Gene enrichment analyses indicated involvement of cytokine signalling in Chagas cardiomyopathy.

Interpretation: The findings demonstrate the potential of circulating miRNAs as diagnostic biomarkers for

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Chagas cardiomyopathy. The associations found with disease severity and immune response may help to improve our knowledge of complications' development in Chagas disease.

Introduction

Around eight to ten million people worldwide are currently affected by Chagas disease, which is caused by infection with the protozoan *Trypanosoma cruzi*.[1–3] Chagas disease is endemic in 21 Latin American countries and is on the World Health Organization list of neglected tropical diseases.[4] The disease is most common in South America, but due to migration and travel, its diagnosis and treatment of patients have become a worldwide challenge.[5] Three phases are distinguished in the course of Chagas disease. In the first (acute) phase, patients usually have mild, non-specific symptoms, hindering swift diagnosis. In the second (indeterminate chronic) phase, which lasts from several years to a lifetime, patients are usually asymptomatic.[3] If the indeterminate chronic phase persists for several decades, up to 40% of patients develop serious complications, including Chagas-associated cardiomyopathy and megacolon; this third phase is referred to as the determinate chronic phase.[3,6] Chagas cardiomyopathy is the predominant chronic complication and represents the leading cause of death in patients with Chagas disease.[4] While Chagas disease is largely diagnosed by serological methods such as enzyme-linked immunosorbent assay (ELISA), Chagas cardiomyopathy is currently detected primarily by electrocardiography (ECG), echocardiography and radiological examinations.[4]

Two drugs are currently authorised for the treatment of Chagas disease patients: nifurtimox and benznidazole.[7] Both treatment regimens are lengthy and can lead to severe side effects such as neuropathy, especially in older patients, which contributes to low treatment compliance.[8] New findings suggest that current treatment regimens can be optimised through intermittent dosing, dose reductions or combination therapies.[9–11] At the same time, new drugs are being tested.[8,10,12] In the acute phase of the disease, nifurtimox and benznidazole have a cure rate of up to 100%, and the antibody response to *T. cruzi* becomes negative relatively soon after treatment.[7,11,13] However, only about 20% of acutely infected patients show symptoms; the majority remain undiagnosed and thus untreated, and the disease progresses to the indeterminate chronic stage.[4] In the chronic phase, the titre of anti-*T. cruzi* antibodies decreases slowly, and confirmation of cure by conversion to seronegativity can take decades after effective treatment, with reported rates ranging from 5% after 5 to 10 years, to 45% after more than 20 years.[9,14] With increasing disease duration, the rate of successful treatment drops to less than 40%.[15,16] Moreover, the older the patients become, the higher the rate of therapy-related side effects.[17,18] For these reasons, treatment is controversial in the chronic phase of Chagas disease. A randomised controlled trial showed that despite a reduction in parasite load, treatment with benznidazoles was unable to prevent cardiac progression of the disease in patients with pre-existing cardiac involvement.[19] In spite of the low success rate of treatment, the risk of therapy-related side effects, and the possibility of symptomless disease progression, treatment is generally recommended in the early stages of chronic disease. As there are currently no predictive biomarkers for disease progression, early intervention remains the only way to prevent severe complications such as Chagas cardiomyopathy.[19]

MicroRNAs (miRNA) appear to be involved in the pathomechanism of Chagas cardiomyopathy.[20–22] These short (approximately 18–25 nucleotides long), non-coding RNAs play an important role in gene regulation by binding transcribed messenger RNAs (mRNA), inducing their degradation and preventing translation.[23] In recent years, miRNAs circulating in serum have been increasingly described as a new diagnostic tool.[24,25] One of the advantages of miRNAs as biomarkers is that they are remarkably stable compared with other candidates in body fluids.[26] In cardiological diseases, miRNAs have already been shown to have diagnostic value. For example, a recent review concluded that several miRNAs are useful for the diagnosis of myocardial infarction, and a signature of six different miRNAs has been proposed as a diagnostic marker of acute myocardial infarction with high sensitivity and specificity.[27] Some studies have even found earlier changes in miRNA expression profiles than in those for troponin, so the former have the potential to perform better than the standard diagnostic tool used.[28]

It has been shown that the expression of many miRNAs in cardiomyocytes differs between healthy donors and patients with Chagas cardiomyopathy.[22] This is particularly true for miRNAs involved in signalling pathways that are important for cardiomyopathy, such as the IFNγ signalling pathway.[22] In addition, a number of miRNAs circulating in serum (miR-1, miR-133a-2, miR-133b, miR-208a, miR-208b, miR-146a-5p, miR-155-5p, miR-19a-3p, miR-21-5p, miR-29b-3p, miR-30a-5p, miR-199b-5p) have been investigated in recent years for their suitability as prognostic biomarkers in small cohorts of Chagas disease patients.[20,21,29–31] These studies focused on previously selected miRNA candidates already known from other heart diseases, such as dilatative cardiomyopathy, and investigated their suitability as markers for the progression of Chagas cardiomyopathy. [20,21,29–31]

The goal of the present study was to predict Chagas cardiomyopathy based on the expression profiles of circulating miRNAs. We performed small-RNA sequencing of serum samples from 228 patients with Chagas disease and investigated the association between miRNA expression and ECG status (normal/abnormal), bundle-branch block on ECG and Kuschnir score to assess the degree of heart dysfunction. The identified miRNAs have the potential to become diagnostic biomarkers for Chagas cardiomyopathy and to improve our understanding of the link between *T. cruzi* infection and Chagas cardiomyopathy.

Materials and methods

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of the Faculty of Medicine, Universidad de Chile, Santiago, Chile; Universidad Mayor de San Simón, Cochabamba, Bolivia; the Lazzaro Spallanzani National Institute for Infectious Diseases, Rome, Italy; and Universidad de Tarapacá and University College London, as described in ref.[32] All study participants provided written informed consent prior to enrolment. Once enrolled, patients were interviewed by local study coordinators, who also collected blood samples and clinical information using standardised case report forms that included clinical involvement (indeterminate, cardiac, gastrointestinal, mixed), ECG findings, the cardiothoracic index on chest radiography, and treatment information (e.g. type of treatment, discontinuation of treatment and adverse events).

Investigated patients and data

The investigated Chagas disease patients were recruited in endemic and non-endemic countries: Chile (n = 99), Bolivia (n = 74)

and Italy (n = 55). They originated from Chile (n = 99), Bolivia (n = 122) and other countries: Brazil (n = 2), Honduras (n = 2), Venezuela (n = 2) and El Salvador (n = 1).

RNA was extracted from serum samples using Qiagen's miRNeasy kit. Small-RNA sequencing was performed using the NEBNext Small RNA kit (cat. no. E7300, New England Biolabs, Ipswich, MA, USA) to produce RNA sequencing libraries, which were then sequenced on a HiSeq 2500 platform (Illumina, San Diego, CA, USA) with an average depth of 18M reads per sample. The RNA sequencing protocol used has been previously described in detail.[33] Briefly, our protocol covered RNA molecules from 17 to 47 nucleotides, allowing us to capture fragments of both miRNA and messenger RNA (mRNA). First, reads from the HiSeq 2500 platform were adapter-trimmed (AdapterRemoval v2.1.7),[34] then adapter-trimmed reads were mapped to the human genome (hg38) by a Bowtie2 v2.2.9 aligner.[35] HTSeq was used to count reads mapped to miRNA regions and mRNA exons in GENCODE v26 annotations.[36,37]

Genomic DNA was extracted from peripheral blood or saliva using standard commercial kits and following standard laboratory procedures. Intraplate and interplate replicates and blinded duplicates (5) were included for quality control. Study participants were genotyped with Illumina's OmniExpress or Global Screening arrays. Both arrays included more than 700,000 single-nucleotide polymorphisms (SNPs). Missing genotypes were imputed using the TOPMed imputation server.[38]

We also used data from the Framingham Heart Study (FHS) to investigate whether the miRNAs identified were specific to Chagas cardiomyopathy or shared with other heart diseases. The miRNAs were measured in whole blood samples from FHS participants as a part of the Systems Approach to Biomarker Research in Cardiovascular Disease initiative (dbGaP accession number: phs000363.v19.p13). The dataset analysed included 491 participants with both miRNA expression and clinical data.[39–41] The FHS study design and details of the miRNA expression profiling and quality control have been described previously.[42] For the present study, we focused on information on right and left bundle-branch blocks obtained from ECG data as part of the Sleep Heart Health Study conducted between 1998 and 2003 within the FHS (dbGaP accession code: pht000745.v3.p13).[43] The clinical data also included information on the study participants' gender and age at first examination (dbGaP accession number: pht003099.v7.p13). The data used in this study can be accessed through the National Center for Biotechnology Information Database of Genotypes and Phenotypes (dbGaP accession number: phs000007.v32.p13). All protocols were approved by Boston University Medical Center Institutional Review Board. All participants had provided written informed consent when joining the FHS.

Statistical analysis

Read counts were log2 transformed, and miRNAs with low expression variability (median absolute deviation [MAD] of read counts equal to 0) were excluded from further statistical analyses. Quantile normalisation was performed first by country and ECG status (normal/abnormal), then by country, and finally for the complete dataset. Principal component analysis (PCA) was performed for an unsupervised examination of global expression and identification of potential samples with outlying expression profiles. Graphs were plotted using the R package "ggplot2".[44,45]

The primary outcome assessed in this study was an abnormal ECG, defined as any ECG finding deviating from a normal sinus rhythm with a normal axis, e.g. sinus bradycardia < 50 beats per minute or right bundle-branch block (RBBB).[46] Secondary outcomes were bundle-branch block on ECG and the Kuschnir score based on clinical symptoms and ECG findings, with higher scores generally requiring more intensive monitoring and treatment.[68] Univariate and multiple robust logistic regression models were fitted to identify miRNAs associated with abnormal ECG findings, adjusting for age, country and sex[47]:

ECG status (normal/abnormal) ~ log2 miRNA expression + age + country + sex

Left bundle-branch block (LBBB) is less common than RBBB in Chagas cardiomyopathy. To examine whether the identified miRNAs are specific to Chagas cardiomyopathy or shared with other cardiopathies, we also fitted robust logistic regression models adjusted for age and gender to investigate the association between miRNA expression and RBBB or LBBB in FHS participants:

RBBB ~ log2 miRNA expression + age + gender

LBBB ~ log2 miRNA expression + age + gender

Robust logistic regression models were fitted using the R package "robustbase", setting the tuning constant c in Huber's psi function equal to 1.2.

Potential target genes of miRNAs associated with abnormal ECG findings were identified using the miRWalk database,[48] and linear regression models were fitted to our own miRNA and mRNA sequencing data to investigate the relationship between miRNA and mRNA expression:

log2 miRNA expression ~ log2 mRNA expression

The mRNAs that showed a negative correlation p-value < 0.05 after Bonferroni correction were declared miRNA targets. We then performed pathway analysis for miRNA targets using the 'enrichr' platform and the 'PANTHER Pathways' dataset.[49–52]

To assess the direction of the identified associations - whether miRNA expression levels are the cause or rather the effect of Chagas cardiomyopathy, reflected by abnormal ECG findings - we also (1) identified SNPs associated with miRNA serum expression in a dataset that included both genome-wide genotype and serum miRNA expression data from 110 participants in two Chilean studies on chronic obstructive pulmonary disease (COPD, n = 22) and Chagas disease (n = 88), and (2) examined the relationship between genotype-based miRNA expression and abnormal ECG findings in an independent set of 194 Chagas disease patients with genotype and ECG information.

The list of SNPs potentially associated with the expression of miRNAs (cis-miRNA-eQTLs) differentially expressed in Chagas patients with normal and abnormal ECG findings was retrieved from the 'miRNA-eQTL' database: http://ibi.hzau.edu.cn/ncRNA-eQTL/miRNA/.[53] We then fitted robust linear regression models adjusting for age, sex and the first 10 genetic principal components:

log2 expression ~ SNP + age + sex + 10PCs

predicted log2 expression = $\Sigma_{i=1 \text{ to } k}\, \beta_i\, A_i$

For each SNP, we compared four penetrance models – Additive (major allele count), Three genotypes (genotype as a categorical variable), Dominant (at least one affect allele vs. the other genotype), Recessive (two affect alleles vs. the other genotypes) – and selected the best prediction model using a robust version of Akaike's information criterion (RAIC). Robust linear regression models were fitted using the function "rlm" in the R package "MASS".[54] The corresponding p-values were obtained using the "rob.pvals" function of the R package "repmod".[55] The RAIC for each model was calculated using the "AIC" function in the R package "AICcmodavg".[56]

After identification of cis-miRNA-eQTL in the 'miRNA-eQTL' database, validation of the association between the cis-miRNA-eQTL

Table 1
Main characteristics of the investigated patients with Chagas disease.

Variable	Level	Chagas patients n	Patients with abnormal ECG n %		OR	95% CI	p
Gender	female	154	81	53%	Ref.		
	male	74	57	77%	3.02	1.60 - 5.69	0.0006
Age	under 38	57	24	42%	Ref.		
(years)	38–47	57	36	63%	2.36	1.11- 5.00	0.005
	48–57	57	35	61%	2.19	1.03 - 4.63	
	over 58	57	43	75%	4.22	1.89 - 9.45	
Country	Chile	99	45	45%	Ref.		
	Bolivia	122	89	73%	3.24	1.84 - 5.69	0.0002
	other	7	4	57%	1.60	0.34 - 7.53	

Other countries: Brazil (n = 2), Honduras (n = 2), Venezuela (n = 2) and El Salvador (n = 1), OR: odds ratio for abnormal ECG findings, CI: confidence interval, p: probability value, Ref.: Reference category

and miRNA serum expression, and selection of the penetrance model, serum miRNA expression was predicted based on individual genotypes using the formula:

$$\text{predicted log2 expression} = \Sigma_{i=1 \text{ to } k}\, \beta_i\, A_i$$

where k is the number of cis-miRNA-eQTLs, β_i are the effect estimates from the linear robust regression model, and A_i are the individual genotypes coded according to the selected penetrance model.

Finally, we fitted robust logistic regression models to assess the association between genotype-based serum miRNA expression and abnormal ECG findings, adjusting for age, sex and the first 10 genetic principal components:

$$\text{ECG status} \sim \text{Predicted log2 serum expression} + \text{age} + \text{gender} + 10\text{PCs}$$

Results

Table 1 shows the main characteristics of the study participants. The median age of the 228 patients with Chagas disease was 47.5 years; 154 (68%) were female and 122 (54%) were from Bolivia. The proportion of patients with abnormal ECG findings was 77% in men versus 53% in women (p = 0.0006), and this increased with age (42% in patients younger than 38 years versus 75% in patients older than 58 years, p = 0.005). The proportion of study participants with abnormal ECG findings was higher among those from Bolivia (73%) than in those from Chile (45%) (p = 0.0002).

Small-RNA sequencing of serum samples identified 2609 miRNAs in total. Of these, 353 had a MAD greater than 0 and were retained for the subsequent data analyses. Fig. 1A shows a PCA plot representing the global expression profiles of the serum samples analysed. The first principal component explained 12% of the variance in miRNA expression, the second principal component a further 11.5%. After excluding potential outliers (the 5% of the samples with the highest statistical depth based on the first two principal components), 212 samples were included. Three separate clusters were observed: samples collected in Chile at the top, Bolivian samples at the bottom left, and Italian samples at the bottom right of the plot. This indicated specific global expression profiles per country of recruitment. However, samples of patients with normal and abnormal ECG findings within each country were well mixed in the plot. Based on these exploratory results, we decided to include country of recruitment in the multiple logistic regression analyses as a potential confounder for the association between miRNA expression and the investigated cardiac outcomes.

The volcano plot in Fig. 1B shows the results of the univariate robust logistic regression for the 353 miRNAs with a MAD greater than 0. The association between expression and an abnormal ECG was particularly strong for three miRNAs: the association p-value exceeded the multiplicity threshold ($0.05/353=1.4\times10^{-4}$) for miR-629-5p, and miR-101-3p and miR-576-3p also showed strong associations ($p < 6\times10^{-4}$). Interestingly, the average expression levels of these three miRNAs were lower in serum samples from Chagas disease patients with abnormal ECG findings.

Fig. 1C shows more detailed information on the relationship between the expression of the three identified miRNAs and the cardiac outcomes analysed. In samples from patients with normal versus abnormal ECG results, the median log2 expression of miR-629-5p decreased from 8.3 to 7.8, that of miR-576-3p from 5.8 to 5.1, and that of miR-101-3p from 11.2 to 10.6 (Table 2). Common ECG alterations in Chagas cardiomyopathy are block patterns, particularly left anterior hemiblock and complete RBBB.[4] Bundle-branch block on ECG was associated with reductions in median log2 expression from 8.8 to 8.2 for miR-629-5p and from 5.8 to 5.7 for miR-576-3p. In contrast, bundle-branch block on ECG was associated with an increase in median log2 expression from 11.2 to 11.8 for miR-101-3p. We also examined the differences in miRNA expression according to Kuschnir score as a marker of cardiomyopathy severity, and found monotonic decreases in serum expression with increasing Kuschnir score for the three identified miRNAs (Fig. 1C; median log2 expression for miR-101-3p: 10.5 [Kuschnir 0], 10.3 [Kuschnir 1], 9.8 [Kuschnir >2]; median log2 expression for miR-576-3p: 5.1 [Kuschnir 0], 4.8 [Kuschnir 1], 4.6 [Kuschnir >2]; median log2 expression for miR-629-5p: 7.8 [Kuschnir 0], 7.6 [Kuschnir 1], 7.5 [Kuschnir >2]). Taken together, these results indicate a negative correlation between the expression of the three miRNAs identified and the severity of Chagas disease. The differences in serum miRNA expression were generally higher in male patients and in patients older than 48 years (Table 2, Table S2).

We then tested whether the miRNAs identified are specific to Chagas cardiomyopathy or shared with other cardiac diseases. For this purpose, we analysed data from the FHS. We fitted robust logistic regression models for RBBB and LBBB as outcomes and the expression of miR-576-3p and miR-629-5p in whole blood as predictors (miR-101-3p expression was not available in this dataset). We found no association (Table S1), suggesting that the miRNAs identified are probably specific to Chagas cardiomyopathy.

We found 50 (SNPs) associated with miR-629-5p expression, 26 SNPs associated with miR-101-3p and no SNPs associated with miR-576-3p in the 'miRNA-eQTL' database (Table S3). Among the list of 76 potential cis-miRNA-eQTLs in this database, only rs11630316 was associated with serum expression of miR-629-5p (Table S4). As shown in Fig. 2A, miR-629-5p expression decreased with the number of T alleles for this SNP (per T allele change in log2 expression −0.3751, p = 0.01). In agreement with the results based on direct quantification of serum miRNA expression based on small-RNA sequencing, miR-629-5p expression based on the rs11630316 SNP was also lower in patients with abnormal ECG findings (Fig. 2B). The reduction in rs11630316-based expression of miR-629-5p did not reach the statistical significance level of 0.05 (OR for abnormal ECG results per log2 expression unit = 0.71, 95% CI = 0.30–1.72, p =

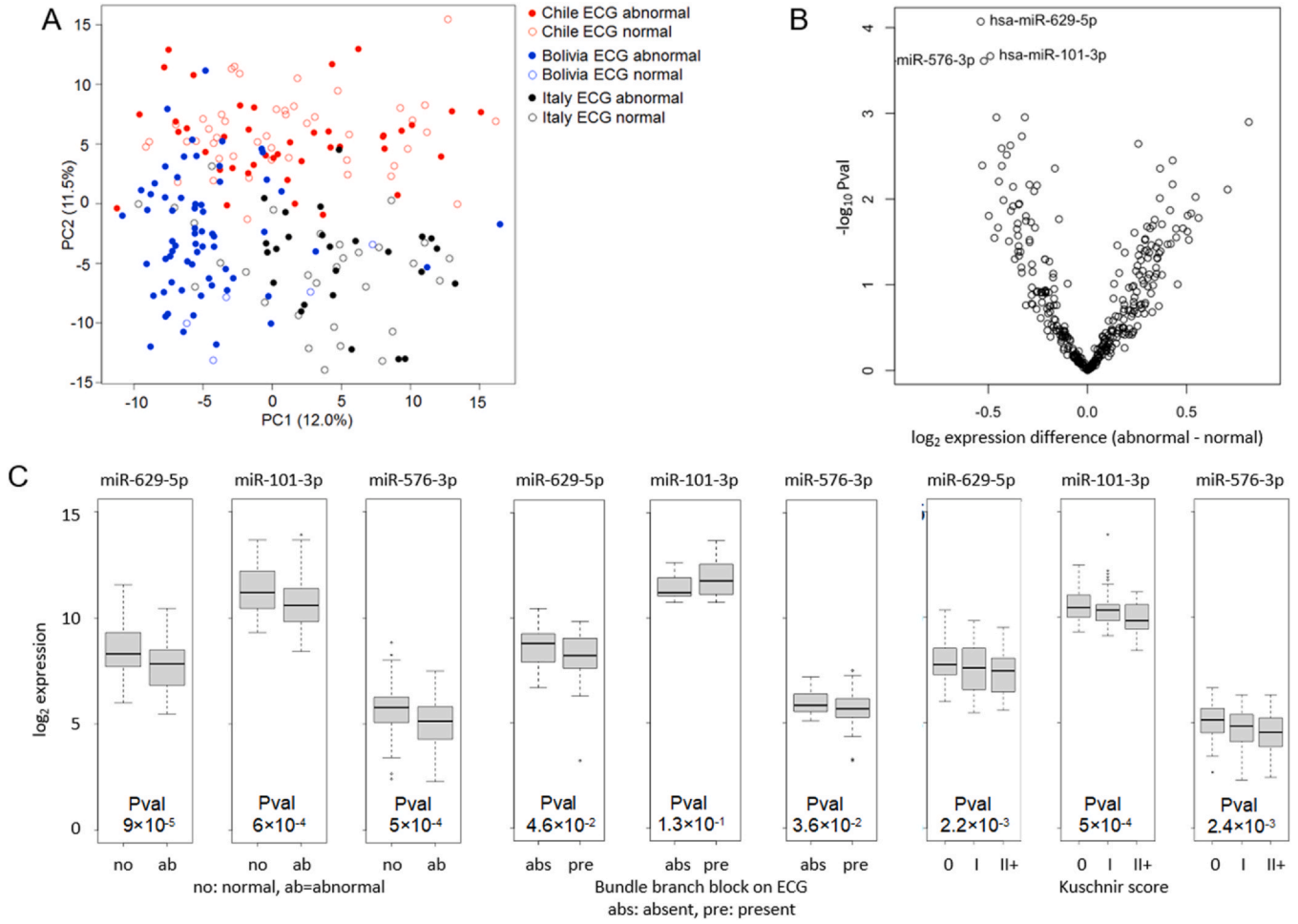

Fig. 1. Principal component analysis plot showing global serum miRNA expression profiles (A), volcano plot for the association between miRNA expression and abnormal ECG findings (B), and box plots of serum miRNA expression by ECG status (normal/abnormal), bundle-branch block on ECG and Kuschnir score (C).

0.45), but suggests an unconfounded relationship between serum miR-629-5p expression and Chagas cardiomyopathy.

We then investigated which genes and pathways were affected by miR-629-5p, miR-101-3p and miR-576-3p. Small-RNA sequencing was performed with a cutoff size on the pippin preparation covering RNA molecules up to 47 nucleotides, which allowed the capture of mRNA fragments. To identify miRNA targets, we selected mRNAs with expression levels negatively correlated with the expression of the three miRNAs identified. In total, 59 mRNAs showed negative correlations with a corresponding p-value < 0.05. Fig. 2C shows the potential target genes for each miRNA. No mRNA showed a negative expression correlation with all three miRNAs simultaneously. Eight genes were potentially targeted by two miRNAs simultaneously: *NPFFR1* by miR-101-3p and miR-576-3p; *SH3BGRL3* and *SHROOM4* by miR-629-5p and miR-101-3p; and *ANKRD13A*, *ARHGEF39*, *DNAH2*, *MEGF8* and *SMC1A* by miR-629-5p and miR-576-3p. A pathway analysis using the 'enrichr' platform and the 'PANTHER Pathways' dataset revealed that miR-629-3p affected the "Inflammation mediated by chemokine and cytokine signalling" pathway.

Among the genes targeted by miR-629-5p was *CCL5*, a gene encoding for the chemokine ligand previously described in the context of Chagas cardiomyopathy.[57,58] We found a negative correlation between miR-629-5p and *CCL5* ($R = -0.37$, $p = 3.7\times10^{-8}$; Fig. 2D). Thus, the decreased expression of miR-629-5p observed in Chagas disease patients with abnormal ECG findings corresponds to an increased expression of *CCL5* indicating inflammatory processes.

Discussion

In the present cross-sectional study, serum samples from over 200 patients with Chagas disease recruited in Chile, Bolivia and Italy (54% Bolivian and 43% Chilean) were subjected to small-RNA sequencing. We investigated for the first time the relationship between abnormal ECG findings as a surrogate marker of Chagas disease progression to cardiomyopathy and unselected miRNAs with detectable expression variability. We found three miRNAs – miR-629-5p, miR-101-3p and miR-576-3p – with reduced expression levels in Chagas disease patients with abnormal ECG, whose expression decreased gradually with increasing Kuschnir score, i.e. with increasing disease severity. After examining these miRNAs in the FHS, we postulate that they are specific diagnostic markers for Chagas cardiomyopathy.

Chagas cardiomyopathy is a terminal complication of *T. cruzi* infection with a poor prognosis.[3] In recent years, little progress has been made in understanding the development and progression of Chagas cardiomyopathy or protective factors, and new avenues of research are urgently needed. Only then will meaningful stratification of the patients on the one hand and targeted investigation of new therapeutic options on the other be possible. A limitation of this study was that practically only Bolivian and Chilean patients were investigated. Validation of the present results in independent samples from other patient populations will permit assessment of the transferability of our findings. Small-RNA sequencing allowed

Table 2
Expression of mi-R-629-5p, miR-576-3p and miR-101-3p stratified by ECG status; unfiltered and filtered for gender, age and country of recruitment.

			Median log_2 expr.	1^{st} – 3^{rd} quartile log_2 expr.	Univariate logistic regression			Multiple logistic regression		
Population	ECG	Patients			OR	95%-CI	p	OR	95%-CI	p
miR-629-5p										
All	normal	81	8.3	7.7–9.3	Ref.		0.00008	Ref.		0.00003
	abnormal	131	7.8	6.8–8.5	0.584	0.446 - 0.764		0.620	0.459–0.835	
Women	normal	66	8.3	7.7–9.3	Ref.		0.005	Ref.		0.007
	abnormal	77	8.0	7.0–8.7	0.629	0.456 - 0.867		0.645	0.453–0.920	
Men	normal	15	8.5	7.5–9.4	Ref.		0.016	Ref.		0.080
	abnormal	54	7.6	6.6–8.3	0.502	0.286 - 0.880		0.566	0.320–1.002	
Less than 48	normal	49	8.5	7.8–9.4	Ref.		0.003	Ref.		0.017
years	abnormal	57	7.8	6.9–8.4	0.574	0.398 - 0.827		0.585	0.396–0.863	
48 years or	normal	32	8.2	7.7–9.2	Ref.		0.013	Ref.		0.012
more	abnormal	74	7.9	6.8–8.5	0.591	0.391 - 0.893		0.731	0.461–1.160	
Bolivians in	normal	27	8.0	7.6–8.7	Ref.		0.407	Ref.		0.652
Italy	abnormal	21	8.4	7.7–8.8	1.320	0.685 - 2.545		1.459	0.724–2.939	
Bolivians in	normal	5	6.1	6.0–7.3	Ref.		0.836	Ref.		0.320
Bolivia	abnormal	65	7.0	6.4–8.0	1.109	0.416 - 2.961		0.358	0.086–1.500	
Chile	normal	49	8.6	8.2–9.4	Ref.		0.040	Ref.		0.067
	abnormal	45	8.3	7.7–9.0	0.643	0.422 - 0.980		0.599	0.384–0.932	
miR-576-3p										
All	normal	81	5.8	5.1–6.3	Ref.		0.0002	Ref.		0.0002
	abnormal	131	5.1	4.3–5.8	0.593	0.449 - 0.784		0.690	0.501–0.951	
Women	normal	66	5.8	5.1–6.2	Ref.		0.007	Ref.		0.026
	abnormal	77	5.2	4.4–5.8	0.641	0.463 - 0.888		0.714	0.497–1.025	
Men	normal	15	5.7	5.0–6.5	Ref.		0.032	Ref.		0.157
	abnormal	54	4.8	4.2–5.7	0.506	0.272 - 0.942		0.578	0.272–1.229	
Less than 48	normal	49	5.9	5.1–6.5	Ref.		0.049	Ref.		0.124
Years	abnormal	57	5.3	4.6–6.1	0.694	0.482 - 0.999		0.749	0.497–1.127	
48 years or	normal	32	5.6	5.0–6.1	Ref.		0.006	Ref.		0.009
more	abnormal	74	4.9	4.1–5.6	0.542	0.349 - 0.842		0.625	0.372–1.050	
Bolivians in	normal	27	5.2	4.7–5.7	Ref.		0.523	Ref.		0.840
Italy	abnormal	21	5.3	5.0–6.0	1.257	0.623 – 2.535		1.288	0.621–2.669	
Bolivians in	normal	5	4.3	4.3–4.5	Ref.		0.787	Ref.		0.596
Bolivia	abnormal	65	4.4	3.8–5.1	0.869	0.313 – 2.409		0.830	0.281–2.449	
Chile and	normal	49	6.1	5.5–6.6	Ref.		0.244	Ref.		0.253
others	abnormal	45	5.7	5.3–6.3	0.783	0.518 - 1.182		0.730	0.470–1.136	
miR-101-3p										
All	normal	81	11.2	10.5–12.2	Ref.		0.0002	Ref.		0.0002
	abnormal	131	10.6	9.8–11.4	0.612	0.472 - 0.794		0.721	0.519–1.003	
Women	normal	66	11.2	10.5–12.3	Ref.		0.008	Ref.		0.033
	abnormal	77	10.7	10.1–11.6	0.663	0.488 - 0.900		0.727	0.492–1.072	
Men	normal	15	11.2	10.3–11.7	Ref.		0.123	Ref.		0.168
	abnormal	54	10.2	9.6–11.0	0.659	0.388 - 1.12		0.703	0.376 –1.313	
Less than 48	normal	49	11.6	10.6–12.3	Ref.		0.015	Ref.		0.098
Years	abnormal	57	10.7	10.0–11.9	0.657	0.469 - 0.921		0.689	0.446–1.064	
48 years or	normal	32	11.0	10.2–11.6	Ref.		0.016	Ref.		0.012
more	abnormal	74	10.5	9.8–11.2	0.600	0.396 - 0.909		0.756	0.450–1.270	
Bolivians in	normal	27	10.5	10.1–11.1	Ref.		0.644	Ref.		0.883
Italy	abnormal	21	10.5	10.2–11.2	1.195	0.562 – 2.538		1.205	0.563–2.580	
Bolivians in	normal	5	10.0	9.5–10.5	Ref.		0.326	Ref.		0.213
Bolivia	abnormal	65	9.8	9.3–10.5	0.640	0.262 – 1.561		0.416	0.154–1.122	
Chile and	normal	49	11.8	11.0–12.6	Ref.		0.406	Ref.		0.449
others	abnormal	45	11.4	11.0–12.3	0.826	0.526 - 1.297		0.843	0.533–1.334	

quantification of the expression of types of small RNAs in serum other than miRNAs (e.g. hairpins and piwi-interacting RNAs), but the sample size was too small to study their association with abnormal ECG findings.

To date, few studies have investigated circulating miRNAs as biomarkers for Chagas cardiomyopathy. They basically explored the hypothesis that biomarkers identified for other cardiac diseases might be relevant to Chagas cardiomyopathy, e.g. miR-1, implicated in heart failure and arrhythmias; miR-133, involved in cardiac hypertrophy and fibrosis; and miR-21, miR-208 and miR-499, each of which contributes to hypertrophy, ischemia and fibrosis.[20,21,29–31] The three miRNAs identified in this study have not been previously described in the context of Chagas cardiomyopathy, but miR-629-5p has been investigated as a possible diagnostic marker for dilated cardiomyopathy,[59] miR-101-3p in the context of endothelial dysfunction and calcific aortic valve disease,[60,61] and miR-576-3p has been shown to be dysregulated in patient with chronic heart failure.[62] In addition, previous studies found an association between *CCL5* expression and the risk of cardiac complications developing in Chagas disease patients, but *CCL5* expression showed no association with general cardiovascular disease.[63,64]

Interestingly, reduced miR-629-5p expression in serum samples from Chagas disease patients with abnormal ECG findings, bundle-branch block on ECG and elevated Kuschnir score appears to be an effect rather than a cause of Chagas cardiomyopathy, although our genotype-based miRNA expression results are far from conclusive. To date, clinical diagnosis and estimation of disease severity using rating systems such as the Kuschnir score rely on cumbersome tests such as ECG and X-ray.[65,66] As we have shown, the serum expression of the three identified miRNAs correlates strongly with disease severity, allowing us to propose a new diagnostic tool for Chagas cardiomyopathy which our data show to be more accurate than any miRNA previously studied for this purpose. This would allow future diagnosis of Chagas disease complications and monitoring of disease

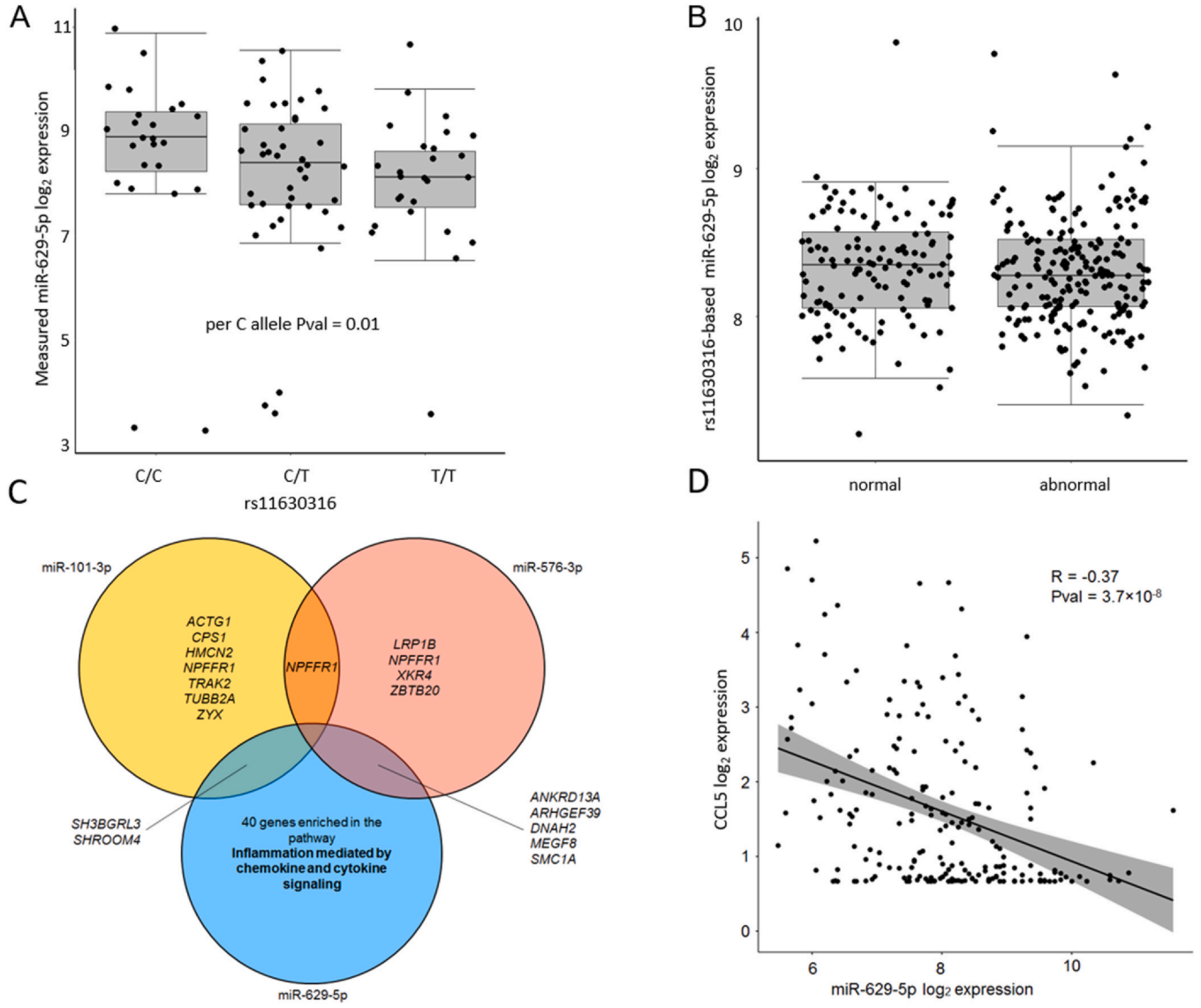

Fig. 2. Relationship between serum miR-629-5p log2 expression and rs11630316 genotype (A), box plots of rs11630316-based miR-629-5p log2 expression according to ECG status (normal/abnormal; B), Venn diagram showing the target genes of miR-629-5p, miR-101-3p and miR-576-3p (C), and scatter plot of the relationship between serum log2 expression of miR-629-5p and *CCL5* (D).

progression from serum samples. Further studies are needed to define, for example, cut-off values as well as the clinical feasibility and utility of the procedure described here.

In addition to circulating miRNAs, differential miRNA and mRNA expression has also been investigated in myocardial biopsies from Chagas cardiomyopathy patients and controls, and differences in miR-146 and miR-155 expression between the two groups have been described.[22] We did not find an association between serum expression of the two miRNAs and abnormal ECG results. In the aforementioned study, however, decreased expression of miR-101-3p was observed. Thus, miRNA-101-3p shows decreased expression in both serum and myocardium in the context of Chagas cardiomyopathy, and the association of the expression of miRNA-101-3p in serum with disease stage and immune response in Chagas cardiomyopathy underscores its potential as a circulating biomarker. Interestingly, altered expression of *CCL5* in particular and increased activity of inflammatory signalling pathways in general has also been described in myocardial tissue.[22] These independent observations add plausibility to our findings. As *CCL5* has been increasingly described as a prognostic marker for Chagas cardiomyopathy in other recent studies,[57,58,63] we hypothesise that this chemokine plays a crucial role in disease progression. It is secreted by activated T cells, monocytes, epithelial cells, fibroblasts and platelets, and is considered a chemokine that leads to the maintenance of inflammation.[67]

Overall, this study contributes to the understanding of Chagas cardiomyopathy. Three miRNAs were identified in patient sera that are associated with disease progression and may be useful as diagnostic tools. Analysis of their target genes revealed that these miRNAs are markers for inflammatory signalling pathways. This suggests that inflammation is the key mechanism of disease progression and that the degree of inflammation can be quantified in patients' serum. This immediately opens up new possibilities in diagnosis, and, in the long term, the results may contribute to the development of new therapeutic concepts.

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Author contributions

DS, IZ, WA, LG, EN and JLB designed the project and obtained funding. MM designed the analysis plan, analysed the data, and wrote the first draft of the manuscript. AB and JLB supervised the data analysis and revised the draft. IZ, WA, LG, LO, EN, MLG, AA and SG carried out patient recruitment, and data and sample collection. DS, NMV, MW and PSA contributed to genotyping, and TBR and HL designed and supervised the small-RNA sequencing and bioinformatics. ML was responsible for the evaluation of the patients' ECGs. All authors revised the manuscript and had final responsibility for the decision to submit it for publication.

Data availability

The source code used for this study and the necessary input files with de-identified data will be made publicly available at www.biometrie.uni-heidelberg.de/StatisticalGenetics/Software_and_Data at the time of publication. dbGaP accession numbers for the Framingham Heart Study are provided in the Material and Methods section.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106613.

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