Real-Time PCR for the Evaluation of Treatment Response in Clinical Trials of Adult Chronic Chagas Disease: Usefulness of Serial Blood Sampling and qPCR Replicates.

Short Title: qPCR as marker of treatment failure in chronic Chagas disease

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Abstract

This work evaluated a serial blood sampling procedure to enhance the sensitivity of duplex real-time PCR (qPCR) for baseline detection and quantification of parasitic loads and post-treatment identification of failure in the context of clinical trials for treatment of chronic Chagas disease, namely DNDi-CH-E1224-001 (NCT01489228) and MSF-DNDi PCR sampling optimization study (NCT01678599). Patients from Cochabamba (N= 294), Tarija (N= 257), and Aiquile (N= 220) were enrolled. Three serial blood samples were collected at each time-point and qPCR triplicates were tested per sample. The first two samples were collected during the same day and the third one seven days later.

A patient was considered PCR positive if at least one qPCR replicate was detectable. Cumulative results of multiple samples and qPCR replicates enhanced the proportion of pre-treatment sample positivity from 54.8 to 76.2%, 59.5 to 77.8%, and 73.5 to 90.2% in Cochabamba, Tarija, and Aiquile cohorts, respectively. This strategy increased the detection of treatment failure from 72.9 to 91.7%, 77.8 to 88.9%, and 42.9 to 69.1% for E1224 low, short, and high dosage regimens, respectively; and from 4.6 to 15.9% and 9.5 to 32.1% for the benznidazole arm in the DNDi-CH-E1224-001 and MSF-DNDi studies, respectively. The addition of the third blood sample and third qPCR replicate in patients with non-detectable PCR results in the first two samples, gave a small, non-statistically significant improvement in qPCR positivity. No change in clinical sensitivity was seen with a blood volume increase from 5 to 10 ml. The monitoring of patients treated with placebo in the DNDi-CH-E1224-001 trial revealed fluctuations in parasitic loads and occasional non-detectable results. In conclusion, serial sampling strategy enhanced PCR sensitivity to detecting treatment failure during follow-up and has the potential for improving recruitment capacity in Chagas disease trials, which require an initial positive qPCR result for patient admission.

Keywords: chronic Chagas disease, ravuconazole, benznidazole, treatment monitoring, real-time PCR.

Introduction

Following years of little progress in research and development of new compounds for treatment of Chagas disease (CD), new chemical classes and alternative treatment regimens have demonstrated encouraging activity against its causative agent, *Trypanosoma cruzi* (1-4). The efficacy of anti-*T. cruzi* compounds has habitually been measured by means of parasite detection or antibody titers. However, in chronically infected patients, traditional parasitological methods lack sensitivity and *T. cruzi*-specific antibody titers don't usually decrease until many years after treatment (5). In this context, molecular methods, such as conventional and real-time PCR (qPCR) assays, have opened promising opportunities for monitoring bloodstream parasitic levels to detect therapeutic failure or response (6–8). Following this approach, multicenter PCR studies have allowed harmonization and validation of standard operating procedures (SOPs) for PCR-based detection and quantification of *T. cruzi* DNA in blood samples (10, 11) coupled with external control quality assurance (12). However, the best performing qPCR methods reached between 60-70% of positivity in untreated chronic Chagas disease patients when a single baseline blood sample was tested (10, 11, 13), a figure which has been verified in different clinical trials (14–16).

In clinical trials in which eligibility criteria for patient enrollment include PCR positivity, such low values of sensitivity require that a larger proportion of seropositive subjects must be screened before being admitted. To overcome this limitation, a PCR sampling optimization study (NCT01678599) was developed by Drugs for Neglected Diseases *initiative* (DND*i*) and Médecins Sans Frontières (MSF) with the aim of evaluating sampling conditions for qPCR monitoring of benznidazole (BZN) treatment, as well as DNDi-CH-E1224-001, a DND*i*-sponsored randomized clinical trial (NCT01489228) to evaluate safety and efficacy of three oral regimens of E1224 (ravuconazole prodrug) in comparison with BZN and placebo, which

planned to collect three serial peripheral blood samples from each patient at each follow-up time point and to perform qPCR in triplicate from each blood sample DNA extract.

This report presents the data obtained in these studies, showing an improvement in qPCR clinical sensitivity for both enrollment and detection of treatment failure in adult patients with chronic Chagas disease.

Methods

Ethics statement

The clinical trials, including the sampling requirements, were approved by the Ethical Review Boards of Universidad Mayor de San Simón, Fundación CEADES, Hospital Clínic and Médecins Sans Frontières, following the principles expressed in the Declaration of Helsinki. Written informed consent forms were signed by the study volunteers (no minor subjects were included in these trials). All samples were anonymized before being processed.

Subjects and samples

Subjects were recruited for two different clinical studies:

i) The DNDi-CH-E1224-001 clinical trial (NCT01489228), designed and sponsored by DND*i*, with a proof-of-concept double-blinded randomized design aiming to evaluate the safety and efficacy of three (high, low, and short) oral regimens of E1224, compared to BZN (5 mg/kg/day) and placebo, during 60 days of treatment of adult patients with chronic indeterminate Chagas disease (17). A total of 560 patients aged 18-50 years and serologically confirmed as having Chagas disease were screened in two study sites of The Platform for a Comprehensive Care of Patients with Chagas disease in Bolivia, one site in the city of Cochabamba and the other in the city of Tarija. Of those screened, 551 patients had PCR

results available for analyses, as a total of 9 patients withdrew consent for participation and no PCR sample was collected.

Samples consisted of peripheral blood mixed with an equal volume of guanidine hydrochloride 6 M EDTA 0.2 M pH 8.0 buffer. A maximum of three 10 mL blood samples were collected at baseline: sample 1 (S1) and sample 2 (S2) were collected during the same day and sample 3 (S3) seven days later, but only if DNA extracts from S1 and S2 gave non-detectable results (as depicted in Figure 1). The qPCR was assayed in duplicate from both S1 and S2 DNA extracts. In cases where both replicates gave non-detectable results, a third replicate was analyzed. When all qPCR replicates from both S1 and S2 gave non-detectable results, S3 was collected and assayed in triplicate. During follow-up, three blood samples were collected at each time-point visit (end of treatment [EOT], and 2, 4, and 10 months post-treatment) and qPCR was assayed in triplicate from each S1, S2, and S3 DNA extract (Figure 1).

ii) The PCR Sampling Optimization study (NCT01678599) launched by DND*i* and MSF aimed to evaluate sampling strategies for qPCR treatment monitoring in adult patients with chronic Chagas disease (with indeterminate or early target organ involvement) treated with BZN (5 mg/kg/day) for 60 days. This study was carried out in 17 communities in the rural locality of Aiquile and did not include a placebo or other comparison treatment group. A total of 220 patients aged 18-60 years with serologically confirmed Chagas disease were recruited for this trial but only those with qPCR results at baseline were considered in this work (N= 205). All houses of patients entering the study were subjected to entomological surveillance.

From each seropositive patient, three blood samples were collected at baseline and at each follow-up visit (EOT, 4, and 10 months post-treatment) (Figure 1). S1 and S2 were collected during the same day and S3 seven days later. S1 and S3 consisted of 10 mL of blood, whereas for S2 5 mL was collected; all samples were mixed with an equal volume of guanidine-EDTA buffer. The qPCR was assayed in triplicate from each S1, S2, and S3 DNA extract (Figure 1).

Only patients with at least one positive result out of a maximum of nine qPCR replicates were enrolled in these trials. In both studies, therapeutic failure was defined as the persistence of parasite DNA, detected in at least one qPCR replicate, at any time-point during post-treatment follow-up.

DNA extraction

The High Pure PCR Template Preparation kit (Roche Diagnostics Corp., Indianapolis, IN) was used to process 300 µL of each guanidine-EDTA-blood (GEB) sample and DNA was eluted in 100 µL elution buffer, as previously described (13).

Quantitative real-time PCR procedure

A duplex qPCR targeted to *T. cruzi* satellite DNA (SatDNA) and an internal amplification control (IAC) were used, as previously described (13). The qPCR reactions were carried out with the use of FastStart Universal Probe Master Mix (Roche Diagnostics GmbHCorp., Mannheim, Germany) with 5 µL DNA extract in a final volume of 20 µL. Cycling conditions were a first step of 10 minutes at 95 ºC, followed by 40 cycles at 95 ºC for 15 seconds, and a final step of 1 minute at 58 °C. The amplifications were carried out in a Rotor-Gene Q (Corbett LifeScience, Cambridgeshire, United Kingdom) real-time PCR device.

For quantification purposes, standard curves were plotted with 1/10 serial dilutions of total DNA obtained from a GEB seronegative sample spiked with 10^5 par. eq./mL LL014-1-R1 Cl1 *T. cruzi* stock (TcV) cultured epimastigotes. One negative control and two positive controls containing 10 and 1 fg/µL *T. cruzi* CL-Brener DNA were included in every run, as recommended (18).

Genotyping of *T. cruzi* discrete typing units

Baseline samples from both clinical studies with SatDNA qPCR Ct (threshold cycle) values below 33 (N= 180) were genotyped using PCR-based strategies targeted to nuclear genomic markers, namely: (1) spliced leader intergenic region (SL-IR) based PCR was used to distinguish TcI (150 bp), and TcII, TcV, and TcVI (157 bp) from TcIII and TcIV (200 bp); (2) heminested SL-IR-I PCR was used to confirm TcI (350 bp) and heminested SL-IR-II PCR was used to confirm TcII, TcV, and TcVI (300 bp); (3) heminested PCR of the 24S alpha-ribosomal DNA (24Sα-rDNA) was used to distinguish TcV (125 bp) from TcII and TcVI (140 bp); and (4) heminested PCR targeted to genomic fragment A10 was used to discriminate TcII (580 bp) from TcVI (525 bp) (19).

Samples that yielded positive results by SL-IR-II PCR but were non-detectable by 24Sα-rDNA PCR were reported as belonging to the TcII/V/VI group. Those samples that amplified the 140 bp of 24Sα-rDNA fragment but had non-detectable results for A10 fragment-based PCR were reported as belonging to TcII/VI group. Those samples amplifying both bands of 125 and 140 bp after 24Sα-rDNA PCR, were interpreted as mixed infections by TcV plus TcII and/or TcVI, as previously described (19).

Statistical analysis

McNemar's test was used to compare the qualitative qPCR results for S1, S2, and S3 samples from Cochabamba, Tarija, and Aiquile cohorts at baseline, and between baseline and follow-up time-point samples from each treatment group in both clinical trials. The Fisher's exact test was used to compare the qPCR sensitivity using two or three replicates, and one, two, or three serial samples, and to compare the qPCR positivity between the baseline samples from Cochabamba, Tarija, and Aiquile cohorts, as well as the cumulative therapeutic failure at the end of 12-month follow-up within each treatment group using one, two, or three serial samples, and between BZN arms from both trials. Kruskal-Wallis non-parametric analysis of variance was used to compare the medians of the parasitic loads of quantifiable samples from Cochabamba, Tarija, and Aiquile cohorts at baseline, and from each treatment group at baseline and follow-up time-points. The Tukey's criterion was used to detect samples with outlier Ct values of IAC (Cts> 75th percentile + 1.5 x interquartile distance of median Ct) (20). All analyses were performed using SPSS Statistics for Windows V17.0 (SPSS, Chicago, IL).

Results

Screening of pre-treated chronic CD patients in DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization studies

Analysis of qPCR replicates in the DNDi-CH-E1224-001 trial

In this trial, qPCR was firstly assayed in duplicate from each S1 and S2 DNA extract. When both replicates gave non-detectable qPCR results from one of these DNA extracts, a third qPCR replicate was analyzed from the corresponding sample. When the third replicate was included, qPCR positivity increased from 54.8 to 60.5% (S1) and from 53.6 to 59.2% (S2) in samples collected from the Cochabamba cohort, and from 59.5 to 63.4% (S1) and from 55.3 to 60.7% (S2) in those collected from the Tarija cohort (Table 1, p> 0.05).

Analysis of serial blood samples

In the DNDi-CH-E1224-001 trial, the comparison of qPCR positivity obtained after testing individual S1 or S2 samples did not give significant differences (Table 1, $p > 0.05$) but qPCR positivity increased when cumulative results from S1+S2 were computed; this was observed in both Cochabamba (60.5 vs 69.7%, $p < 0.05$) and Tarija cohorts (63.4 vs 73.9%, $p < 0.05$).

When S1 and S2 gave non-detectable qPCR results, a third sample (S3) was taken seven days later. The analysis of PCR positivity obtained using three serial samples (S1+S2+S3) compared to that obtained from individual samples demonstrated higher sensitivity for both Cochabamba (60.5 vs 76.2%, $p < 0.001$) and Tarija cohorts (63.4 vs 77.8%, $p < 0.001$). Finally, qPCR positivity obtained after testing S1+S2 versus that obtained after testing S1+S2+S3 increased by 6.5% (N= 19/294) in Cochabamba and 3.9% (N= 10/257) in Tarija cohorts (Table 1, $p > 0.05$).

On the other hand, no statistical difference was observed in qPCR positivity by testing individual S1, S2, or S3 samples in the MSF-DNDi PCR sampling optimization study (Table 1, $p > 0.05$). Computing the cumulative qPCR positivity obtained for S1+S2 (85.1%) in comparison to the positivity obtained for S1 (10 mL of blood, 73.5%, $p < 0.01$) or S2 alone (5 mL of blood, 76.9%, $p < 0.05$) increased sensitivity. This was also true for the cumulative qPCR positivity obtained for S1+S2+S3 (90.2%) compared to that obtained for the individual samples (S1, $p < 0.001$; S2, $p < 0.001$; and S3, 72.7%, $p < 0.001$). Comparison of the cumulative qPCR positivity obtained from S1+S2+S3 with respect to S1+S2 showed an increase of 5.1% (Table 1, $p > 0.05$).

Analysis of T. cruzi DTUs and parasitic loads

It is worth noting the higher qPCR positivity obtained in patients from Aiquile (90.2%) compared to those recruited from Cochabamba (76.2%, $p< 0.001$) and Tarija (77.8%, $p< 0.001$); whereas no difference was found between both E1224 trial cohorts (Table 1, $p> 0.05$). Because both studies used the same qPCR method performed in the same laboratory, a hypothesis for this geographical variability in qPCR positivity could be related to diversity of *T. cruzi* strains or parasitic loads in the populations studied, and/or to a higher endemicity and exposure to the vector in Aiquile, and therefore a potential risk of reinfection. In order to investigate this, the distribution of *T. cruzi* DTUs was analyzed by genotyping the 180 qPCR positive samples from these localities with the highest parasitic loads.

DTUs could be identified in 31 samples: 23 patients were infected with parasite populations belonging to the group TcII/V/VI, six patients were infected with TcI and two presented mixed infections by TcI plus TcII/V/VI (Table 2). TcI was five times more frequent in Cochabamba and Tarija in comparison to Aiquile, although the low number of genotyped samples preclude determination of its significance. TcIII and TcIV were not detected.21

The parasitic loads of baseline samples from the three different cohorts are shown in Fig 2. In Aiquile, 33.0% of samples had parasitic loads above the qPCR Limit of Quantification (LOQ) of 1.53 par. eq./mL, whereas in Cochabamba and Tarija the percentage of quantifiable samples was 19.6% and 24.5%, respectively (Table 1). The median and interquartile range values of the quantifiable parasitic loads were 2.6 [2.0-3.5], 2.6 [2.0-3.6], and 3.0 [2.0-4.7] par. eq./mL, for Cochabamba, Tarija, and Aiquile cohorts, respectively (Table 1, $p> 0.05$).

Follow-up of treated chronic CD patients in DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization studies

Analysis of qPCR positivity and parasitic loads

Table 3 shows the cumulative qPCR findings obtained from all three serial blood samples during screening and monitoring of all treatment arms in both clinical trials.

The qPCR positivity of the placebo group from the DNDi-CH-E1224-001 clinical trial was significantly higher at baseline (100%, as per study entry criteria) than at the follow-up time-points (2 months, 73.9%, $p< 0.01$; 4 months, 80.4%, $p< 0.01$; 6 months, 87.0%, $p< 0.05$; 12 months, 78.3%, $p< 0.01$), whereas no differences were found between follow-up time-points (Table 3, $p> 0.05$). Out of the patients who received placebo, 27 were persistently qPCR positive, 15 had intermittently positive and non-detectable results, and four became persistently qPCR undetectable during follow-up.

In both clinical trials, the treated cohorts showed a drastic reduction in PCR positivity at EOT. (E1224 LD, 10.4%; E1224 SD, 8.9%; E1224 HD, 16.7%; DNDi-CH-E1224-001BZN, 6.8%; DND*i*-MSF sampling optimization study BZN, 23.1%) (Table 3, p< 0,001). In the E1224 treatment arms, qPCR positivity increased during post-treatment follow-up, reaching its highest value at the end of the study (E1224 LD, 76.6%, $p< 0.001$; E1224 SD, 84.4%, $p< 0.001$; E1224 HD, 56.1%, $p< 0.01$), whereas in the cohorts treated with BZN, the proportion of qPCR-positive cases diminished at the end of follow-up (DNDi-CH-E1224-001 BZN, 4.5%, $p> 0.05$; DND*i*-MSF sampling optimization study BZN, 5.2%, $p< 0.01$).

Interestingly, all treatment arms showed statistically significant differences between the proportion of positive qPCR results at baseline and end of follow-up (E1224 LD, $p< 0.01$;

E1224 SD, p< 0.05; E1224 HD, p< 0.001; DNDi-CH-E1224-001 BZN, p< 0.001; DND*i*-MSF sampling optimization study BZN, p< 0.001).

The number of patients with quantifiable qPCRs results from the placebo group of the E1224 trial ranged between 14-16 during follow-up; except at 4 months when, as at baseline, nine patients rendered quantifiable qPCR results (Table 3). Out of the nine patients enrolled in the placebo group of DNDi-CH-E1224-001 who showed quantifiable parasitic loads at baseline , five had quantifiable results throughout follow-up, two patients alternated between quantifiable and non-quantifiable results, and the two remaining showed persistent detectable but non-quantifiable qPCR results throughout follow-up. No significant differences were found among the medians of parasitic loads at baseline and follow-up time-points in the placebo group of the DNDi-CH-E1224-001 trial (Fig 3A, p> 0.05).

Patients treated with E1224 showed non-quantifiable parasitic loads at the end of treatment, but this increased later on; indeed, 12 cases reached quantifiable loads for E1224 low dose (LD) and short dose (SD) regimes and six in E1224 high dose (HD) regime at the end of follow-up; whereas in BZN treated groups only one patient had parasitic loads higher than 1.53 par. eq./mL during follow-up (Table 3). Statistically significant differences were observed between parasitic loads at baseline and 6 months for E1224 LD (3.5 [2.6-7.0] and 2.1 [1.7-2.4] par. eq./mL, respectively Fig 3B, p< 0.05), and between baseline and 12 months (2.5 [1.9-3.4]) and2.0 [1.9-2.2] par. eq./mL) for E1224 HD Fig 3D, p< 0.05).

Analysis of cumulative therapeutic failure

Fig 4 compares the cumulative qPCR positivity as a measure of treatment failure obtained for each treatment group in both clinical trials from EOT until the end of follow-up.

In the DNDi-CH-E1224-001 trial, the multi-sampling strategy (S1+S2+S3) increased detection of treatment failure at the end of follow-up by up to 91.7% for E1224 LD (Fig 4B, $p< 0.05$), 88.9% for E1224 SD (Fig 4C, $p> 0.05$), 69.1% for E1224 HD (Fig 4D, $p< 0.05$), and 15.9% for BZN (Fig 4E, $p> 0.05$) arms. No significant differences were found between the cumulative treatment failure detected for single S1 (72.9, 77.8, 42.9, and 4.6%), S2 (70.8, 86.7, 50.0, and 4.6%), and S3 (72.9, 82.2, 45.2, and 6.8%) samples, and comparing S1+S2 (81.3, 88.9, 61.9, and 9.1%) versus S1+S2+S3 for E1224 LD, SD, and HD, and BZN arms, respectively (Fig 4, $p> 0.05$).

In the MSF-DND*i* PCR sampling optimization study, the strategy involving serial sampling analysis allowed an increase in detection of treatment failure of up to 32.1% (S1+S2+S3) at the end of follow-up in comparison to that detected from individual samples (S1, 9.5%, $p< 0.001$; S2, 19.0%, $p< 0.05$; S3, 11.0%, $p< 0.001$). Significant difference was found between the cumulative treatment failure of S1 and S2 ($p< 0.05$), whereas no differences were found between S3 and S1 or S2 (Fig 4F, $p> 0.05$). There was an increase of 7.3% in cumulative treatment failure detected after testing S1+S2+S3 versus that detected after testing S1+S2 (24.8%) (Fig 4F, $p> 0.05$).

Analysis of cumulative therapeutic failure among the different groups of treatment of E1224 trial did not show significant differences among placebo and E1224 LD and SD arms (Fig. 4, $p> 0.05$). In contrast, the E1224 HD arm showed lower treatment failure than placebo ($p< 0.05$) and E1224 LD ($p< 0.01$) and SD ($p< 0.05$). In addition, the DNDi-CH-E1224-001 BZN group showed lower treatment failure than placebo and E1224 arms ($p< 0.001$).

No statistically significant differences were observed between the cumulative therapeutic failure of BZN-treated cohorts enrolled in DNDi-CH-E1224-001 and MSF-DND*i* PCR sampling optimization studies (Figure 4; p> 0.05).

Discussion

Impact of serial sampling strategies on qPCR sensitivity

In recent years, several clinical trials to evaluate anti-parasitic treatments for CD were carried out using different sampling strategies and PCR protocols, and variable rates of PCR positivity were obtained (14, 15, 21).

The present analyses shows that qPCR sensitivity was significantly improved at baseline in the DNDi-CH-E1224-001 trial when two blood samples were collected and each DNA extract was analyzed in duplicate by qPCR. The addition of the third blood sample and third qPCR replicate in the subset of patients who had non-detectable PCR results for S1 and S2, gave a small but non-statistically significant improvement in positivity. The limited data available thus far is insufficient to determine the clinical relevance of this small increase in qPCR sensitivity in the evaluation of treatment response. In fact, the samples with only one out of three PCR positive results were non-quantifiable. As treatment was expected to reduce further the parasite burden in those patients with non-quantifiable baseline qPCR results, reducing the chance of detecting treatment failure, three blood samples and qPCR triplicates were tested during post-treatment follow-up.

In the MSF-DND*i* PCR sampling optimization study, the use of 5 mL of blood, instead of 10 mL as starting sample for qPCR analysis, as well as the collection of a third blood sample

seven days after the first two samples instead of few minutes later, did not modify the overall clinical sensitivity.

In conclusion, these findings support the use of lower volume of blood, collected during the same visit, for qPCR testing purposes.

Distribution of DTUs and parasitic loads

TcV was the prevailing DTU, in agreement with findings reported by Martinez-Perez et al. (2016) (22), who found TcV in the 55.2% of Bolivian CD patients living in Madrid, Spain. However, TcIV, usually associated with the sylvatic cycle and occasional oral outbreaks (23, 24) but found as the second predominant DTU in Bolivian patients (22), was not detected.

Differences in qPCR positivity between Cochabamba or Tarija cohorts compared with Aiquile could be attributed to different distribution of parasite DTUs in these localities, such as it was observed for TcI (Table 2), although the low number of genotyped samples precluded assessment of the significance of this finding.

Median parasitic loads were higher in Aiquile than in Cochabamba or Tarija, although the differences did not reach statistical significance (Table 1 and Fig 1). This could be due to the rural nature of the Aiquile area compared to the cities of Cochabamba and Tarija. In a recent study of pregnant women from Bolivia, it was observed that the differences in seroprevalence for *T. cruzi* infection were above all related to the area in which the patients lived most of their lives. Hyper-endemic hotspots were observed where prevalence surpassed 60% and one of the affected areas was the municipality of Aiquile, with 66% seroprevalence (25). In areas where vector infestation was higher, the seroprevalence of CD was also higher (25).

Dynamics of bloodstream parasite burden in chronic CD

The monitoring of samples from patients treated with placebo in the DNDi-CH-E1224-001 trial allowed follow-up of the natural history of human chronic *T. cruzi* infection in adult patients for a period of one year. The results showed that a proportion of patients had fluctuations of parasitic loads, which, in some cases, fell below the LOQ (1.53 par. eq./mL) of the qPCR method (13), and even gave non-detectable results, reflecting the fluctuations of parasitemia observed in chronic CD patients using traditional parasitological methods (24). Such findings underscore the need for serial sampling and qPCR replicates analysis for the evaluation of therapeutic failure in chronic CD.

qPCR as surrogate marker of therapeutic failure in CD clinical trials

The qPCR-based study of the DNDi-CH-E1224-001 clinical trial demonstrated that BZN was a better parasiticidal drug than E1224 in monotherapy, and that in turn, E1224 HD had higher efficacy than the other E1224 regimens (Fig 3). Treatment with BZN gave a better parasitological response in the urban cohorts of the DNDi-CH-E1224-001 trial than in the rural patients from the MSF-DNDi PCR sampling study, although no significant differences were found. This could be due to the more controlled conditions of treatment administration and follow-up in the DNDi-CH-E1224-001 trial, rather than to a higher risk of re-infection in the rural community of Aiquile, since the houses of all patients enrolled in the MSF-DNDi PCR sampling study were under entomological surveillance.

Finally, this report demonstrates the usefulness of serial blood sampling and qPCR replicates analysis not only for enhancing the capacity to recruit chronic CD adult patients for clinical trials, in which the inclusion criteria require at least one qPCR positive result at baseline, but

399 more importantly for increasing sensitivity to detect treatment failure in this population. At
400 the same time, this work highlights the importance of standardized methods for monitoring
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402

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552 **Table 1. Accumulative qPCR findings in pre-treated chronic Chagas disease patients from DNDi-CH-E1224-001 and MSF-DNDi PCR**
553 **sampling optimization clinical studies, using one, two and three serial samples and two or three qPCR replicates per sample.**

Clinical trial	Locality	Parameters	S1 qPCR(1+2)	S1 qPCR(1+2)+3	S2 qPCR(1+2)	S2 qPCR(1+2)+3	S1+S2	S3 qPCR(1+2+3)	S1+S2+S3
DNDi-CH-E1224-001	CBBA	N	294	294	289	289	294	74	294
		Positives	161 (54.8%)	178 (60.5%)	155 (53.6%)	171 (59.2%)	205 (69.7%)	19 (25.7%)	224 (76.2%)
		Quantifiables	31 (19.3%)	31 (17.4%)	26 (16.8%)	26 (15.2%)	44 (21.5%)	0 (0.0%)	44 (19.6%)
		Median [IQR] (par. eq./mL)	2.6 [1.9-3.4]	2.6 [1.9-3.4]	2.7 [2.0-3.9]	2.7 [2.0-3.9]	2.6 [2.0-3.5]	---	2.6 [2.0-3.5]
	Tarija	N	257	257	257	257	257	53	257
		Positives	153 (59.5%)	163 (63.4%)	142 (55.3%)	156 (60.7%)	190 (73.9%)	10 (18.9%)	200 (77.8%)
		Quantifiables	37 (24.2%)	37 (22.7%)	32 (22.5%)	33 (21.2%)	49 (25.8%)	0 (0.0%)	49 (24.5%)
		Median [IQR] (par. eq./mL)	2.4 [2.0-3.4]	2.4 [2.0-3.4]	3.0 [2.4-3.6]	3.0 [2.2-3.6]	2.6 [2.0-3.6]	---	2.6 [2.0-3.6]
MSF-DNDi Sampling Study	Aiquile	N	---	196	---	195	201	176	205
		Positives	---	144 (73.5%)	---	150 (76.9%)	171 (85.1%)	128 (72.7%)	185 (90.2%)
		Quantifiables	---	34 (23.6%)	---	40 (26.7%)	51 (29.8%)	29 (22.7%)	61 (33.0%)
		Median [IQR] (par. eq./mL)	---	2.4 [1.9-4.5]	---	2.9 [1.9-4.9]	2.8 [1.9-4.8]	3.2 [2.0-4.8]	3.0 [2.0-4.7]

554 S1-3: samples 1-3; qPCR1-3: qPCR replicates 1-3; CBBA: Cochabamba; N: number of samples; IQR: interquartile range; par. eq./mL: parasite
555 equivalents in 1 mL of blood.
556

Table 2. Direct identification of *T. cruzi* DTUs in blood samples of pre-treated chronic Chagas disease patients from DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization clinical studies.

Clinical trial	Locality	*T. cruzi* DTU			
		TcI	TcI+II/V/VI	TcII/V/VI	TcV/VI
DNDi-CH-E1224-001	CBBA-Tarija	5	1	10	1
MSF-DNDi Sampling Study	Aiquile	1	1	11	1

DTU: Discrete Typing Unit; CBBA: Cochabamba.

562 **Table 3. qPCR findings during baseline and follow-up of the different groups of**
563 **treatment of DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization clinical**
564 **studies.**

Clinical trial	Group of treatment	Parameters	BL (S1+2+3)	2M (S1+2+3)	4M (S1+2+3)	6M (S1+2+3)	12M (S1+2+3)
DNDi-CH-E1224-001	Placebo	N	46	46	46	46	46
		Positives	46 (100%)	34 (73.9%)	37 (80.4%)	40 (87.0%)	36 (78.3%)
		Quantifiables	9 (19.6%)	15 (44.1%)	9 (24.3%)	14 (35.0%)	16 (44.4%)
		Median [IQR] (par. eq./mL)	2.2 [2.0-4.1]	2.2 [1.9-4.3]	3.3 [2.1-4.1]	3.1 [2.1-3.7]	2.7 [1.9-5.3]
	E1224 LD	N	48	48	48	48	47
		Positives	48 (100%)	5 (10.4%)	18 (37.5%)	32 (66.7%)	36 (76.6%)
		Quantifiables	14 (29.2%)	0 (0.0%)	6 (33.3%)	10 (31.3%)	12 (33.3%)
		Median [IQR] (par. eq./mL)	3.5 [2.6-7.0]	---	2.1 [1.9-2.5]	2.1 [1.7-2.4]	2.2 [2.1-4.3]
	E1224 SD	N	45	45	44	43	45
		Positives	45 (100%)	4 (8.9%)	31 (70.5%)	33 (76.7%)	38 (84.4%)
		Quantifiables	9 (20.0%)	0 (0.0%)	8 (25.8%)	5 (15.2%)	12 (31.6%)
		Median [IQR] (par. eq./mL)	2.3 [2.0-2.9]	---	2.5 [1.9-3.5]	2.2 [1.9-2.6]	2.8 [2.1-4.5]
	E1224 HD	N	42	42	41	41	41
		Positives	42 (100%)	7 (16.7%)	9 (22.0%)	14 (34.1%)	23 (56.1%)
		Quantifiables	11 (26.2%)	0 (0.0%)	0 (0.0%)	1 (7.1%)	6 (26.1%)
		Median [IQR] (par. eq./mL)	2.5 [1.9-3.4]	---	---	1.8	2.0 [1.9-2.2]
	BZN	N	44	44	43	43	44
		Positives	44 (100%)	3 (6.8%)	0 (0.0%)	2 (4.7%)	2 (4.5%)
		Quantifiables	11 (25.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		Median [IQR] (par. eq./mL)	2.1 [1.9-2.7]	---	---	---	---
MSF-DNDi Sampling Study	BZN	N	137	121		115	116
		Positives	137 (100%)	28 (23.1%)		11 (9.6%)	6 (5.2%)
		Quantifiables	47 (34.3%)	0 (0.0%)	---	1 (9.1%)	0 (0.0%)
		Median [IQR] (par. eq./mL)	2.8 [1.9-4.6]	---		2.2	---

565 BL: baseline; 2M, 4M, 6M and 12M: 2, 4, 6 and 12 months from the beginning of the study;
566 S1-3: samples 1-3; LD, SD and HD: low, short and high dosages; BZN: benznidazole; N:
567 number of samples; IQR: interquartile range; par. eq./mL: parasite equivalents in 1 mL of
568 blood
569

Fig 1. Study diagram and schedule of qPCR assessments

Fig 2. Distribution of parasitic loads in peripheral blood samples of pre-treated chronic Chagas disease patients from DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization clinical studies. par. eq./mL: parasite equivalents in 1 mL of blood; CBBA: Cochabamba.

Fig 3. Distribution of parasitic loads during baseline and follow-up of the different groups of treatment of DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization clinical studies. A: E1224-Placebo arm; B: E1224-Low Dose arm; C: E1224-Short Dose arm; D: E1224-High Dose arm; E: Benznidazole arm from the DNDi-CH-E1224-001 trial; F: Benznidazole arm from the MSF-DNDi PCR sampling optimization study; par. eq./mL: parasite equivalents in 1 mL of blood; BL: baseline; 2M, 4M, 6M and 12M: 2, 4, 6 and 12 months from the beginning of the study

Fig4. Cumulative therapeutic failure during the follow-up of the different groups of treatment of DNDi-CH-E1224-001 and MSF-DNDi PCR sampling optimization clinical studies. A: E1224-Placebo arm; B: E1224-Low Dose arm; C: E1224-Short Dose arm; D: E1224-High Dose arm; E: Benznidazole arm from the DNDi-CH-E1224-001 trial; F: Benznidazole arm from the MSF-DNDi PCR sampling optimization study; S1-3: samples 1-3; * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$

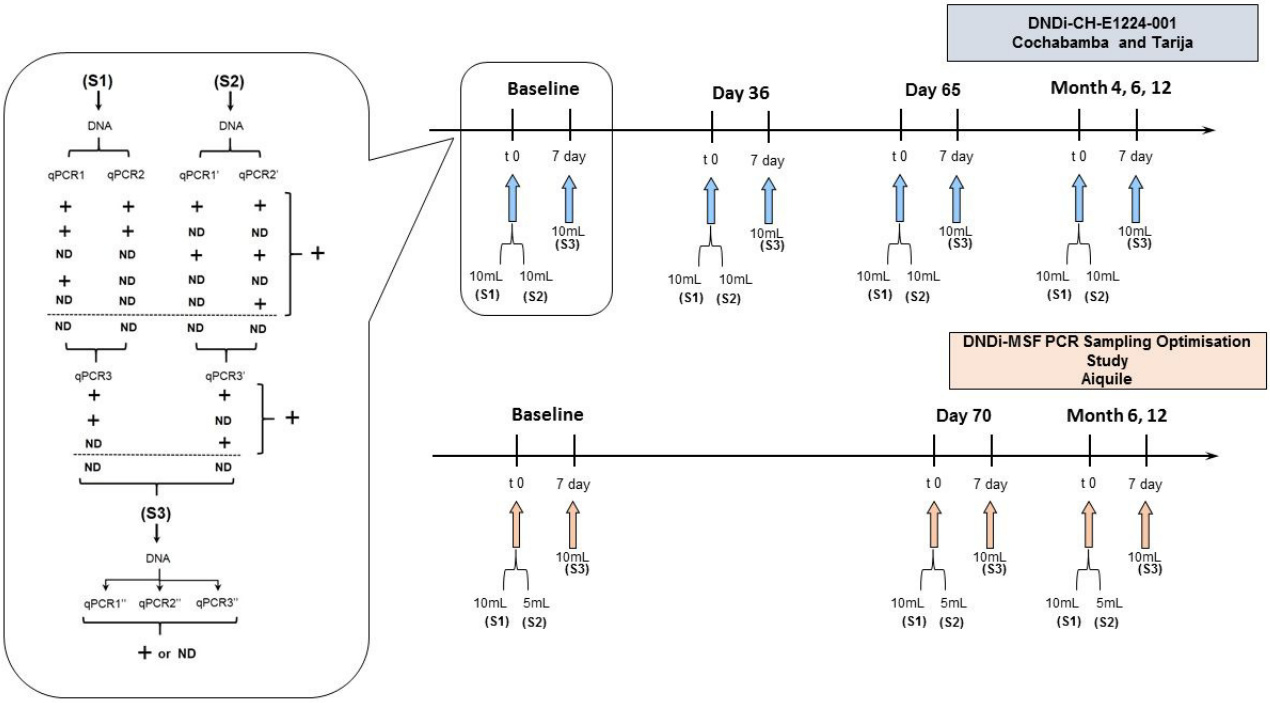

Figure 1.