

## Genetic polymorphism of *Trypanosoma cruzi* bloodstream populations in adult chronic indeterminate Chagas disease patients from the E1224 clinical trial

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**Background:** The role that the genetic diversity of natural *Trypanosoma cruzi* populations plays in response to trypanocidal treatment of chronic Chagas disease (CD) patients remains to be understood. We analysed the genetic polymorphisms of parasite bloodstream populations infecting chronic CD patients enrolled in the E1224 clinical trial.

**Methods:** A total of 506 baseline and post-treatment follow-up samples from 188 patients were analysed. *T. cruzi* satellite DNA (satDNA) was amplified and sequenced using cruzi1/cruzi2 primers, and samples with TcI/III, TcII, TcIV or hybrid satDNA sequences were identified. Minicircle signatures were obtained after kinetoplast DNA amplification using 121/122 primers and restriction enzyme digestion. Genetic distances between baseline and post-treatment minicircle signatures were estimated using the Jaccard coefficient.

**Results:** At baseline, 74.3% TcII, 17.9% hybrid and 7.8% TcI/III satDNA sequences were found, whereas at the end of follow-up the distribution was 55.2% TcII, 35.2% hybrid and 9.5% TcI/III. The placebo arm was the treatment group with the highest variation of satDNA sequences between baseline and post-treatment follow-up. Genetic distances between baseline and post-treatment minicircle signatures were similar among all treatment arms. No association between minicircle signature variability and satDNA type distribution was found.

**Conclusions:** Genetic variability of *T. cruzi* bloodstream populations during post-treatment follow-up did not differ from that observed during chronic infection in the absence of treatment, suggesting that there were no selection events of E1224-resistant parasite populations. This is the first report documenting the genetic polymorphism of natural *T. cruzi* populations in chronic patients in the context of clinical trials with trypanocidal drugs.

### Introduction

Chagas disease (CD), ‘the most neglected of neglected diseases’, is caused by the protozoan *Trypanosoma cruzi*.<sup>1</sup> CD has been treated as an endemic disease in tropical and subtropical areas of South America, Central America and Mexico, where it still represents a major challenge for public health. However, it is an emerging global disease since migration of affected people to non-endemic areas has been increasing, leading to the introduction of the

disease in urban areas and non-endemic countries, and its perpetuation through vertical transmission, blood transfusion or organ transplantation from infected donors.<sup>2,3</sup>

The natural populations of *T. cruzi* have a complex multiclonal structure, with rare events of sexual recombination that account for the high genetic diversity found within the species.<sup>4–6</sup> Unravelling the populational diversity of *T. cruzi* is crucial to understanding CD epidemiology, developing control strategies and

assessing associations between parasite genotype, pathogenesis and clinical prognosis. Moreover, genetic diversity could play a role in the differential response to trypanocidal drugs observed in different geographical regions.<sup>7,8</sup>

The *in vitro* drug susceptibility of *T. cruzi* stocks may not predict the therapeutic outcome of treated patients<sup>9</sup> and it is relevant to discriminate between primary resistance observed in natural isolates and acquired resistance due to selective drug pressure.<sup>6,8,10</sup>

Parasite genetic diversity may be characterized at different levels. Natural populations of *T. cruzi* have been classified into seven discrete typing units (DTUs; TcI–TcVI and Tcbat), with diverse geographical distribution and transmission cycles.<sup>11,12</sup> All DTUs cause CD, but may exhibit different histotropism, leading to diverse clinical forms and severity.<sup>4,6</sup> Different DTUs have distinct DNA content and gene dosage, and a high degree of intra-DTU variability has been reported.<sup>13–15</sup>

Genetic markers for the identification of DTUs are mostly based on the use of polymorphic middle repetitive nuclear genes, such as the spliced-leader intergenic region, the D7 domain of the 24S $\alpha$  rRNA gene or the 18S rRNA gene.<sup>12</sup> MLST of polymorphic microsatellite loci and maxicircle genes has also been used.<sup>16</sup> Due to the scarcity and intermittency of *T. cruzi* bloodstream populations during the chronic phase of infection, all these genotyping strategies have limited sensitivity when applied directly to clinical or biological specimens, particularly when methods targeting single or low-copy-number genes are used. In contrast, highly repetitive sequences, such as the nuclear satellite DNA (satDNA) and the kinetoplast minicircle DNA, allow the assessment of genetic diversity in bloodstream and tissue specimens from infected patients.<sup>17–20</sup>

No association studies between natural *T. cruzi* population diversity and treatment outcome have yet been carried out in patients enrolled in clinical trials with trypanocidal drugs. Accordingly, we aimed to characterize *T. cruzi* bloodstream populations in cohorts of chronic CD adult patients treated with benznidazole and three E1224 dosing regimens in the context of a proof-of-concept, randomized, placebo-controlled trial,<sup>21</sup> using nucleic acid amplification-based typing strategies to compare their genetic polymorphisms at baseline and during post-treatment follow-up.

## Materials and methods

### Ethics statement

This study was approved by the Ethical Review Boards of the participating institutions (Universidad Mayor de San Simón and Fundación CEADES, Cochabamba, Bolivia; and Hospital Clinic, Barcelona, Spain), following the principles expressed in the Declaration of Helsinki. Written informed consent forms were signed by the study volunteers (no minor subjects were included). All samples were anonymized before being processed.

### Subjects and samples

Subjects were recruited for the E1224 clinical trial (NCT01489228), a proof-of-concept, randomized, placebo-controlled trial aiming to evaluate safety and efficacy of three oral regimens of E1224 [high-dose (HD): 4000 mg in 8 weeks; low-dose (LD): 2000 mg in 8 weeks; and short-dose (SD): 2400 mg in 4 weeks], a ravuconazole prodrug, compared with benznidazole (300 mg/day) and placebo, during 60 days of treatment.<sup>21</sup>

Two cohorts of Bolivian chronic CD patients from Cochabamba ( $N = 116$ ) and Tarija ( $N = 115$ ) cities were enrolled. In order to prevent reinfections

during the trial, the residences of the recruited patients were under entomological surveillance until the end of the study.

Peripheral blood samples were collected during baseline and post-treatment timepoint visits at the end of treatment, and after 4, 6 and 12 months of follow-up. Samples consisted of 10 mL of blood mixed with an equal volume of 6 M guanidine hydrochloride + 0.2 M EDTA pH 8.0 buffer. DNA was extracted from guanidine-EDTA-blood samples and analysed by quantitative real-time *T. cruzi* DNA PCR (qPCR), as previously described.<sup>22</sup> Those positive DNA samples with threshold cycles (Ct) below 35 [corresponding to blood samples with more than 0.1 parasite equivalents per mL (par. eq./mL)] were used for genetic polymorphism analysis.

### SatDNA sequence analysis

SatDNA-based analysis was performed from baseline samples and, if applicable, the last positive follow-up sample from each patient. A hot-start PCR procedure targeting *T. cruzi* satDNA was carried out using 5  $\mu$ L DNA samples in 50  $\mu$ L final volume. Reaction mix final concentrations were: 3 mM MgCl<sub>2</sub>, 250  $\mu$ M each deoxyribonucleotide triphosphate (dNTP), 2.5  $\mu$ M cruzi1 and cruzi2 primers<sup>23</sup> and 1 U GoTaq DNA polymerase (Promega, Madison, WI, USA). Amplifications were carried out in an MJR PTC-100 thermocycler (MJ Research Inc., Waltham, MA, USA) as follows: one step of 3 min at 94°C; 44 cycles at 94°C for 45 s, 66°C for 45 s and 72°C for 45 s; and a final extension step at 72°C for 10 min. Two PCR replicates were run per sample and, after confirmation by agarose-ethidium bromide gel electrophoresis, PCR products were pooled, purified using the PCR Purification Kit (Dongsheng Biotech Co., Guangzhou, China) and sequenced (Macrogen, Seoul, Korea).

Sequences were aligned using Clustal X v2.1<sup>24</sup> and edited with BioEdit v7.0.<sup>25</sup> Samples were classified into four *T. cruzi* satDNA types (TcI/III, TcII, TcIV or hybrid) according to the prevalence of SNPs corresponding to DTUs TcI/III, TcII, TcIV or TcI/III + TcII, respectively, as previously described.<sup>20</sup> Phylogenetic analyses were performed, grouping sequences by treatment arm and using a set of *T. cruzi* satDNA sequences from Ramirez *et al.*<sup>20</sup> as reference (Table S1, available as [Supplementary data](#) at JAC Online). The phylogenetic trees were built by maximum likelihood with IQ-TREE v1.6<sup>26</sup> and an appropriate substitution model according to the Akaike information criterion, estimated with jModelTest v2.1.<sup>27</sup> Analyses were run on the CIPRES Science Gateway server<sup>28</sup> and confidence was assessed by ultrafast bootstrap approximation (10 000 replicates).<sup>29</sup>

### Kinetoplast minicircle DNA signatures

Kinetoplast DNA (kDNA) minicircle signatures were characterized from positive samples collected at baseline and at the different timepoint visits of post-treatment follow-up. A hot-start PCR procedure targeting the variable region of the kDNA minicircle was carried out using a 5  $\mu$ L DNA sample in 50  $\mu$ L final volume. Reaction mix final concentrations were: 3 mM MgCl<sub>2</sub>, 250  $\mu$ M each dNTP, 2.5  $\mu$ M 121 and 122 primers<sup>18</sup> and 1 U GoTaq DNA polymerase (Promega). Amplifications were carried out in an MJR PTC-100 thermocycler as follows: one step of 3 min at 94°C; 6 cycles at 94°C for 45 s, 68°C for 45 s and 72°C for 45 s; 38 cycles at 94°C for 45 s, 64°C for 45 s and 72°C for 45 s; and a final extension step at 72°C for 10 min. Three PCR replicates were run per sample and, after confirmation by agarose-ethidium bromide gel electrophoresis, PCR products were pooled and purified using the PCR Purification Kit (Dongsheng Biotech Co.). RFLP-PCR profiling was performed from 1  $\mu$ g of purified PCR products digested with 1 U HinfI, MspI and RsaI restriction enzymes at 37°C for 3 h. The digestion products were visualized after 10% PAGE and SYBR Gold staining (Invitrogen, Carlsbad, CA, USA).

Genetic distances between baseline and post-treatment minicircle signatures from each patient were estimated using the Jaccard coefficient (JC).<sup>30</sup> JC was calculated with the formula  $JC = 1 - [a/(a + b + c)]$ , where  $a$  is the number of bands that are common to the two compared profiles,  $b$  is the number of bands that are present in the first profile and absent in the

second, and c is the number of bands that are absent in the first profile and present in the second.

**Statistical analysis**

The Wilcoxon test was used to analyse the differences of satDNA types between baseline and follow-up samples for each treatment group. Non-parametric analysis of variance was used to compare the JC values, grouped by treatment arm and follow-up timepoint visit. Multivariate analysis with logistic regression was used to determine the variables (parasitic load, satDNA type and kDNA signature differences between baseline and 12 month follow-up samples for each patient) associated with the placebo or E1224 arms. Statistical analyses were performed using SPSS Statistics v17.0 (SPSS Inc., Chicago, IL, USA) and InfoStat v2018 (Centro de Transferencia InfoStat, Cordoba, Argentina).

**Results**

A total of 506 qPCR-positive samples with Ct values below 35 (188 collected at baseline and 318 during post-treatment follow-up) were analysed. Table 1 shows the distribution of samples according to the treatment group and follow-up timepoint visit.

**SatDNA sequence analysis**

Two hundred and eighty-four satDNA sequences from baseline (179) and post-treatment follow-up (105) samples were amplified and sequenced. Comparison of satDNA sequences between baseline and post-treatment samples was carried out for the placebo and E1224 arms, but not for the benznidazole arm, because the only post-treatment qPCR-positive (Ct < 35) sample from this group could not be sequenced (sample collected at 6 months of follow-up, Table 1).

The analysis of *T. cruzi* satDNA from clinical samples allowed the detection of three types of sequences in the study population: TcI/III, TcII and hybrid (Figure 1). At baseline, samples had predominantly TcII satDNA (133; 74.3%), followed by hybrid (32; 17.9%) and TcI/III (14; 7.8%) satDNA sequences. On the other hand, at the end of follow-up, 58 (55.2%), 37 (35.2%) and 10 (9.5%) samples presented TcII, hybrid and TcI/III satDNA sequences, respectively.

The satDNA classification of the samples was assessed by performing a phylogenetic analysis of their sequences with those

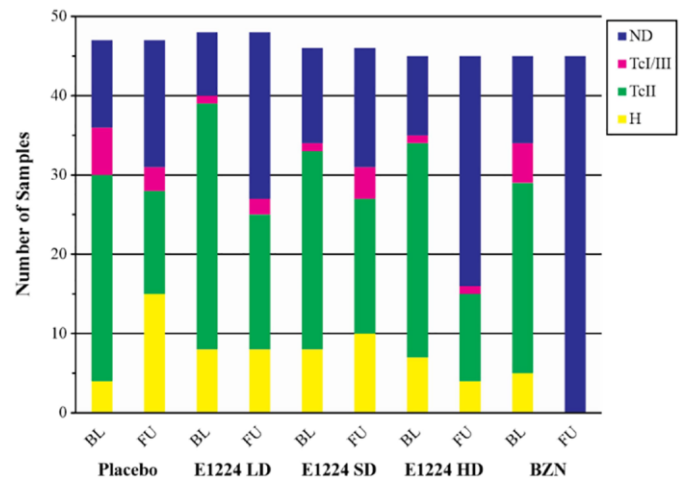
**Table 1.** Groups of treatment and follow-up timepoint visits distribution of DNA samples from the E1224 clinical trial used in this study

Treatment group	Number of qPCR-positive samples with Ct < 35				
	BL	EOT	4M	6M	12M
Placebo	42	29	35	35	30
E1224 LD	41	0	12	26	30
E1224 SD	34	1	28	25	34
E1224 HD	37	0	4	10	18
BZN	34	0	0	1	0
Total	188	30	79	97	112

BL, baseline; EOT, end of treatment; M, months of follow-up; BZN, benznidazole.

from reference *T. cruzi* stocks representative of DTUs TcI, TcII, TcIII and TcIV (Figure S1). Phylogenetic trees showed that sequences classified as TcI/III or TcII satDNA types were grouped in or close to TcI/III and TcII clusters, respectively. Hybrid sequences, instead, were distributed into, close to or between TcI/III and TcII clusters, according to the prevalence within their sequences of specific SNPs for TcI/III or TcII satDNA types, respectively (Figures S2 to S6).

Table 2 describes the variation of satDNA types between baseline and follow-up paired samples for patients enrolled in the placebo and E1224 arms. Surprisingly, the placebo arm, which represents the natural history of chronic infection during 1 year of follow-up, was the treatment group with the highest variation of satDNA sequences between baseline and post-treatment



**Figure 1.** Type of *T. cruzi* satDNA sequences in baseline and post-treatment samples from the E1224 clinical trial. ND, not done; H, hybrid; BL, baseline; FU, follow-up; BZN, benznidazole. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

**Table 2.** Variation of satDNA types of *T. cruzi* bloodstream populations from baseline and post-treatment paired samples of the E1224 clinical trial

Treatment group	satDNA type	Follow-up			
		BL	TcI/III	TcII	H
Placebo	TcI/III	3	0	2	1
	TcII	22	3	9	10
	H	3	0	0	3
E1224 LD	TcI/III	1	0	0	1
	TcII	20	2	13	5
	H	4	0	3	1
E1224 SD	TcI/III	1	0	0	1
	TcII	19	1	13	5
	H	6	2	3	1
E1224 HD	TcI/III	1	0	1	0
	TcII	11	0	8	3
	H	3	1	1	1

BL, baseline; H, hybrid.

follow-up. The number of samples with hybrid satDNA sequences was increased from baseline (3) to the end of follow-up (14), mostly due to the decrease of samples with TcII sequences between baseline (22) and post-treatment follow-up (11) ( $P < 0.05$ ).

In the case of E1224 arms, most TcII populations detected at baseline persisted at the end of follow-up: 13 out of 20 (65.0%) for the E1224 LD arm, 13 out of 19 (68.4%) for the E1224 SD arm and 8 out of 11 (72.7%) for the E1224 HD arm ( $P > 0.05$ ). For all treatment groups, the patients with baseline samples harbouring TcI/III sequences did not show this type of satDNA at the end of follow-up, although TcI/III sequences were detected at the end of follow-up in patients showing TcII or hybrid satDNA sequences at baseline.

### Kinetoplast minicircle DNA signatures

Minicircle signatures from 381 qPCR-positive samples (148 from baseline and 233 from post-treatment follow-up) were characterized by RFLP-PCR analysis to estimate the JC as a measure of the genetic distance between *T. cruzi* bloodstream populations at baseline and follow-up timepoint visits (Table 3). At the end of treatment, this analysis could only be done for patients from the placebo and E1224 SD arms, because the remaining ones gave negative qPCR results or had Cts  $\geq 35$ . For each treatment group, a higher number of patients became qPCR positive from Month 4 until the end of follow-up, except for the benznidazole arm, with only one qPCR-positive sample with Ct  $< 35$  at 6 months of follow-up.

Interestingly, the highest range of genetic distances between baseline and post-treatment minicircle signatures was observed within the placebo arm, ranging from 0 (identical parasite populations) to 0.85, at 4 months of follow-up (Table 3). No genetic distances equal to zero between baseline and post-treatment samples were found within the E1224 or benznidazole arms. The lowest range of JC values (0.33–0.53) was detected within the E1224 HD arm at 4 months of follow-up. On the other hand, the single refractory case of the benznidazole arm had a genetic distance of 0.82 between the minicircle signatures of its baseline and post-treatment samples. No statistical differences in JC values were observed within and between the placebo and E1224 arms for the different follow-up timepoint visits ( $P > 0.05$ ).

Figure 2 shows the dynamics of genetic distance between baseline and post-treatment minicircle signatures for 66 patients with more than one positive follow-up sample. There was a high variability in JC values from one follow-up timepoint visit to another, with patients increasing, decreasing or remaining around the same kDNA genetic distance between their baseline and post-treatment samples along follow-up, in the placebo (26) as well as in the E1224 LD (16), SD (17) and HD (7) treatment arms.

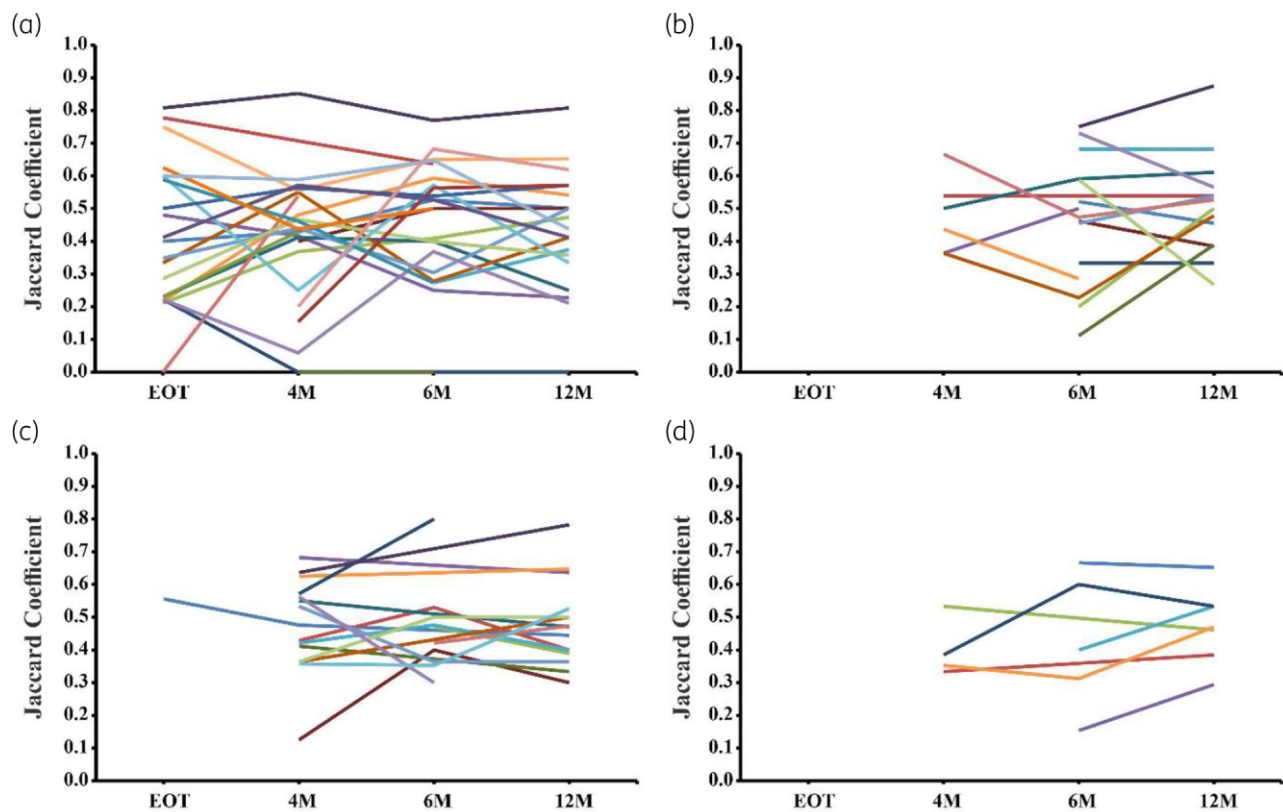
Finally, multivariate analysis was performed for 62 patients from the placebo (19) and E1224 LD (15), SD (18) and HD (10) arms, with parasitic load, satDNA type and kDNA minicircle signature characterizations for baseline and 12 month follow-up samples. The analysis showed that neither kDNA genetic distance nor satDNA type change between baseline and follow-up samples were associated with the placebo or E1224 arms ( $P > 0.05$ ).

**Table 3.** JC as a measure of the genetic distance between *T. cruzi* minicircle signatures of baseline and post-treatment samples from the E1224 clinical trial

Treatment group	Parameters	JC			
		EOT	4M	6M	12M
Placebo	N	22	27	25	21
	Median	0.41	0.44	0.50	0.47
	IQR	0.23–0.60	0.32–0.54	0.30–0.57	0.36–0.57
	Min–max	0.00–0.81	0.00–0.85	0.00–0.77	0.00–0.81
E1224 LD	N	0	7	19	18
	Median	—	0.44	0.47	0.49
	IQR	—	0.36–0.52	0.32–0.59	0.38–0.56
	Min–max	—	0.29–0.67	0.11–0.75	0.27–0.88
E1224 SD	N	1	16	10	21
	Median	0.56	0.50	0.45	0.44
	IQR	—	0.40–0.56	0.37–0.49	0.39–0.53
	Min–max	—	0.13–0.68	0.30–0.80	0.14–0.78
E1224 HD	N	0	4	6	12
	Median	—	0.37	0.43	0.47
	IQR	—	0.35–0.42	0.33–0.56	0.37–0.53
	Min–max	—	0.33–0.53	0.15–0.67	0.29–0.65
BZN	N	0	0	1	0
	Median	—	—	0.81	—
	IQR	—	—	—	—
	Min–max	—	—	—	—

EOT, end of treatment; M, months of follow-up; BZN, benznidazole; N, number of baseline and follow-up paired samples.





**Figure 2.** Dynamics of the genetic distance between *T. cruzi* minicircle signatures of baseline and post-treatment samples from 66 patients of the E1224 clinical trial with at least two qPCR-positive follow-up samples. (a) Placebo arm ( $N=26$ ); (b) E1224 LD arm ( $N=16$ ); (c) E1224 SD arm ( $N=17$ ); and (d) E1224 HD arm ( $N=7$ ). EOT, end of treatment; M, months of follow-up. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

## Discussion

This study attempted to search associations between the genetic diversity of natural *T. cruzi* populations detectable in the bloodstream of chronic CD patients, in the context of a proof-of-concept, randomized, placebo-controlled trial that included one benznidazole arm and three different E1224-dosing regimens.<sup>21</sup>

There is an association between *T. cruzi* satDNA types and DTUs.<sup>20,31,32</sup> TcI and TcIII strains harbour TcI/III satDNA type; TcII and TcIV strains harbour TcII and TcIV satDNA types, respectively; and TcV and TcVI hybrid strains harbour both TcI/III and TcII satDNA sequences, in agreement with the ‘three ancestor’ evolutionary model of *T. cruzi* hybrid lineages.<sup>20,33</sup>

However, direct classification of satDNA types in clinical samples does not allow the presence of hybrid lineages (TcV and TcVI) and coinfections of TcII with TcI or TcIII strains to be distinguished.<sup>20</sup> Therefore, satDNA typing results should be analysed in the context of the epidemiological setting of the region where patients come from. On the other hand, the low parasitic load of the chronic patients enrolled in the E1224 trial, with 73.3% of qPCR-positive samples with less than 1.5 par. eq./mL,<sup>34</sup> and the different rates of satDNA TcI/III and TcII sequence distribution among hybrid strains<sup>20</sup> could lead to samples from patients infected with TcV or TcVI strains being erroneously classified as TcI/III or TcII satDNA type. Although we have attempted to minimize this limitation by pooling two independent PCR replicates per

sample for sequencing analysis, the variations observed in satDNA types between baseline and post-treatment samples could be due in part to stochastic effects, particularly in those samples with parasitic loads close to the limit of detection of the PCR assay.

In the studied population, TcII satDNA sequences followed by hybrid (TcI/III + TcII) and TcI/III sequences were found at baseline, whereas TcIV satDNA was not found. These findings are consistent with the distribution of DTUs previously identified in the same cohort of patients.<sup>34</sup> Indeed, out of the 17 genotyped samples, DTUs TcII/V/VI were found in 11, but TcII, TcV and TcVI lineages could not be accurately distinguished due to the low parasitic loads of the patients. Besides, 5 out of those 17 samples were classified as infected by TcI populations and 1 was a mixed infection of TcI + TcII/V/VI,<sup>34</sup> which is compatible with the distribution of samples with TcI/III and hybrid satDNA sequences identified in this study. The DTU distribution obtained from this cohort was similar to that reported in 2016 by Martínez-Pérez *et al.*,<sup>35</sup> who found TcII/V as predominant in Bolivian migrants living in Madrid, Spain.

Interestingly, samples from the placebo arm showed the highest variation of satDNA sequences from baseline to the end of post-treatment follow-up. This observation is in agreement with a 10 day interval DTU typing analysis of 54 Colombian asymptomatic patients, which revealed that bloodstream parasite populations may vary drastically in a matter of days.<sup>36</sup> Moreover, this finding supports the clonal-histotropic model

and the multiclonal structure of *T. cruzi* populations proposed in 2004 by Macedo *et al.*<sup>4</sup>

Restriction pattern analysis of the hypervariable regions of kDNA minicircle using RFLP-PCR has been applied to identify the high degree of parasite polymorphism in clinical samples,<sup>18,19</sup> as well as to develop typing algorithms to complement other DTU typing strategies.<sup>37–39</sup> In our study, this strategy was used to characterize the genetic variability of bloodstream parasite populations detected at baseline and during follow-up timepoint visits.

As for satDNA sequences, the highest variation in kDNA minicircle signatures was observed within the placebo arm, which represents the natural history of the chronic infection during 1 year of follow-up. However, no significant differences were found between *T. cruzi* genetic distances in the placebo and E1224 arms, indicating a high genetic variability between baseline and post-treatment parasite populations for both treated and non-treated groups of patients. On the other hand, it is worth noting the high genetic distance (JC = 0.82) of the single refractory case of the benznidazole arm analysed in this work. One plausible explanation for this finding is that it might be due to drug selective pressure on the parasite post-treatment population.

The absence of differences between the genetic polymorphism of baseline and post-treatment samples of the three E1224 regimens in comparison with the placebo arm suggests that there was no drug selection of resistant parasite clones. This is in contrast with a recent study on the genetic constitution of TcI populations involved in an oral CD outbreak in Venezuela affecting patients who did not respond to benznidazole treatment. In that setting, JC values between baseline and post-treatment minicircle signatures decreased during the follow-up.<sup>40</sup> None of the post-treatment minicircle signatures was identical to its baseline counterpart population in the same patient, suggesting selection of parasite subpopulations between primary oral infection and post-treatment follow-up. Accordingly, the post-treatment decrease of intra-TcI diversity found in these patients might have been a consequence of the transition from acute to chronic phase, as well as the persistence of natural resistant subpopulations selected during benznidazole treatment. We cannot rule out that the prevalence of different DTUs, TcI in the above-mentioned setting and TcII/V/VI in the present study, as well as the evaluation of different trypanocidal drugs may account for the different findings of both studies.

To our knowledge, this is the first report addressing the genetic variability of natural parasite populations infecting chronic CD patients enrolled in a clinical trial with trypanocidal drugs. Further studies will be necessary to fully understand the role of the genetic polymorphism and the multiclonal population structure of *T. cruzi* in treatment failure.

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## Transparency declarations

None to declare. The funding sponsors had no role in the design of the study, the collection, analysis, or interpretation of data, the writing of the manuscript, or the decision to publish the results.

## Supplementary data

Supplementary sequence data, Table S1 and Figures S1 to S6 are available as Supplementary data at JAC Online.

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